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Transferrin receptor expression in the sheep reticulocyte: Biosynthesis and fate during reticulocyte maturation

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Abstract

The transferrin receptor (TfR) is a membrane protein whose function is to deliver transferrin-bound iron into the cell. Erythroid precursor cells and reticulocytes require huge amounts of iron for hemoglobin synthesis and express large numbers of TfRs. However, as the reticulocyte matures into an erythrocyte, TfRs are lost from the cell, as are a number of other membrane proteins. Nearly a decade ago, it was shown that TfRs are released from the sheep reticulocyte in vesicular form (in exosomes) during culture. Exosome formation may be the mechanism for remodelling of the plasma membrane during reticulocyte maturation. In this thesis, we determine whether the sheep reticulocyte has the capacity to sythesize TfRs and whether TfR biosynthesis or externalization is controlled directly by iron. Although the sheep reticulocyte incorporates [^{35}S]methionine into the TfR, the labelled TfRs are incompletely glycosylated and are not transported to the cell surface. TfR synthesis is regulated by heme, rather than directly by iron. Newly synthesized TfRs are not externalized; however, pulse-chase experiments showed that they are lost slowly by an iron-dependent mechanism. Externalization of preexisting TfRs is stimulated by hemin but not iron. We propose that hemin catalyzes an oxidative change in the TfR which may facilitate the removal of the TfR from the recycling pathway and allow it to be targeted to multivesicular bodies and ultimately released in exosomes. We suggest that the TfR ectodomain can be cleaved from the released vesicles and propose that exosomal membranes are the source of the soluble truncated TfRs found in the circulation.

Résumé

Le récepteur de la transferrine (TfR) est une protéine membranaire qui a la fonction de transporter le fer lié à la transferrine à l'intérieur de la cellule. Les précurseurs érythroides et les réticulocytes ont besoin d'énormément de fer pour la synthèse de l'hémoglobine et contiennent beaucoup de TfRs. Mais quand le réticulocyte se transforme en érythrocyte, les TfRs disparaissent, ainsi qu'un nombre d'autre protéines membranaires. Il y a presque dix ans, il a été démontré que pendant la culture des réticulocytes de mouton, les TfRs sont libérés de la cellule sous une forme vésiculaire (dans les exosomes). La formation d'exosomes peut être le mécanisme pour le remodelage de la membrane plasmique durant la maturation du réticulocyte. Dans cette thèse, nous voulons déterminer si le réticulocyte de mouton a la capacité de synthétiser les TfRs et si la synthèse ou la libération sont affectées directement par le fer. Bien que le réticulocyte incorpore la [^{35}S]méthionine dans le TfR, les TfRs marqués sont incomplètement glycosylés et ils ne sont pas transportés à la surface cellulaire. La synthèse du TfR est regulée par l'hémin et non directement par le fer. Les TfRs nouvellement synthétisés ne sont pas libérés; cependant, nos expériences ont demontré que ces TfRs sont perdus lentement par un mécanisme dépendant du fer. La libération des TfRs préexistant est stimulée par l'hémin et non par le fer. Nous proposons donc que l'hémin catalyse un changement d'oxydation dans le TfR lequel facilite l'élimination du TfR de la chaîne de recyclage et lui permet d'être ciblé aux corps multivésiculaires et ultérieurement liberé dans les exosomes. Nous suggérons que l'ectodomaine du TfR peut être coupé des vésicules libérées et nous croyons que les membranes vésiculaires sont sources des TfRs tronqués solubles que l'on retrouve dans la circulation.

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Foreword

Parts of this thesis have already been published or submitted for publication. Chapter 3 (with the exception of section 3.2) and parts of Chapter 4 (including Figures 18, 25, 26, and 28) have been published as a paper with the title "Maturation-associated loss and incomplete de novo synthesis of the transferrin receptor in peripheral sheep reticulocytes: response to heme and iron." [Ahn, J., and Johnstone, R.M. (1989) *J. Cell. Physiol.* 140, 107-118]. Sections 4.1, 4.2, 4.3 (Figure 27), and 4.5 of Chapter 4 have been published as a paper titled "Intracellular localization of newly synthesized transferrin receptors in the peripheral sheep reticulocyte" [Ahn, J., and Johnstone, R.M. (1991) *Arch. Biochem. Biophys.* 291, 154-160]. Chapter 5 has been submitted under the title "The origin of a soluble truncated transferrin receptor." [Ahn, J., and Johnstone, R.M. (1992)].

The experiment shown in Figure 26 of Chapter 3 was performed by Claire Turbide and subsequently repeated by me. The data for Table 9 and Figures 31, 34, and 35 of Chapter 5 were contributed by Francine Nault. The ^{35}S incorporation experiment with chicken reticulocytes (Chapter 4) was performed in collaboration with Anu Mathew.

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Finally, I wish to thank my parents for their constant support.

Contributions to Original Knowledge

1. The newly synthesized TfR of sheep reticulocytes is incompletely glycosylated and not expressed at the cell surface. It appears that the reticulocyte has lost some component(s) of the Golgi complex that is/are required for the transport of nascent proteins from the ER to the Golgi and between Golgi cisternae.

2. Unlike the preexisting TfR, the newly synthesized TfR is not externalized during reticulocyte maturation, but remains in an intracellular membrane compartment where it is lost slowly ($t_{1/2}$ ~20 h) by an iron-dependent mechanism.

3. Hemin stimulates the loss of preexisting TfRs and nucleoside transporters (NBMPR binding sites) from reticulocytes while increasing the amount of these proteins and the 70 kDa protein in exosomes that are released from the cells during reticulocyte maturation. Therefore, heme may accelerate the maturation process.

4. Although sheep reticulocytes can maintain their ATP levels when depleted of oxygen, TfRs and nucleoside transporters are not externalized under this condition, suggesting that there is an absolute requirement of oxygen for reticulocyte maturation.

5. Truncated non-vesicular TfRs (M_r ~80 kDa) are released by cultured human HL60 myeloid cells. A portion of the TfRs released by reticulocytes may also be truncated.

6. A 17 kDa cytoplasmic domain of the TfR is present in sheep exosomes, suggesting that soluble truncated TfRs arise from cleavage of exosomal TfRs.

Publications

Ahn, J. and Johnstone, R.M. (1989) Maturation-associated loss and incomplete de novo synthesis of the transferrin receptor in peripheral sheep reticulocytes: response to heme and iron. *J. Cell. Physiol.* **140**, 107-118.

Johnstone, R.M. and Ahn, J. (1990) A common mechanism may be involved in the selective loss of plasma membrane functions during reticulocyte maturation. *Biomed. Biochim. Acta* **49**, S70-S75.

Ahn, J. and Johnstone, R.M. (1991) Intracellular localization of newly synthesized transferrin receptors in the peripheral sheep reticulocyte. *Arch. Biochem. Biophys.* **291**, 154-160.

Ahn, J. and Johnstone, R.M. (1991) Synthesis of the transferrin receptor in peripheral sheep reticulocytes: evidence for incomplete oligosaccharide processing. *Adv. Exp. Med. Biol.* **307**, 3-13.

Ahn, J. and Johnstone, R.M. (1992) The origin of a soluble truncated transferrin receptor. (submitted)

Abstracts

Ahn, J. and Johnstone, R.M. (1987) Incomplete synthesis and plasma membrane translocation of the transferrin receptor in peripheral sheep reticulocytes. Eighth International Conference on Proteins of Iron Transport and Storage, May 10-14, Montebello, Canada, p. 112.

Ahn, J. and Johnstone, R.M. (1988) Hemin stimulates turnover of newly synthesized transferrin receptor in sheep reticulocytes and promotes shedding of pre-existing receptor during reticulocyte maturation. 4th International Congress of Cell Biology, Aug. 14-18, Montreal, Canada, p. 246.

Ahn, J. and Johnstone, R.M. (1990) Incomplete processing of membrane proteins in the sheep reticulocyte. Third European Congress on Cell Biology, Sept. 2-7, Florence, Italy. *Cell Biology International Reports* **14**, 115.

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Abbreviations

anti-cdTfR	antibody to the cytoplasmic domain of the transferrin receptor
ATP	adenosine triphosphate
CMP	cytidine monophosphate
Con A	concanavalin A
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DOH	4,6-dioxoheptanoate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
endo H	endoglycosidase H
endo F	endoglycosidase F/N-glycosidase F
ER	endoplasmic reticulum
FVA	Friend virus-anemia strain
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GT	galactosyltransferase
HA	hemagglutinin
HAO	hemagglutinin precursor
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IRE	iron responsive element
IRE-BP	IRE binding protein
LDL	low density lipoprotein
M6P	mannose-6-phosphate
MAb	monoclonal antibody
MEM	minimal essential medium
MES	2-(N-morpholino) ethanesulfonic acid
MVB	multivesicular body
NBMPR	nitrobenzylthioinosine
NEM	N-ethylmaleimide
NSF	N-ethylmaleimide sensitive factor
PAGE	polyacrylamide gel electrophoresis
PBS	20 mM sodium phosphate, 140 mM NaCl (pH 7.0 unless noted otherwise)

PEG	polyethylene glycol
PES	post-exosome supernatant
PIH	pyridoxal isonicotinoyl hydrazone
PMSF	phenylmethanesulfonylfluoride
POPOP	1,4 bis(5-phenyloxazole-2-yl)benzene
PPO	2,5-diphenyloxazole
SD	standard deviation
SDS	sodium dodecyl sulfate
SIH	salicylaldehyde isonicotinoyl hydrazone
TCA	trichloroacetic acid
TFMS	trifluoromethanesulfonic acid
Tf	transferrin
TfR	transferrin receptor
TGN	trans Golgi network
Tris	Tris(hydroxymethyl)aminomethane
TX-100	Triton X-100
UDP	uridine diphosphate
UTR	untranslated region

Chapter 1 Introduction

The central question under study is what determines the expression or distribution of specific membrane proteins in the compartmentalized cell, i.e. how are these proteins directed? This question arises during protein biosynthesis when nascent proteins are targeted to different destinations, during receptor-mediated endocytosis when receptors are either recycled or degraded, and during membrane remodelling associated with cell maturation when selective proteins are eliminated. Is the information contained in the structure of the protein? How do cellular components process this information? Are accessory proteins, cytosolic factors and/or external factors involved? How is this membrane trafficking and protein sorting regulated? The transferrin receptor (TfR) has been studied by many researchers interested in these questions. The TfR, whose function is to transport iron into the cell, is expressed by many different cells since nearly all cells require iron for the synthesis of a number of important proteins, including hemoglobin, cytochromes, ribonucleotide reductase, and catalase. Its biosynthesis, endocytic pathway, and fate during reticulocyte maturation have been well characterized over the last decade; however, many questions at the molecular level have yet to be resolved.

A model which can be used to approach some of these questions is the maturing reticulocyte. The reticulocyte expresses a high concentration of TfRs but at the same time, its intracellular structure is simplified due to the maturation-associated reduction in ER, Golgi, mitochondria, and lysosomes. For this reason, reticulocytes have been used for the study of endocytosis [1-3] and isolation of clathrin coated vesicles [4,5]. In fact, the existence of TfRs on the cell surface [6] and the recycling of transferrin (Tf) were first proposed for reticulocytes [7]. As the reticulocyte matures into an erythrocyte, there is a

decline in TfR number [8-12] accompanied by an overall remodelling of the plasma membrane and loss of endocytic vesicles. Understanding how TfRs are processed in these cells will give us insight into the fate of other membrane proteins which are known to disappear during reticulocyte maturation. Although it is well known that reticulocytes are active in hemoglobin synthesis, their ability to synthesize and process functional membrane proteins has not been studied in detail. This thesis examines the biosynthetic pathway for membrane proteins in the maturing reticulocyte and the possible roles of iron and heme in TfR expression during reticulocyte maturation.

1.1 Erythroid Cell Differentiation

All the highly differentiated cells in a multicellular organism are derived from a small number of multipotent stem cells, e.g. hepatocytes originate from liver stem cells and nerve cells originate from nerve stem cells. Multipotent stem cells have the choice to either proliferate (undergo self-renewal) or differentiate and become specialized to carry out specific functions. To be defined as a stem cell, the cell must be capable of self-renewal, i.e. to produce an exact copy of itself with the same potential to either proliferate or differentiate. The red blood cell is derived from the bone marrow stem cell (hemopoietic stem cell) which gives rise to all the blood cells in the circulation (Fig. 1). Less than 1% of the cells in bone marrow are multipotent stem cells which continuously divide throughout the lifespan of the animal to replenish the blood cell supply [13]. The remainder are precursors and immature cells of the erythroid and other hemopoietic (white blood cell and platelet) lineages, all of which are derived from multipotent stem cells. Differentiation involves commitment to a specific lineage, reduced proliferative capacity, and the expression of a specific

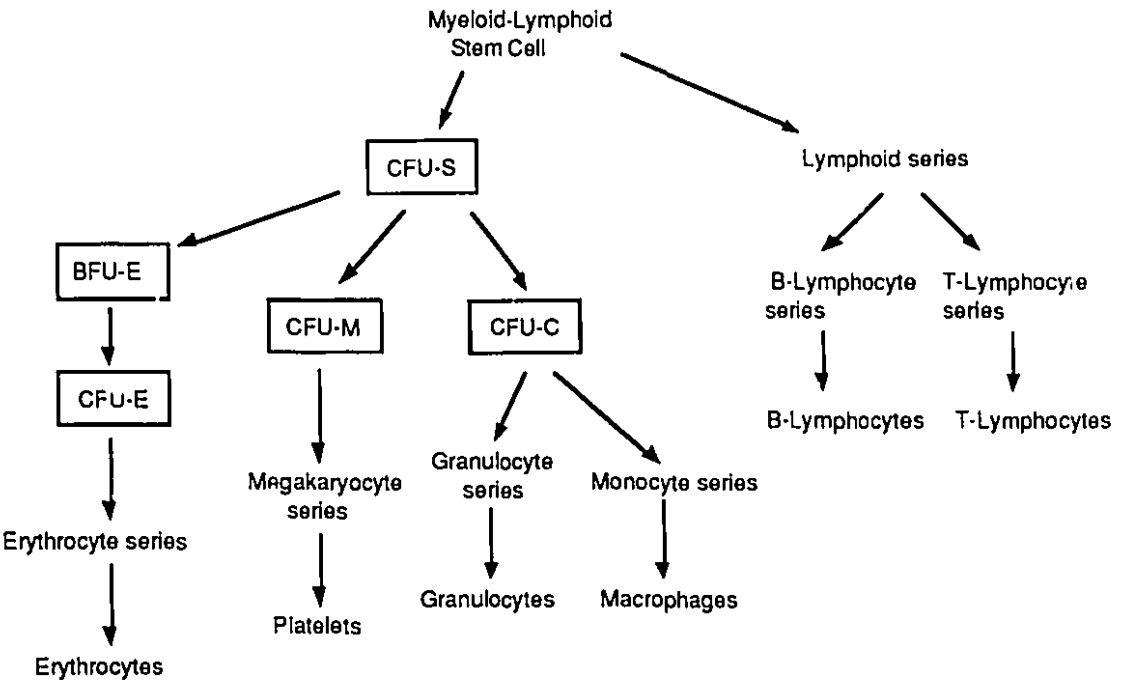


Fig. 1. Model of hemopoietic stem cell differentiation

The cell classes indicated within rectangles can be detected by assays for colony formation. CFU-S, pluripotent myeloid stem cell; BFU-E, early erythrocyte progenitor; CFU-E, late erythrocyte progenitor; CFU-M, megakaryocyte progenitor; CFU-C (also called CFU-GM), granulocyte, monocyte, macrophage progenitor. Taken from Till and McCulloch [13].

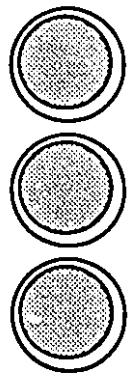
set of proteins. Protein expression is regulated at many levels, including transcription and translation of mRNA, modulation of activity after translation, and protein degradation. The differentiation of bone marrow cells has been studied extensively and serves as a model for the differentiation of other types of stem cells.

1.1.1 Erythropoiesis

Erythropoiesis, or the production of red blood cells, takes place mainly in the bone marrow. Figure 2 outlines the stages of the erythroid cell, a result of studies aided by several models of red cell differentiation (section 1.1.2). The stem cells and committed progenitor cells look similar and therefore cannot be easily identified while the erythroid precursor cells of the next four stages of differentiation have distinct morphological and biochemical characteristics. The method used to identify morphologically unrecognizable progenitor cells is the clonal assay. A single cell when cultured will develop into a colony of several different cells, many of which will have recognizable characteristics but restricted proliferative potential. For example, the colony-forming unit (CFU)-GEMM gives rise to granulocytes, erythrocytes, macrophages and megakaryocytes [16,17]. The earliest cell to be identified by this specific assay was the spleen colony-forming unit (CFU-S) for mouse [13] and mix-CFC (or CFU-GEMM) for man. The burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) are the erythroid progenitors. BFU-Es produce large clusters of 8-30 colonies and CFU-Es give rise to 8-50 erythroblasts after several days in culture [18,19].

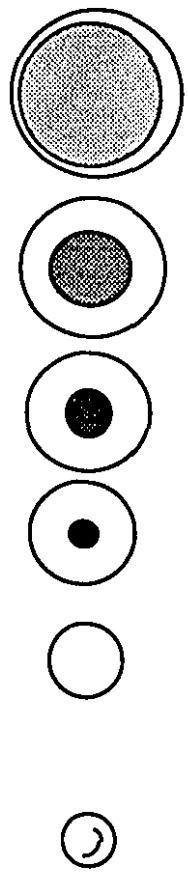
The growth of erythroid progenitor cells depends on several hemopoietic growth factors as well as non-lineage specific growth factors [20].

ERYTHROID PROGENITORS



CFU-S	pluripotent stem cells forming spleen colonies
CFU-GEMM BFU-E	commitment to erythroid lineage
CFU-E	erythropoietin receptors 10^6-10^7 TfRs per cell

ERYTHROID PRECURSORS



pronormoblast	limited proliferative capacity increased globin gene transcription synthesis of glycophorin A and spectrin; $8x10^6$ TfRs per cell
basophilic normoblast	synthesis of hemoglobin, band 3, and band 4.1 $3x10^6$ TfRs per cell
polychromatophilic normoblast	peak of globin gene transcription ~10^6 TfRs per cell
orthochromatic normoblast	peak of heme, globin, and band 3 synthesis loss of nucleus
reticulocyte	~10^5 TfRs per cell; loss of TfR and other membrane proteins synthesis of lipoxygenase cessation of protein synthesis
ERYTHROCYTE	peak of hemoglobin content

Fig. 2. Erythroid Cells

The morphological and biochemical changes associated with erythroid cell differentiation are outlined. Iron uptake is proportional to the number of TfRs in developing erythroid cells [8]. At least 8-10 cell divisions take place between the pronormoblast and reticulocyte stages. Adapted from Koury et al.[14] and Telen [15].

Erythropoietin is made in fetal liver and adult kidney in response to hypoxia. Erythropoietin increases the proportion of committed erythroid precursor cells and accelerates the rate of terminal differentiation. Erythropoietin stimulates mature BFU-Es or CFU-Es (or more mature cells) but not immature BFU-Es or undifferentiated stem cells (CFU-S). Recently, TGFα was implicated in the growth of chicken erythroid progenitor cells [21]. Interleukin-3 and hemin may also play a role in the growth of erythroid progenitor cells (see section 1.1.2).

The morphologically recognizable erythroid precursors are pronormoblast (proerythroblast), basophilic normoblast, polychromatophilic normoblast, orthochromatic normoblast, and reticulocyte (Fig. 2). The erythroid cell, at each stage of differentiation, is identified by its overall size, the size of its nucleus, and the expression of specific proteins. TfRs are synthesized early during erythroid cell differentiation with maximal TfR expression at the pronormoblast stage [8]. Recently, high numbers of TfRs (10^6-10^7 receptors/cell) were found to be expressed on CFU-Es purified from normal human peripheral blood [22] suggesting that TfR synthesis occurs even earlier.

At the orthochromatic normoblast stage, the nucleus is lost (in mammals) or inactivated (in chicken). Electron micrographs of the nuclear extrusion process show the nucleus localized to one side of the normoblast and surrounded by a portion of the plasma membrane which eventually wraps the nucleus completely and separates it from the cytoplasm of what will become the reticulocyte [23]. Since colchicine, a microtubule-depolymerizing agent, increases the number of normoblasts arrested at the extrusion stage, microtubules are probably involved in the process [24]. Some membrane remodelling may take place during enucleation. Most of the sialoglycoproteins and spectrin remain in the reticulocyte [25] whereas concanavalin A binding glycoproteins are enriched in the plasma membrane surrounding the nucleus [26,27]. It has been

suggested that these plasma membrane proteins are involved in the recognition of extruded nuclei by macrophages which leads to degradation of the nuclei. However, membrane protein profiles of erythroblasts, bone marrow reticulocytes and erythrocytes from anemic rabbits show that the major change in the composition of the plasma membrane takes place between reticulocyte and erythrocyte, i.e. after and not during nuclear extrusion [28]. One would expect changes to take place at the cell surface as the reticulocyte is released from the bone marrow environment into the circulation. Loss of the fibronectin receptor and cell adhesion has been associated with the release of reticulocytes from the bone marrow into the peripheral blood [29]. Deformability increases as reticulocytes mature and may also control the release of bone marrow reticulocytes [24,30].

Cytoskeletal structures including microtubules and intermediate filaments (e.g. vimentin) are absent in mature mammalian red blood cells. Vimentin is lost at the early to mid-erythroblastic stage before enucleation [31]. Chicken erythrocytes, however, retain their microtubules (marginal band) and vimentin which could account for their ellipsoidal shape.

Before the maturation of reticulocytes is discussed in detail, a brief description of the models for erythroid cell differentiation will be given since many studies have been carried out using these cells.

1.1.2 Models for the study of erythroid cell differentiation

(a) Normal bone marrow cultures

Normal marrow-derived multipotent stem cells (CFU-GEMM) can be cultured in semi-solid media using methylcellulose, fibrin or plasma clots, or agar to maintain the cells in a suspended state and exposed to the required

concentration of nutrients and growth factors. In order to define and characterize the growth factors and components responsible for colony formation in vitro, it was necessary to develop "serum-free" cultures with a defined nutrient medium, a process which has been difficult with these hemopoietic cell progenitors which have a high serum requirement for optimal growth. In "serum-free" methylcellulose clonal cultures, both interleukin-3 and hemin, but not erythropoietin, were required for colony growth [32]. Hemin also stimulated erythroid colony formation by more mature bone marrow and fetal liver cells (BFU-Es and CFU-Es) [33,34].

Red cell precursors at defined stages of maturation may be obtained with relative ease from the circulating blood of developing chick embryos. At day 4, most of the red cells are polychromatophilic erythroblasts of the primitive series. At day 10, 25% are mature primitive erythrocytes, 35% are mid- to late polychromatophilic erythroblasts of the definitive series and 35% are mature definitive erythrocytes. At day 15, ~75% are mature definitive erythrocytes [35].

(b) Proerythroblasts from virus-infected mice and chicken

Viral infection can enhance the production of a large homogeneous population of erythroid progenitor cells. Proerythroblasts from the spleens of mice infected with the anemia strain of Friend virus (FVA) respond to erythropoietin by synthesizing erythroid-specific proteins, i.e. hemoglobin, spectrin, band 3, protein 4.1, and by increasing TfR expression [36]. Enucleation can occur after 48 hrs [14].

The avian erythroblastosis virus (AEV) induces erythroleukemia in chicken in vivo and transforms chicken erythroblasts in vitro. AEV is a retrovirus carrying the v-erbA and v-erbB oncogenes. v-ErbA blocks

differentiation of chicken erythrocytic progenitors at the CFU-E stage. v-ErbB codes for a mutated form of the EGF receptor which transduces a mitogenic signal even in the absence of EGF. The homologous cellular gene is c-erbB whose product is the receptor for EGF and TGFα. After examining these virus-infected erythroblasts, it was found that normal chick embryo bone marrow cells also express the c-erbB product and require TGFα for growth in culture [21]. The normal cells appeared to be between the BFU-E and CFU-E stages and differentiated into erythrocytes upon the addition of anemic chicken serum.

(c) Erythroleukemia cell lines

1. human (K562, HEL)

K562 and HEL cells are erythroid-like in that they can synthesize hemoglobin and glycophorin A [37]. Induction of hemoglobin synthesis in K562 cells requires the addition of hemin, sodium butyrate, actinomycin D, or hydroxyurea, and takes 4-5 days for maximum hemoglobin accumulation [38]. The induction is reversible and hemoglobin synthesis ceases upon removal of hemin [39]. Unlike normal erythroid cells, hemin-induced K562 cells do not make adult hemoglobin (only embryonic and fetal hemoglobin [$\alpha_2\gamma_2$]), do not express band 3, and do not lose their proliferative capacity. Recently it was found that anthracycline drugs could act synergistically with hemin to promote the terminal differentiation of K562 cells [40]. About 40% of these induced cells developed the morphological characteristics of orthochromatic normoblasts, including decreased proliferation. However, these cells still could not synthesize β-globin chains and therefore do not make hemoglobin A ($\alpha_2\beta_2$). Transforming growth factor-β (TGF-β) also induces hemoglobin accumulation in HEL and K562 cells. When both TGF-β and hemin were added to HEL cultures, a

synergistic decrease in proliferation and increase in glycophorin A expression was observed [41].

2. murine (MEL)

Friend virus transformed erythroleukemic cells [42] are proerythroblast-like cells (CFU-E) which are induced to synthesize hemoglobin and differentiate in the presence of dimethylsulfoxide (DMSO), sodium butyrate, N-methylacetamide, or actinomycin D. In DMSO-treated MEL cells, the enzymes for heme synthesis are fully induced [43] and TfR number and iron uptake increase [44,45]. Intermediate filaments (vimentin) are found in undifferentiated cells but decrease in amount during differentiation with DMSO [31]. Four days after DMSO induction, MEL cells grown in suspension cultures reach the late erythroblast stage (orthochromatic normoblast) and hemoglobin accounts for up to 25% of the protein being synthesized. After 4 days, these cells lyse and therefore do not reach the reticulocyte or erythrocyte stages. MEL cells can be made to differentiate further and even enucleate if they are induced by DMSO on fibronectin-coated dishes [46], in the presence of high concentrations of albumin [47], or in the presence of cytochalasin B [48]. However, only 8% of the induced cells differentiate completely to resemble erythrocytes [46]. Hemin will also induce hemoglobin accumulation in MEL cells [49] but unlike DMSO, hemin by itself will not commit the cells to terminal differentiation.

(d) Reticulocytes from anemic animals

It is very difficult to obtain a homogeneous population of erythroid precursor cells in large quantities from the bone marrow or spleen unless they are first infected with a virus. Erythroleukemia cell lines have the advantage of being easily handled, but these cells, like erythroblasts from FVA-infected mice,

are not normal cells. Of the models described above, only MEL cells and erythroblasts from the spleens of FVA-infected mice terminally differentiate, but only a small fraction of these cells become erythrocytes. Reticulocytes can be obtained in a large quantity from the circulation of animals made anemic by repeated bleeding. Under anemic stress, larger and more immature reticulocytes are released into circulation (which under non-anemic conditions are found in the bone marrow), increasing the reticulocyte count from a few % to as much as 30% of the cells in the peripheral blood. These reticulocytes develop into erythrocytes after two days of culture. The main limitation of using reticulocytes is that they are at the last stage of erythroid differentiation and one cannot study events before this stage. However, for the purpose of studying plasma membrane remodelling, the reticulocyte is an appropriate model, taking into account that considerable changes occur during reticulocyte maturation.

1.1.3 Reticulocyte maturation

As the reticulocyte matures into an erythrocyte, the cell loses all of its intracellular organelles, the protein synthetic machinery, some cytosolic enzymes, and as already mentioned, selected plasma membrane proteins [50]. The mechanisms used by the reticulocyte to rid itself of these functions are proving to be fascinating. The emphasis of earlier studies concerning the expression of proteins (in cells of all types) has been on their synthesis with little attention paid to their turnover. More recently, the selective removal of proteins has been found to play an important role in regulating the number (and hence activity) of cellular proteins. Proteins can be (1) degraded by ATP-dependent and ubiquitin-dependent proteolysis, (2) targeted to lysosomes, (3) degraded in

non-lysosomal compartments (ER?), or (4) extruded from the cell. Each of the these routes uses a different set of proteins and proteolytic enzymes.

(a) Mitochondria and other organelles

There are very few organelles in the mammalian reticulocyte compared to erythroid progenitor cells. Reticulocytes are capable of respiration, synthesis of membrane-bound proteins, and endocytosis which suggest that they have mitochondria, ER, and endosomes [50]. There are also morphological data supporting the existence of residual organelles. Erythrocytes, however, do not have any intracellular membranes. Only the loss of mitochondria has been studied in detail. How the other organelles disappear is largely unknown.

The main mechanism for the loss of mitochondria is degradation by a reticulocyte-specific lipoxygenase [51] and ubiquitin-dependent proteases. Lipoxygenase is a 78 kDa glycoprotein containing non-heme iron and is the major non-globin protein synthesized by the reticulocyte [52]. The lipoxygenase mRNA is transcribed in the normoblast before the loss of the nucleus but the mRNA is masked and not translated until the late reticulocyte stage. Extrusion of mitochondria by maturing reticulocytes has also been reported [53,54].

(b) Protein synthetic machinery

The reticulocyte has proved to be a useful system for the study of translational regulation of protein synthesis, using either intact cells or cell-free lysate. Reticulocytes are responsible for 30-50% of the total hemoglobin synthesis that takes place in immature red cells [55], as well as for the synthesis of lipoxygenase and other proteins. However, the protein synthetic machinery is lost during reticulocyte maturation and erythrocytes do not synthesize proteins.

The total RNA content declines with a half-time of 6-12 h during in vitro maturation of sheep reticulocytes [56]. Initiation and elongation factors also appear to be maturation-dependent. Transfer RNAs appear to be more stable during maturation ($t_{1/2}$ ~50 h) than the other components and probably are not the limiting factors of protein synthesis [50]. Messenger RNAs have different stabilities and could determine which proteins are still synthesized in the reticulocyte. The heme-controlled repressor (HCR) which inhibits initiation of protein synthesis in reticulocyte extracts is also found in mature human and rabbit erythrocytes and has been suggested to play a role in shutting down protein synthesis during reticulocyte maturation [57]. However, no definitive evidence to substantiate this view is available.

(c) Loss of cytosolic enzymes

The glucose consumption and glycolytic enzymes (hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase) decline during reticulocyte maturation [58,59]. The several isoforms of hexokinase (Ia, Ia*, and Ib in rabbit reticulocytes) share similar kinetics, but differ in their intracellular distributions and rates of loss during reticulocyte maturation [60]. Ia and Ib are soluble whereas Ia* is bound to mitochondria. Ia* makes up half of the total hexokinase in reticulocytes but disappears quickly during reticulocyte maturation. Only Ia and a small amount of Ib are present in mature erythrocytes. The decay of soluble but not mitochondrial hexokinase activity was ATP- and ubiquitin-dependent [59]. The Ia* isoform may become a substrate for this proteolytic system as the mitochondria are degraded during reticulocyte maturation. Others [61] found that hexokinase was lost in the absence of ATP and suggested that the lysosomal proteolytic system was involved. Loss of hexokinase and other

cytosolic enzymes (glucose phosphate isomerase, glucose-6-phosphate dehydrogenase) during maturation of sheep reticulocytes was not ATP-dependent [62].

(d) Protein degradation

A nonlysosomal proteolytic system requiring ATP and ubiquitin has been described in reticulocytes [reviewed in ref. 63]. Ubiquitin is a small, heat-stable polypeptide (76 amino acids) which is highly conserved between species and present in all eukaryotic cells. The C-terminal glycine of ubiquitin is covalently attached to the ε-amino group of lysines in proteins to be degraded. The ATP-dependent attachment of ubiquitin to target proteins is catalyzed by three cytosolic proteins: ubiquitin-activating enzyme (E1), ubiquitin carrier proteins (E2), and ubiquitin-protein lyase (E3). Ubiquitin-conjugated proteins are then degraded by cytosolic proteases. Two large cytosolic proteases which degrade both abnormal globins (containing amino acid analogues or puromycin) and normal globin have been purified from reticulocytes [64]. One has a molecular weight of 1500 kDa and is likely to be a multicomponent enzyme system. This protease requires ATP and ubiquitin and is inhibited by hemin and NEM. The second protease has a molecular weight of 670 kDa and is not required for ATP-dependent proteolysis. Liver and muscle cell extracts also contain enzymes which conjugate ubiquitin and degrade ubiquitin-protein conjugates, including a 1500 kDa protease [65]. How proteins are specifically recognized for ubiquitin conjugation is not known.

The level of ATP-dependent and ubiquitin-dependent proteolysis declines during reticulocyte maturation [66]. Erythrocytes contain high levels of ubiquitin and the amount of ubiquitin-activating enzyme (E1) is comparable to

that in reticulocytes. The E2 carrier proteins, however, disappear during reticulocyte maturation and E3 ligase is partially reduced in the erythrocyte [67]. One can restore ATP-dependent proteolysis in erythrocyte extracts by adding E2 and E3. Erythrocytes contain other ligases which attach one or a few ubiquitin molecules, a step requiring E1. Monoubiquitination of histone and certain receptors may modify their function in other cells. The function of monoubiquitinated proteins in erythrocytes is not known. Conjugation of multiple ubiquitins is necessary for protein degradation [63].

(e) Plasma membrane remodelling

During reticulocyte maturation, there is a decrease in the activities of many plasma membrane proteins (Table 1); however, the anion transporter (band 3), glycophorin A, and components of the membrane skeleton are retained by erythrocytes of all species. The different transporters decrease in number as the need for glucose, nucleosides, and amino acids (especially glycine for heme biosynthesis) are reduced in the mature red cell. Na^+-dependent transport of glycine [68,69] and Na^+-independent exchange diffusion of histidine, methionine, and leucine [70] decrease during maturation of sheep reticulocytes. Alanine transport (both Na^+-dependent and independent [71,72]) and Na^+-independent transport of lysine and other cationic amino acids [71] also decrease during reticulocyte maturation but with different schedules. The alanine transporter is lost much more rapidly than the lysine transporter [71]. The nucleoside transporter is lost more slowly [69,72,73,99].

The degrees to which these membrane activities decrease is species-dependent. For example, the pig but not human reticulocyte loses all its glucose transporters. The pig erythrocyte, however, has nucleoside transporters and uses

Table 1. Plasma membrane activities which decrease during reticulocyte maturation

Function	Ref.
transferrin receptor	10-12
amino acid transporters	68-72
nucleoside transporter (NBMPR binding)	69,72,73
insulin receptor	74,75
glucose transporter	76.77
Na,K-ATPase (ouabain binding)	69,78,378
β-adrenergic receptor	79,80
fibronectin receptor	81
acetylcholinesterase	82

There is a species difference in the loss of certain functions. Human reticulocytes retain most of their nucleoside and glucose transporters. Pig reticulocytes also retain their nucleoside transporters. There was no change in acetylcholinesterase activity during maturation of rabbit reticulocytes [378].

nucleosides as an energy source [83]. The TfR is a membrane protein which is lost by reticulocytes of all species [8-12]. In immature red cells, the TfR is essential for hemoglobin production, but as the cells mature, the need for new iron disappears and the loss of this protein during maturation thus avoids cellular accumulation of excess iron which can be toxic. The basis for the selectivity during membrane remodelling is not known but any mechanism would have to take into account this selectivity. The differences in quantitative losses between species would depend on species differences in the proteins or the cellular machinery which process these membrane proteins.

1.1.4 A mechanism for the loss of membrane proteins during reticulocyte maturation

Early studies have proposed that internalization of the cell membrane is associated with remodelling of the plasma membrane during reticulocyte maturation [84-86]. Spectrin-free invaginations and vesicles formed in the maturing reticulocyte and it was suggested that these vesicles are exocytosed with the help of the spleen [25]. With the characterization of proteolytic systems in reticulocytes, some investigators have suggested that lysosomal proteases or ATP- and ubiquitin-dependent proteolysis play a role in the loss of proteins during reticulocyte maturation. However, no one has, to date, directly shown that membrane proteins are degraded intracellularly by either of these two proteolytic systems.

(a) TfRs are externalized in vesicular form

Pan et al. [12] raised reticulocyte-specific antibodies in order to follow the loss of membrane proteins during reticulocyte maturation. Rabbits were injected

with sheep reticulocytes and the antiserum was absorbed with mature sheep cells. The major antigen, present on the reticulocyte surface but not on the erythrocyte, was identified as the TfR.

The TfR is a transmembrane protein which is lost by reticulocytes of all species and is a useful marker for the study of reticulocyte maturation. Both functionally active (Tf binding) and structurally intact (antibody binding) receptors can be measured. The fate of the TfR was followed during in vitro maturation of sheep reticulocytes and the protein was shown to be externalized from the maturing reticulocyte in 50 nm vesicles [87,88], later named exosomes. Rather than budding directly from the cell surface, these exosomes were formed intracellularly in multivesicular sacs and were released after fusion of the sacs with the plasma membrane (Fig. 3). Harding et al [89,90] have also demonstrated a pathway for receptor shedding in rat reticulocytes. The rat vesicles could also be retrieved by centrifugation of the cell-free medium and were similar in size to those observed in the multivesicular endosomes (62nm ±11 SD).

(b) Exosomes contain several proteins

Exosomes contain two prominent proteins in equal amounts detectable by Coomassie blue staining of electrophoretic gels. One protein, the TfR, migrates as two bands (the reduced monomer at 94 kDa and the residual dimer at 190 kDa). The same pattern is seen when immunoprecipitates of cellular TfR are run on reducing gels. The cellular and exosomal TfRs have identical [^{125}I]iodotyrosyl maps [91] and bind transferrin with similar affinities. The only difference found to date between the cellular and exosomal TfRs is that the exosomal TfR is a poor substrate for endogenous kinases or exogenous protein kinase C [92]. TfR immunoprecipitates prepared from reticulocytes, but not

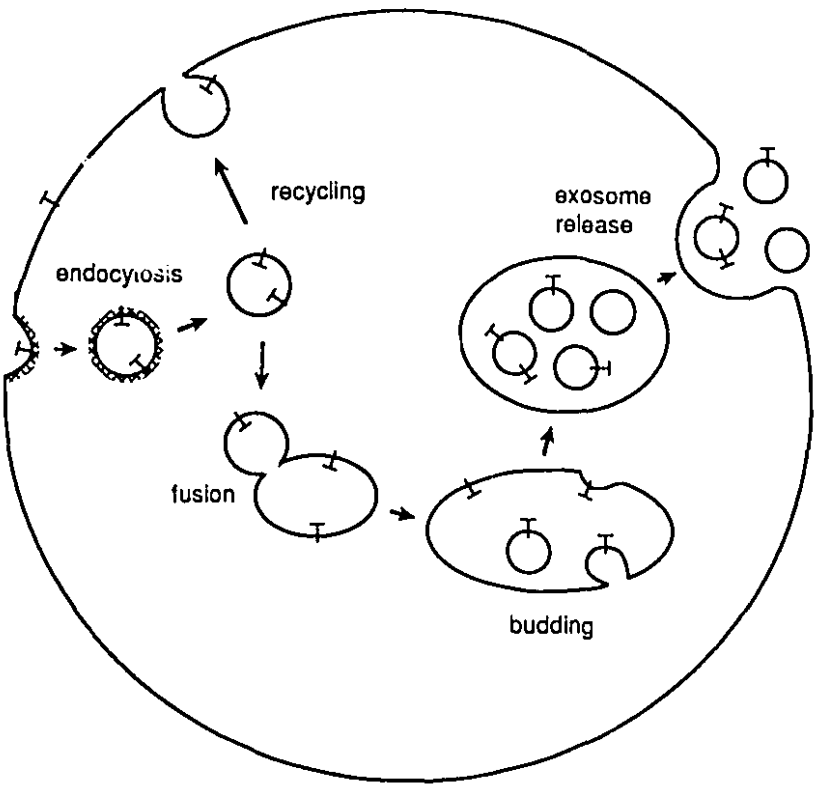


Fig. 3. Externalization of the TfR in vesicles

After internalization of the TfR (T) in coated vesicles, the clathrin dissociates, resulting in an uncoated vesicle (also called an endocytic vesicle). Instead of recycling to the cell surface, the uncoated vesicle fuses with other vesicles to form a large endosome. Vesicles form inside the endosome by budding from the endosomal membrane. When this multivesicular body (MVB) fuses with the plasma membrane, its contents (exosomes) are released. Note that the TfR in the exosome has its extracellular domain oriented outward.

immunoprecipitates from exosomes, could be phosphorylated by protein kinase C. This observation led to the hypothesis that a structural change in the N-terminal domain (containing the protein kinase C phosphorylation site) could act as a signal for routing these TfRs into multivesicular bodies for their subsequent externalization [92].

The other major protein found in the exosomes is a 71-72 kDa doublet which has been identified as the clathrin uncoating ATPase from its immunological properties, iodotyrosyl peptide map and ATPase activity [93]. The 71 kDa polypeptide is probably a cleaved product of the 72 kDa polypeptide and the 71-72 kDa doublet will be referred to as the 70 kDa protein. Unlike the TfR, this protein is not exposed at the exosome surface and is not labelled after surface iodination. As its name implies, the clathrin uncoating ATPase dissociates clathrin from coated vesicles following receptor-mediated endocytosis [94]. It is a member of the 70 kDa family of heat shock proteins (HSP70s) which behave as molecular chaperones by binding to unfolded proteins and preventing undesirable interactions or disaggregating abnormal complexes to allow proper refolding [95]. HSP70s may be involved in the translocation of nascent proteins into the ER and mitochondria during their biosynthesis [96]. Although the 70 kDa protein does not co-immunoprecipitate with the TfR, it responds similarly to agents which increase or decrease TfR release from cells.

The exosomes contain other proteins but in lesser amounts. Activities which have been detected in the exosomes are phosphatase, cholinesterase, glucose transport, nucleoside transport, and amino acid transport [77]. All of these plasma membrane activities are known to diminish during reticulocyte maturation (Table 1). Lysosomal enzyme activities were also found in the exosome fraction. Enzymes of cytosolic origin (lactate dehydrogenase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, 6-

phosphogluconic acid dehydrogenase) or mitochondrial enzymes (succinate dehydrogenase, monamine oxidase) were not detectable in the exosomes. A report has appeared suggesting that partially degraded mitochondria are extruded during maturation of rat reticulocytes [54]. These membranous components were centrifuged at 12,000*g* and are distinct from the 100,000*g* pellet (exosomes) containing smaller and more uniform vesicles.

The question was asked whether a homogeneous population of vesicles is released during reticulocyte maturation, i.e. whether all these activities are present in the same exosome or different exosomes carry different activities. The activities recovered in externalized vesicles are lost from the cell at different rates, e.g. most of the TfR is externalized in 24 h whereas NBMPR binding sites and lysosomal activities are lost more slowly with ~50% of the activity remaining in the cell after 24 h of culture [98,99]. Therefore, exosomes produced at earlier times would be expected to contain more TfRs and less NBMPR binding sites. Lysosomal enzymes which have a cellular site of origin different from that of plasma membrane proteins might be packaged separately. This was shown to be the case when magnetic beads coated with mouse anti-TfR antibody were used to separate exosomes containing TfRs from those which did not contain TfRs [97]. Half of the externalized NBMPR binding sites were in TfR-containing exosomes. The externalized lysosomal activities (N-acetyl-β-glucosaminidase, β-glucuronidase), however, were absent from the TfR-containing exosomes. The 70 kDa protein was also present in the TfR-containing exosomes. These results show that different exosomes contain a different mixture of proteins which are shed during reticulocyte maturation.

(c) Exosome release requires ATP and elevated temperature

Exosome formation and TfR release by reticulocytes during maturation is dependent on energy and temperature [91,98]. When reticulocytes are cultured in the presence of metabolic inhibitors (NaCN, NaF, Na_2AsO_4) which cause depletion of cellular ATP, or at 4°C, externalization of TfRs is inhibited. Alkalinizing reagents such as chloroquine and methylamine which inhibit endocytosis also reduce exosome formation [91].

(d) Exosomes are found in vivo in several species

TfR-containing vesicles have been found in the circulation of anemic animals of several species, strongly suggesting that this is a process which occurs in vivo [99]. The presence of the characteristic multivesicular sacs and/or circulating exosomes has now been demonstrated for nine species of immature red cells from anemic animals, including man [100]. The anemias in which the sacs and/or vesicles were detected were naturally occurring (special breeds of naturally anemic dogs and cats, newborn piglets, and embryonic chicken), or induced by phlebotomy (rabbit, sheep, rat and man) or phenylhydrazine treatment (rat and guinea pig). These observations indicate that exosome formation is not due to excessive bleeding and the formation of "stress reticulocytes".

While mammalian reticulocytes lose all their organelles during maturation, chicken red cells retain their nuclei and mitochondria. The fact that embryonic chicken reticulocytes also shed the TfR in vesicles in vivo and in vitro suggests that plasma membrane remodelling occurs independently of the mechanism for the loss of intracellular organelles [99].

(e) Fate of vesicular TfR and release of a soluble truncated TfR

The fate of the exosomes after release from the cell is not known, but the presence of truncated TfRs in the circulation [101,102] has raised the possibility that TfRs are cleaved from the exosomes following their release from maturing erythroid cells. The level of serum TfRs is increased in hemolytic and iron-deficiency anemias and may be used clinically to assess erythropoiesis [103,104].

1.2 Synthesis of Membrane Proteins

Plasma membrane proteins, secretory proteins, and resident proteins of the ER, Golgi, and lysosome, share a common biosynthetic pathway before they are sorted and localized to their respective membrane compartments. While membrane proteins remain anchored to the membrane, secretory and lumenal proteins lose their membrane anchor.

1.2.1 Translocation of nascent proteins into the ER membrane

Membrane proteins have a somewhat hydrophobic sequence (usually ~15 amino acids) which signals their insertion into the ER membrane [reviewed in ref. 105]. This sequence was originally found on the N-terminus of membrane and secretory proteins and called a signal sequence. As soon as this sequence is translated, the signal recognition particle (SRP) binds to it and causes a temporary arrest in translation of the remainder of the protein. The SRP-mRNA-ribosome complex attaches to the SRP receptor (docking protein) in the ER membrane and the SRP dissociates from the signal sequence of the nascent protein. A major ER protein which can be crosslinked to the signal sequence of the nascent protein has been identified as the signal sequence receptor (SSR [106]). The SSR has been suggested to be a component of the protein conducting channel which mediates translocation of the nascent polypeptide across the ER

membrane. SRP-independent translocation requiring hsp70 is known to occur [107].

Integral membrane proteins can be classified into four types depending on their topology and mode of insertion into the ER membrane (Fig. 4). The nascent protein may be inserted as a hairpin loop structure with both N and C termini in the cytoplasm [109]. The transmembrane domains of membrane proteins which span the lipid bilayer several times may act as start or stop sequences, mediating translocation into the ER membrane. The majority of proteins are inserted into the ER cotranslationally. An exception is the erythrocyte glucose transporter which spans the membrane up to 12 times and can be synthesized on cytosolic ribosomes and inserted into the ER posttranslationally in vitro [110].

Processing of the nascent protein is cotranslational. As soon as the protein is inserted into the ER, high-mannose oligosaccharides are transferred from dolichol precursors to asparagine residues in the polypeptide and protein folding commences. An ER lumen protein, signal peptidase, cleaves the signal sequence of type I proteins.

1.2.2 Processing in the ER

Processing, which normally takes place in the ER, includes the acquisition of function, proper folding, disulfide bond formation, and subunit assembly. The ER is also the site of glucosidases and mannosidases which remove the glucoses and one mannose residue from each oligosaccharide chain (Fig. 5). The receptors for EGF, insulin, and acetylcholine appear to have a common mechanism for the acquisition of ligand-binding function which involves rearrangement of disulfide bonds formed during or immediately after translation

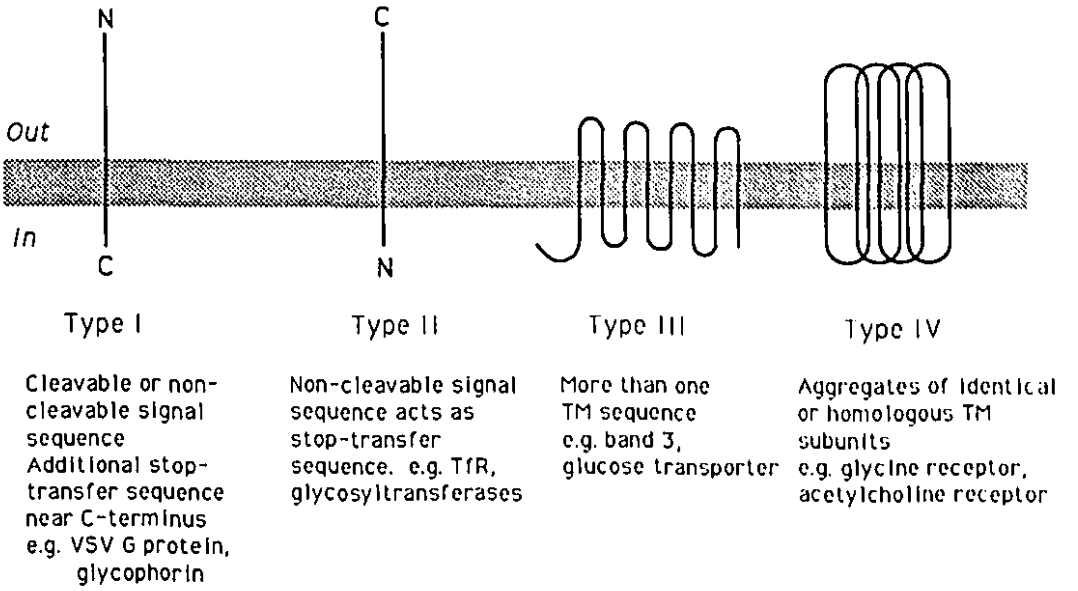


Fig. 4. Insertion of membrane proteins into the ER membrane

These are the four types of integral membrane proteins as proposed by Singer et al.[108]. Type I and type II membrane proteins have a single transmembrane (TM) sequence, but differ in the orientation of their N (amino) and C (carboxyl) termini. Type III proteins may have their N or C termini on either side of the membrane. *Out*, extracellular or lumenal face of membrane; *In*, cytoplasmic face.

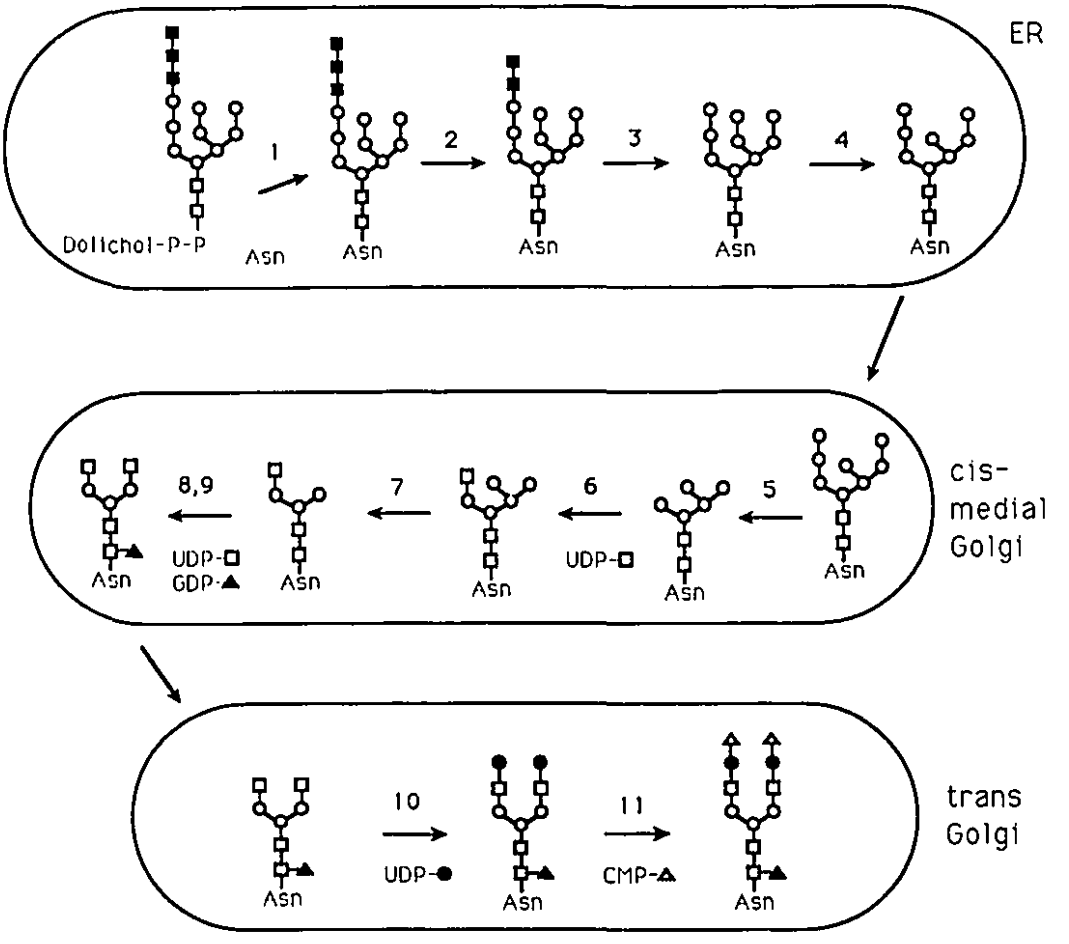


Fig. 5. Processing of asparagine-linked oligosaccharides

This schematic diagram shows an example of oligosaccharide processing. Key: ❑, N-acetylglucosamine; O, mannose; ■, glucose; ●, galactose; Δ, sialic acid; ▲, fucose; (1) oligosaccharyltransferase; (2) α-glucosidase I; (3) α-glucosidase II; (4) ER α-1,2-mannosidase; (5) Golgi α-mannosidase I; (6) N-acetylglucosaminyltransferase I; (7) Golgi α-mannosidase II; (8) N-acetylglucosaminyltransferase II; (9) fucosyltransferase; (10) galactosyltransferase; (11) sialyltransferase. Adapted from Kornfeld and Kornfeld [111].

The following inhibitors of oligosaccharide processing are known to act at the site(s) indicated in parentheses: deoxynojirimycin (2: glucosidase I), castanospermine (2,3: glucosidases I and II), deoxymannojirimycin (5: mannosidase I), swainsonine (7: Golgi mannosidase II).

[112]. For insulin and nicotinic acetylcholine receptors, acquisition of ligand binding function takes up to 45 min and precedes subunit assembly. The TfR also acquires the ability to bind transferrin in the ER [113]. Proteins must be properly folded and assembled before they can exit the ER [114-121]. For example, secretion of heavy chains of immunoglobulins requires the binding of light chains before leaving the ER [118]. An ER lumen protein, BiP (immunoglobulin heavy chain binding protein) binds unassembled heavy chains and is released when two heavy chains and two light chains assemble [119]. BiP is identical to grp78, a protein which is induced by glucose starvation and inhibits glycosylation. BiP also interacts with unglycosylated proteins which are frequently misfolded. In virus-infected cells, the viral proteins, hemagglutinin and G protein, must form homotrimers before leaving the ER. BiP can also associate with hemagglutinin monomers and retain them in the ER until trimerization [115,120,121].

1.2.3 The Golgi apparatus

Proteins with the proper tertiary and quaternary structure are transported to the Golgi where they are further processed, concentrated, and sorted. Transport from the ER to the Golgi and through the Golgi compartments is mediated by nonclathrin coated vesicles and requires cytosol and GTP [122]. An N-ethylmaleimide-sensitive fusion protein (NSF) [123,124], SNAPs (soluble NSF attachment proteins) [125] and small GTP-binding proteins (the rab proteins [126]) are also involved in vesicular transport. These components were identified by using cell-free assays to dissect the steps involved in transporting newly synthesized proteins through the Golgi cisternae. Transport between the ER and Golgi and from the Golgi to the plasma membrane have also been

reconstituted in vitro or in semi-intact cells where the cell is broken gently so that it retains the intracellular structure while allowing soluble factors to pass freely through the perforated cell membrane.

In the Golgi complex some mannose sugars are removed and sugars such as galactose and neuraminic acid are attached to the oligosaccharides. These processed oligosaccharides are resistant to endoglycosidase H (Fig. 5). Other post-translational modifications like O-linked glycosylation, acylation, phosphorylation, and sulfation are known to occur in the Golgi apparatus. Some proteins may undergo conformational changes during transport through the Golgi. For example, an increase in accessibility of the cytoplasmic domain of the G protein to proteases occurs in the trans Golgi after trimerization and may be of functional significance [127].

1.2.4 Sorting of newly synthesized proteins

Newly synthesized proteins with different destinations must be distinguished from each other as well as from resident ER and Golgi proteins. Examples of proteins which are sorted are lysosomal enzymes, lumenal ER and Golgi proteins, constitutive and regulated secretory proteins, and apical and basolateral plasma membrane proteins.

The general belief is that most proteins are transported by bulk flow (default route) to and from the plasma membrane. The rate of bulk flow from the ER to the cell surface is fast enough to explain the rapid appearance of newly synthesized proteins at the cell surface. Proteins are selectively sequestered from the constitutive pathway by a signal which is dictated by the structure of the protein. It appears that all the information pertaining to the protein's destination is encoded in the primary structure of the protein. The unravelling of sorting

signals and the machinery which translates these signals into actions is important for understanding how a cell functions. Several sorting signals are emerging but very little is known about the mechanism of sorting.

Lysosomal enzymes are usually phosphorylated on terminal mannoses. Specific receptors which recycle between the trans Golgi and prelysosomal compartment recognize proteins with the phosphorylmannose and target the protein to the lysosome. There are two different mannose-6-phosphate receptors, a 275 kDa cation-independent M6P receptor which also binds insulin-like growth factor II (IGF-II) and a 46 kDa cation-dependent M6P receptor [reviewed in ref. 128].

Soluble ER proteins (e.g. the glucose-regulated proteins grp78 [BiP] and grp94, and protein disulfide isomerase) have a Lys-Asp-Glu-Leu (KDEL) sequence on the carboxy-terminus which is required for retention in the ER (HDEL in yeast). Deletion of this sequence from these proteins resulted in their secretion [129]. Addition of the KDEL sequence to the carboxy-terminus of a secreted protein, lysozyme, resulted in its retention in the ER [129]. Since ER lumenal proteins have undergone modifications by enzymes which are localized in the Golgi, these proteins are first transported to the Golgi [130], then return to the ER via a putative recycling KDEL receptor [131] or the ERD2 gene product in yeast [132]. A second mechanism for the retention of proteins in the ER against a bulk flow is the retention of unfolded and incompletely assembled proteins in the ER (section 1.2.2 above). Here, the tertiary or quaternary structure of the protein acts as the signal for ER retention.

Less clear is the nature of the signal for retention of resident Golgi proteins. The membrane spanning domain (10 amino acids of the lumenal half) of β-1,4-galactosyltransferase was sufficient to localize most of a hybrid invariant chain (normally found in endocytic compartment and cell surface) to

the *trans*Golgi [133]. In addition, a cytoplasmic domain (either galactosyltransferase or invariant chain) was required for complete retention. In another study, replacement of the transmembrane domain of galactosyltransferase with that of the TfR resulted in transport of the hybrid protein to the cell surface [134]. These results indicate that the membrane spanning domain is required for Golgi retention.

Secretion of proteins may be constitutive or regulated. Regulated secretion involves transport of proteins from the Golgi to secretory storage vesicles and appears to be signal-dependent. There are two models for the sorting of constitutive and regulated secretory proteins. The receptor model is analogous to the targeting of lysosomal enzymes. A protein or sortase (like the mannose-6-phosphate receptor) binds secretory proteins in the TGN and targets them to a pre-secretory vesicle. The sortase then returns to the TGN. However, no sortase or pre-secretory vesicle has been found. There is some evidence for the second model in which regulated secretory proteins are selectively aggregated in the TGN. A decrease in pH and increase in calcium were sufficient to trigger the selective aggregation of granins in the TGN [135]. The granins were thus segregated from the constitutive secretory proteins. If there are factors responsible for this aggregation, they have not yet been found.

The apical and basolateral surfaces of a polarized epithelial cell may be considered as two different membrane compartments. Basolateral membrane proteins are transported directly to the basolateral surface from the TGN. However, the localization of apical membrane proteins appears to depend on the type of cell [136]. Apical proteins may be transported directly to the apical surface (usually in kidney) or they may first be inserted into the basolateral membrane, then transported to the apical surface by transcytosis (in hepatocytes). Both direct and indirect pathways may exist in other cells, i.e. CaCo2 cells.

Once these proteins have reached the correct surface, some proteins may be stabilized by association with the cytoskeleton or basement membrane, thus membrane polarity is maintained. Delivery to both membrane domains may be signal-mediated. There is evidence that targeting of apical proteins depends on a lumenal signal [137].

It becomes apparent that the number of possible sites of regulation of the biosynthetic pathway for secretory and membrane proteins is great compared to that for cytosolic proteins. Although cytosolic proteins must also fold properly, assemble, and acquire post-translational modifications to become functional, these events take place in the cytoplasm without the need for transport between membrane compartments. Membrane proteins in the ER must fold properly and subunits must often assemble (in the case of multisubunit proteins) before they are transported out of the ER. Once the proteins have been transported to the Golgi, they must be modified and sorted correctly to be expressed at their target sites. If these requirements are not met, the newly synthesized protein is held up in an intracellular pool or degraded in a nonlysosomal compartment [138].

1.3 Receptor Mediated Endocytosis

1.3.1 Internalization and sorting of receptors

Cells take up certain nutrients and respond to growth factors by binding these ligands to specific cell surface receptors and internalization of the receptor-ligand complexes. Toxins and viruses also gain entry into the cell by receptor-mediated endocytosis.

(a) Clustering into clathrin-coated pits

While the binding of some ligands (epidermal growth factor [EGF], insulin) to their receptors induces clustering and internalization of the ligand-receptor complex, others like the TfR and low density lipoprotein receptor (LDL-R) cluster in coated pits and internalize in the absence of ligand [139-143]. Coated pits occupy 1-2% of the total plasma membrane area but may contain over 10% of the cell surface receptors [144] (>50% of LDL-Rs on fibroblasts). Although TfRs do not induce the formation of coated pits [145], they may promote the formation of lattices (possible precursors to coated pits) since the number of lattices increased when the TfR was overexpressed in fibroblasts [146].

(b) Internalization

Internalization of the receptor-ligand complex from the coated pit is rapid (minutes) and requires ATP [139,147,148]. Comparison of the cytoplasmic domains of several constitutively endocytosed receptors has revealed that the signal for rapid internalization may consist of a 4-6 amino acid sequence containing an aromatic amino acid and a bulky hydrophobic amino acid (e.g. $Y_{20}XRF_{23}$ in the TfR) [149-151]. Equivalent signals from the LDL and mannose-6-phosphate receptors could be transplanted into the TfR to promote the rapid internalization of the TfR in chicken embryo fibroblasts [152]. However, the tyrosine internalization signal from the LDL receptor did not cause rapid internalization of the TfR when expressed in CHO cells, making it possible that the signal for internalization consists of as many as 27 amino acids in the cytoplasmic domain of the TfR [153].

Following internalization, the clathrin dissociates from the coated vesicles and the endocytic vesicle containing the receptor-ligand fuses with the early

endosome (Fig. 6). The majority of receptor-ligand complexes dissociate due to the decrease in pH.

(c) Sorting of receptors and ligands in the endosome

From the early endosome, several routes are possible: (1) Some receptors (EGF-R, a portion of insulin receptors) and their ligands are targeted to the lysosome (or some other compartment) where they are both degraded [154,155]. (2) Other receptors (LDL-R, asialoglycoprotein receptor) recycle to the cell surface while only their ligands are transported to the lysosome [156,157]. (3) Two ligands (Tf and hemopexin) remain bound to their receptors while the iron (of Tf) or heme (of hemopexin) is released in the endosome. Both the receptor and ligand recycle to the cell surface where the ligand is released and both proteins are re-utilized [157,158-165]. Recycling receptors can travel between the cell surface and endosome many times and deliver large amounts of ligand.

A fraction (5-15%) of some recycling receptors (TfR, M6P receptor, asialoglycoprotein receptor) may be transported to the Golgi complex before returning to the plasma membrane. Cell surface TfRs which have been synthesized in the presence of deoxymannojirimycin (inhibitor of mannosidase I) and therefore lacked endo H-resistant sugars acquired terminal sugars when the cells were re-incubated in the absence of inhibitor [166], suggesting that these TfRs recycled through the *trans*Golgi. Asialotransferrin was taken up and resialylated [167]. There are also electron microscope and cell fractionation studies to support the view that the Golgi or transGolgi network (TGN) are part of the cell surface receptor recycling pathway [168]. Some TfRs may pass through multivesicular bodies (MVBs) during their recycling to the cell surface

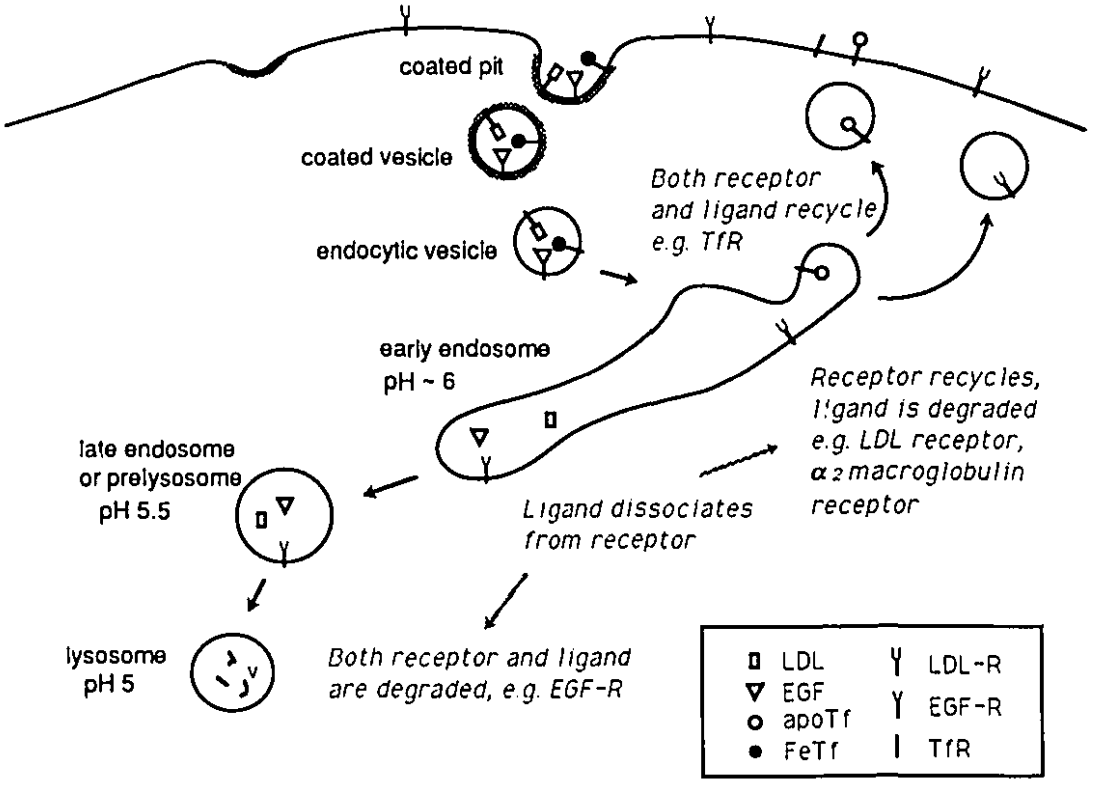


Fig. 6. Schematic diagram showing the routes taken by receptors and their ligands during receptor-mediated endocytosis

[89]. These alternate pathways may be involved in regulating the distribution of receptors at the cell surface and their recycling time.

It appears that the recycling pathway is the default route and receptors, like the EGF receptor, which are degraded inside the cell possess a lysosome targeting signal. The targeting of the EGF-R to lysosomes requires kinase activity since a kinase-negative mutant recycles to the cell surface for re-utilization [169]. Unoccupied EGF receptors also undergo recycling. When specific antibodies or crosslinked ligands are added to surface receptors which are normally recycled, the receptors form dimers which are endocytosed and degraded inside the cell. When K562 cells were exposed to monoclonal antibodies, TfRs were redistributed to intracellular sites where they were degraded [170]. TfRs in mouse lymphoma cells also responded to antibodies by decreasing cell surface expression and degrading TfRs [171]. Therefore, recycling of receptors appears to be the default route and those ligands and receptors which possess a signal (e.g. receptor divalency, kinase activity) are targeted for degradation.

1.3.2 Iron Uptake

Iron is required by all cells since it is a component of many important enzymes, including ribonucleotide reductase, cytochrome oxidase, peroxidases, succinic dehydrogenase, aconitase and xanthine oxidase. Iron is required for heme which itself could have regulatory functions (section 1.4).

Iron (Fe^{2+} the form used by cells) is never found free in solution at neutral pH since it would get oxidized to Fe^{3+}. The latter would be rapidly hydrolyzed or precipitated, forming large complexes that are toxic to cells. When not incorporated into heme or an enzyme (e.g. list above), iron is bound to

Tf, chelated by low molecular weight compounds such as citrate and ATP, or stored in ferritin. Plants and bacteria take up iron by absorption of chelated iron [172]. In mammals, the iron transport protein, transferrin, carries Fe^{3+} in the serum and interstitial fluid. Transferrin contains two high affinity iron-binding sites which are independent of each other and have slightly different affinities for Fe^{3+}. Bicarbonate or some other anion is required for Fe^{3+} binding. Iron binding causes a change in the Tf molecule, making it extremely stable and resistant to denaturation. Diferric Tf also binds more strongly to its receptor than apotransferrin [173,174].

Although reticulocytes may take up iron from Tf-bound to Sepharose, suggesting that Tf does not have to enter the cell in order to deliver its iron [175,176,86], the established means of iron uptake involves TfR recycling (Fig. 6). There are many microscopic (fluorescent and EM) and biochemical studies showing that both Tf and its receptor are internalized during iron delivery [158-164,177].

(a) Recycling of the TfR

The advantages of transporting iron after endocytosis of FeTf over direct transport of Fe across the plasma membrane should be considered. The process of clustering and internalization of TfRs concentrates the FeTf in coated pits and results in compartmentalization of the Tf-TfR in a different environment, i.e. an endosome with a pH lower than that of the extracellular milieu. The lower pH plays a significant part during iron delivery [174,178-180] and weak bases (ammonium chloride or chloroquine) block iron uptake [147,181]. The TfR appears to have a pH-dependent conformation which alters its affinity for Tf. At pH 7.4, the affinity of the receptor for FeTf is higher than that for apoTf.

However, at pH 6.0, Fe is released from Tf and apoTf binds more tightly to the receptor than at pH 7.4 [173,179]. Therefore, after Fe delivery, apoTf remains bound to the receptor inside the endosome and recycles to the cell surface where it is replaced by FeTf [89,160]. Fe transport across the endosome membrane could also depend on acidification [181].

(b) Release of iron and transport across the membrane

When the Tf-TfR complex reaches an endosome of low pH (<6.5), the iron dissociates from Tf. The release of Fe is an early step in the Tf cycle [182], occurring within one minute of internalization in reticulocytes [147]. In addition to transporting the FeTf inside the cell, the TfR appears to have a role in Fe release. At pH 7.4, Fe binds more tightly to Tf in the presence of TfR, but at low pH, Fe is released 5 times more rapidly from Tf-TfR than from Tf [183]. Therefore, at the cell surface, binding of FeTf to its receptor secures the Fe in place until it is transported to the endosome, where due to low pH, the Fe is efficiently released while the apoTf remains bound to its receptor.

After it is released from Tf, the iron must be transported across the endosome membrane into the cytosol, then transported into the mitochondrion for heme synthesis. Not much is known about the steps between the release of iron from Tf and the incorporation of the iron into heme or ferritin. At some point, the iron must be reduced and bound to a chelator. It has been proposed that a reductase/channel in the endosome membrane translocates the iron across the membrane [184]. Receptors for FeATP have been found on the mitochondria [185]. Calcium which is required for iron uptake [186,187] may increase transmembrane iron transport by a mechanism dependent on membrane fluidity [187].

(c) Tf-independent uptake of iron

Cultured cells can oe grown in serum-free medium containing ferrous sulfate or ferric citrate [188-190]. Iron may be taken up by a saturable transport system [188] or by non-specific processes such as fluid endocytosis [191]. Normally, less than 1% of the plasma iron is not bound to Tf. However, the liver may extract iron more efficiently from non-Tf-bound iron (e.g. Fe-ascorbate) and these sources of iron may be important in patients with iron overload where as much as 35% of the plasma iron is not bound to Tf [189].

1.3.3 Regulation of Iron Uptake

(a) Regulation of TfR number by iron

In growing cultured cells, TfR expression is increased by intracellular iron chelators (picolinic acid or desferrioxamine) [192-195] and decreased by iron sources ($Fe(NH_4)$citrate or hemin) [196-199]. The effect of iron on TfR number is through protein synthesis and is accompanied by changes in the level of TfR mRNA. There is a 3-fold difference in transcription of TfR mRNA in cells treated with hemin or desferrioxamine, but this cannot account for the 25-fold difference in mRNA levels observed in these cells [202]. It appears that intracellular iron influences the stability of the TfR mRNA by modulating the binding of a cytoplasmic protein (IRE-binding protein [IRE-BP] or iron regulatory factor [IRF]) [200,201] to iron-responsive elements (IREs) in the mRNA [202]. Iron chelators stimulate binding of the IRE-BP to mRNA 25-fold and stabilize the TfR mRNA whereas iron salts decrease mRNA binding and increase degradation of the TfR mRNA [201]. IRE-BP may be the first example of a repressor of mRNA degradation. In addition to the five

homologous palindromic sequences (IREs), a stem-loop structure in the 3' untranslated region (UTR) of the TfR mRNA is necessary for the regulation of TfR mRNA by iron [203].

The iron storage protein, ferritin, is known to be upregulated by iron at the translational level. An IRE similar to the five IREs of TfR mRNA is found in the 5' UTR of ferritin mRNA [204] and can compete with the TfR mRNA for the binding of IRE-BP. Therefore, the synthesis of two metabolically related proteins, ferritin and TfR, is regulated in a concerted manner (Fig. 7) [205]. Low intracellular iron concentration stimulates high affinity binding of IRE-BP to the 3' UTR of TfR mRNA and to the 5' UTR of ferritin mRNA. This stabilizes TfR mRNA so that it is translated into protein which will increase the transport of FeTf into the cell. Binding of IRE-BP inhibits translation of ferritin mRNA. At high intracellular iron concentrations, binding of IRE-BP is prevented, thus TfR mRNA is degraded and TfR synthesis ceases. At the same time, ferritin mRNA may be translated into protein which will be available for storage of excess intracellular iron. Recently, an IRE was found in the 5' UTR of erythroid δ-aminolevulinate (ALA) synthase [206], an enzyme required for heme synthesis. Therefore, the IRE mechanism may regulate iron utilization in erythroid cells by controlling the translation ALA synthase [207] in addition to regulating iron uptake and storage.

Cloning of the human IRE-BP cDNA has revealed 30% amino acid identity with the Krebs cycle enzyme aconitase from mitochondria and conservation of all 18 active-site residues [208,209]. The 98 kDa IRE-BP may be the cytosolic aconitase since purified IRE-BP also had aconitase activity [210]. The crystal structure of aconitase has been solved and may provide clues for the iron-sensing mechanism of IRE-BP. Aconitase contains a 4Fe-4S cluster which can undergo a reversible conversion to 3Fe-4S (inactive form) in vitro. IRE-BP

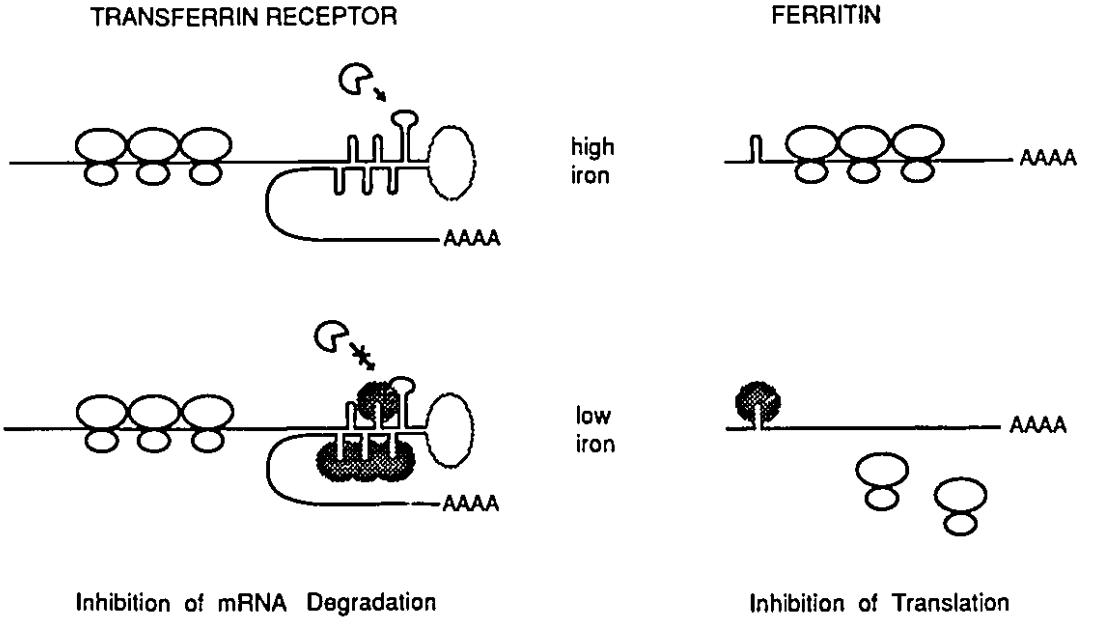


Fig. 7. Model for coordinate regulation of transferrin receptor and ferritin expression

The mRNA and ribosomes are represented by their conventional symbols. The IRE-BP is indicated in dark grey. The RNAase, postulated to attack the stem-loop in human TfR mRNA is indicated as an open circle. Reproduced from Müllner et al [201].

could also have an Fe-S complex containing a labile Fe which can alter the protein conformation depending on the intracellular iron concentration.

TfR expression in human macrophages may respond differently to iron since they store large amounts of ferritin. Even though the intracellular iron concentration is high, there is translation of TfR mRNA [359]. Lowering the intracellular iron concentration with iron chelators decreases mRNA levels. It is not known whether erythroid cells which must accumulate large amounts of iron for heme synthesis regulate TfR synthesis by the post-transcriptional mechanism described above. K562 cells have some erythroid characteristics but they are abnormal and do not terminally differentiate. In undifferentiated MEL cells, addition of hemin (an iron source) decreased TfR expression. However, when MEL cells were grown for 4 days in the presence of DMSO and hemin, the TfR binding capacity increased [211].

(b) Regulation of TfR number by the proliferation status or differentiation level of the cell

Rapidly proliferating cells express high numbers of TfRs and a decrease in proliferation is often accompanied by a decrease in the number of TfRs. For example, when HL60 cells are induced to terminally differentiate by the addition of DMSO or phorbol diesters, cell proliferation decreases and TfRs are lost [212,213]. TfRs are expressed by activated lymphocytes and lymphoblastoid cell lines but not by resting lymphocytes [214,215]. Since deletion of the 3' UTR of TfR mRNA does not affect the decrease in TfRs during growth arrest, growth-related expression of TfRs may be regulated by another mechanism, e.g. transcription [216].

The number of TfRs in erythroid cells is determined by the stage of cell differentiation (see Fig. 2), with pronormoblasts expressing 20-fold more TfRs than reticulocytes [217]. Immature reticulocytes express more TfRs than reticulocytes at a later stage of maturation.

(c) Acute regulation of TfRs by growth factors and mitogens: rate of endocytosis and exocytosis

Growth factors and phorbol esters can influence cell surface expression of TfRs and iron uptake, depending on the treatment and cell type. Insulin-like growth factor I (IGF-I), insulin, and EGF cause a rapid increase of TfRs at the cell surface in fibroblasts and A431 cells but do not always increase iron uptake [218-221]. IGF-I increased surface TfRs in A431 cells by decreasing the rate of endocytosis [220] while insulin and EGF caused redistribution in adipocytes and A431 cells, respectively, by increasing the rate constant for exocytosis [220,221].

Phorbol esters, which are specific activators of protein kinase C, increase phosphorylation of the TfR [92,226-231] and alter its distribution in some cells but have little effect on other cell types. In J774 mouse tumour macrophage-like cells [222], CHO fibroblasts or CHO transfected with human TfR [223], and 3T3 fibroblasts [224,225], phorbol esters (phorbol myristate acetate [PMA]) increased TfRs on the cell surface by increasing the rate of exocytosis. However, in HL60 cells [226,228], K562 cells [227], and lymphoblastoid cells [229], phorbol esters decreased the number of TfRs at the cell surface without a change in iron uptake or total cellular TfR number. PMA did not affect the distribution of TfRs in primary cultures of normal chick embryo myogenic cells; however, iron uptake was increased by 15-30% probably due to increased TfR recycling [230]. There

is only one report where treatment of K562 cells with a phorbol ester produced a change in the total number of TfRs. TPA (12-O-tetradecanoyl phorbol-13-acetate) decreased the total TfR content in K562 cells to 10% and this was due to both a specific decrease in TfR synthesis and decrease in half-life of the TfR from 15 h to 5 h [231].

The effect of phorbol esters on CHO cells [223] and mouse 3T3 fibroblasts [224,225] does not require phosphorylation of serine-24 since the expression of a mutant TfR with Ser_{24} replaced by a non-phosphorylable amino acid is affected in the same way as the wildtype TfR. It appears that phorbol esters perturb endocytosis by another mechanism and not by activating protein kinase C to specifically phosphorylate the TfR. It has been suggested that phosphorylation of protein(s) apart from the TfR could regulate TfR internalization in some cells [223].

Although TfRs can undergo recycling in the absence of Tf, some studies have suggested, to the contrary, that endocytosis and release of internalized ^{125}I-Tf (especially in cells which express high numbers of TfRs, e.g. K562 cells) is dependent on the presence of Tf in the medium [36,227,232]. More recent studies have shown that although Tf is not required for internalization, FeTf can alter the kinetics of TfR recycling by increasing the rate constants for endocytosis and exocytosis [233,234]. In cells with more than one route for TfR recycling, i.e. a fast recycling route between early endosomes and the plasma membrane, and a slower recycling route which includes multivesicular endosomes or the TGN, TfRs bound to ligand may have a tendency to recycle through the faster pathway and show a higher kinetic rate. However, transferrin is unlikely to play a role in modulating TfR recycling and iron uptake in vivo since the Tf concentration in serum and tissues is much higher than that required for saturation of TfRs.

(d) Efficiency of iron release

Less data are available on the regulation of iron transport subsequent to Tf endocytosis. Differentiating MEL cells take up iron more efficiently from Tf than undifferentiated cells which release part of the internalized iron into the medium [235]. It has been known for some time that heme inhibits iron uptake by reticulocytes [236,237], but the mechanism is not entirely clear. At low concentrations, heme blocks delivery of iron from Tf following endocytosis and before insertion into protoporphyrin IX [238], but at higher concentrations, it also inhibits endocytosis of Tf [237,239]. Inhibiting heme synthesis with 4,6-dioxoheptanoate (DOH) stimulates iron uptake and TfR recycling by reticulocytes [237,240,241] This increased delivery of iron in the presence of DOH results in accumulation of intracellular and intramitochondrial non-heme iron [237,240,241]. In other cell types, a rise in the level of intracellular iron would decrease iron uptake by decreasing TfR synthesis. However, in reticulocytes, free heme regulates iron uptake by acting on preexisting TfRs. Heme also inhibits iron uptake by DMSO-treated Friend cells [242], and erythroid cells from fetal mouse and rat liver [237]. Hemin does not inhibit iron uptake by K562 cells [11] and uninduced Friend cells [242].

1.3.5 Structure of the TfR

TfRs from a wide variety of cells have been studied [243-250]. Erythroid cells (reticulocytes) which synthesize enormous amounts of hemoglobin [245-247], placental trophoblasts [248,249] and rapidly dividing cells [250] have a great demand for iron and express high numbers of TfRs. The TfRs from these

cells are particularly well characterized and have been purified using affinity chromatography and chemical methods [247-249,251,252].

The TfR is a transmembrane glycoprotein having two identical subunits (90-100 kDa) which are covalently linked by one or more disulfide bridges [12,253-256]. Full-length human, mouse, and chicken cDNA clones have been isolated and sequenced [257-260]. Fig. 8 shows the significant amino acid sequences and post-translational modifications of the TfR. The human and sheep TfRs have been shown to be acylated [253,261] but this posttranslational modification is not required for TfR internalization and iron uptake [262].

(a) Dimerization

The two 90 kDa subunits appear to associate and form dimers even in the absence of disulfide bonds. When the cysteines at positions 89 and 98 were changed to serines, the two monomers could be crosslinked suggesting that the mutant subunits associated as a dimer [263]. Covalent dimerization is not required for cell surface expression nor Tf binding [256]. Enns et al.[264] found that during TfR biosynthesis in A431 cells, intersubunit dimer formation is a slow event occurring after acquisition of Tf binding function. Furthermore, when the extracellular domains are removed by trypsin distal to the disulfide bonds, the 70 kDa truncated receptors form noncovalent dimers [252] and bind Tf [159,252,255]. Therefore, the cytoplasmic and membrane domains and the disulfide bridges are not required for Tf binding.

(b) Glycosylation

The human TfR has three N-linked oligosaccharides [253,254] as well as O-linked oligosaccharides [265]. One of the N-linked oligosaccharides is

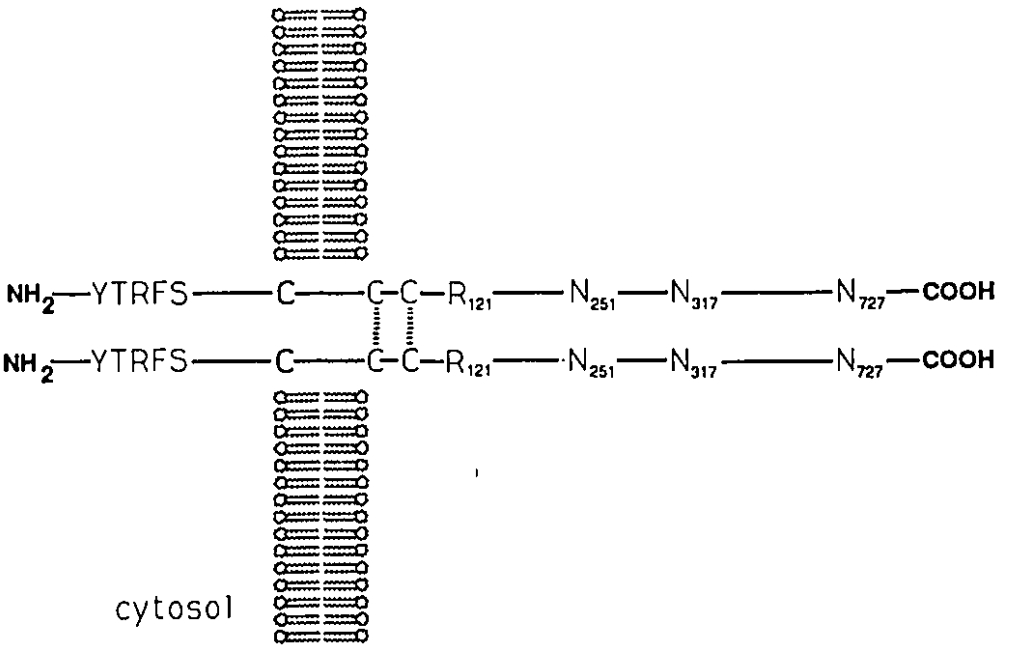


Fig. 8. Structure of the human TfR

Of the 760 amino acids of the human TfR, those considered to be of significance are denoted by their one-letter codes. The disulfide bonds (C""C) and trypsin cleavage site (R) are located close to the extracellular/lumenal surface of the membrane. The YTRF sequence, Ser_{24} phosphorylation site (S), Cys_{62} acylation site (C), and the three Asn glycosylation sites (N) are conserved in the mouse and chicken TfRs [252,256-260].

processed to a complex type oligosaccharide while the other two remain as high-mannose forms in the mature TfR [253,254]. One role of the oligosaccharides is probably, as in other membrane glycoproteins, to mediate proper folding of the protein in the ER and allow its association with other subunits. These steps are required for transport of the nascent protein through the Golgi and to the cell surface. Biosynthetic studies on the TfR support this view. When A431 cells were incubated with tunicamycin, a drug which inhibits the synthesis of dolichol-oligosaccharides and therefore inhibits N-linked glycosylation, the unglycosylated TfR did not form a dimer, did not bind Tf, and was not transported to the cell surface [266]. In other tunicamycin-treated cells, some unglycosylated TfR did reach the cell surface, but at a slower rate [253,267], and was able to form dimers and bind Tf-Sepharose [267,268]. These results indicate that different cells may process unglycosylated receptors differently and/or that tunicamycin may have secondary effects on some cells.

Inhibitors of oligosaccharide processing, such as castanospermine and swainsonine which block the removal of terminal glucoses and mannoses, respectively, have no effect on the transport of TfRs to the cell surface [264,268]. Therefore, high-mannose oligosaccharides do not have to be processed to the complex forms for the TfR to be transported to the cell surface. However, these receptors were reported to have a reduced affinity for Tf [264]. Removal of the terminal sialic acids also appeared to change the affinity of the TfR for Tf [267].

Tunicamycin inhibits the glycosylation of all proteins including resident ER and Golgi proteins and therefore, it may perturb the entire processing machinery. If site-specific mutations are made in the glycosylation sites of TfRs, only the TfR would be unglycosylated. Mutation of the asparagine glycosylation sites resulted in decreased Tf binding, slower transport to the cell surface, and

association with BiP [269]. In another study, replacement of Asn_{251} with a Gln resulted in cleavage of the TfR to yield a 73 kDa soluble polypeptide which was degraded with a half-time of 1 h before entering the Golgi [270]. Therefore, the removal of as few as one N-linked oligosaccharide results in protein misfolding and a block in the transport of the TfR to the cell surface. In one case, the misfolded TfR could not bind Tf and in the second case, a protease sensitive site was exposed.

(c) Phosphorylation

The TfR can be phosphorylated on a serine in the cytoplasmic domain [254,271] by cAMP-dependent protein kinase [92] and protein kinase C [92,226-228,272]. These studies have suggested that phosphorylation of the TfR acts as a signal to target the receptor to different pools, i.e. between the cell surface and internal pool of TfRs [239,273] or between recycling and non-recycling pools of TfRs [271,92]. But more recent studies have shown that mutation of Ser_{24} abolishes phosphorylation without an effect on the rate of TfR internalization [223-225,274]. Dephosphorylation of the TfR appeared to have no effect on its affinity for Tf [267].

1.4 Effect of Heme on Cellular Functions

Heme (ferrous protoporphyrin IX)[1] is a structural component of several important proteins, including hemoglobin and cytochromes. It is also apparent

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[1] Hemin = ferric protoporphyrin IX chloride

from the previous sections that free heme is involved in many cellular processes, especially in erythroid cells. Heme regulates iron uptake, cell growth, gene transcription, protein translation, and protein degradation.

1.4.1 Heme biosynthesis

In the liver cell, heme is a negative inhibitor of δ-aminolevulinate (ALA) synthase at the level of (1) transcription, (2) translation, (3) transport of cytosolic precursors into mitochondria, (4) enzyme activity [38]. ALA synthase, the first enzyme of the heme biosynthetic pathway, catalyzes the formation of δ-aminolevulinic acid (ALA) from glycine and succinyl CoA (Fig. 9).

Control of heme synthesis changes during erythroid cell differentiation. Heme stimulates its own biosynthesis in erythroid precursor cells by increasing the expression of the enzymes of the heme biosynthetic pathway [43]. In uninduced Friend cells, as in liver cells, ALA stimulates heme synthesis by bypassing the rate-limiting enzyme ALA synthase. But in Friend cells induced to differentiate with DMSO, ALA has no effect since the rate-limiting step in these cells is the utilization of iron from transferrin [275]. Heme synthesis in these cells and in reticulocytes is stimulated by adding FeSIH which bypasses the transferrin cycle and provides the cell with larger amounts of iron than that which is normally supplied by FeTf [276]. In reticulocytes, hemin inhibits heme synthesis mainly by decreasing the rate of iron uptake [238,277] and limiting the incorporation of iron into protoporphyrin IX. The activity of enzymes of the biosynthetic pathway are not affected.

The difference in regulation of heme synthesis in liver and erythroid cells was somewhat clarified by the discovery of two distinct isozymes of δ-ALA synthase, encoded by two different genes in chicken and man [206,278]. The

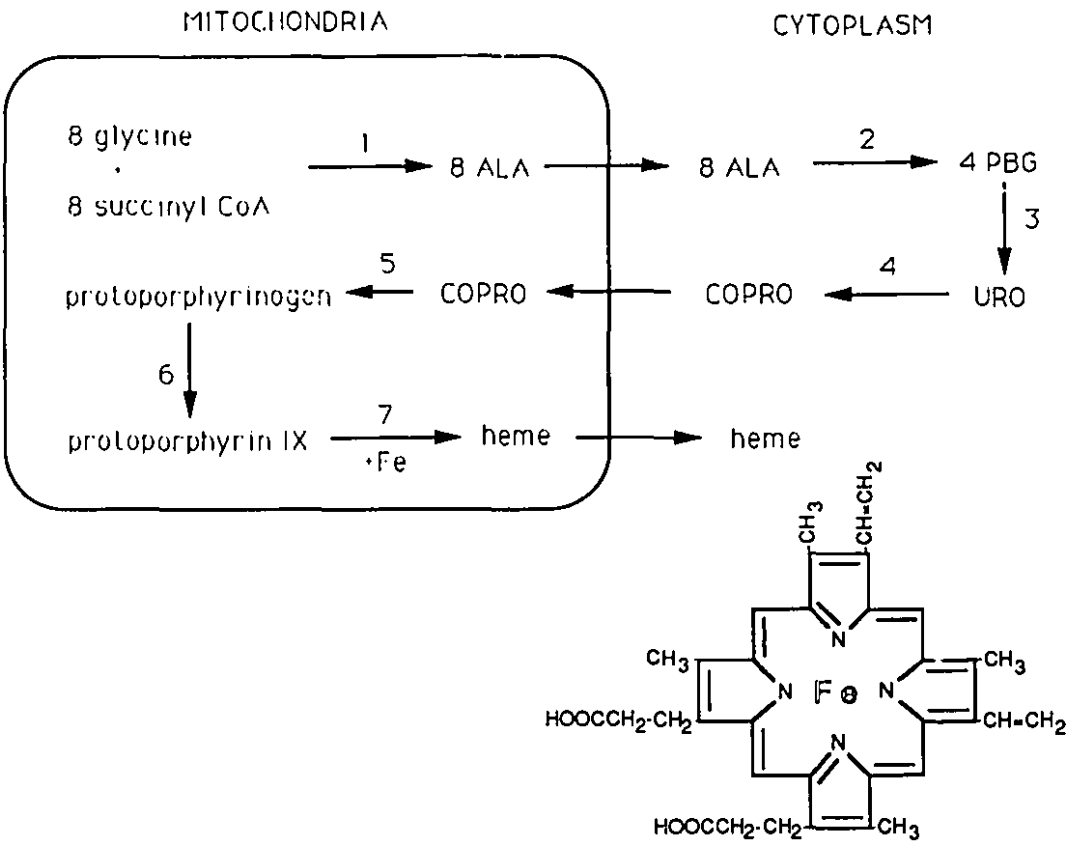


Fig. 9. Biosynthesis of heme

ALA, δ-aminolevulinic acid; PBG, porphobilinogen; URO, uroporphyrinogen III; COPRO, coproporphyrinogen III; (1) ALA synthase; (2) ALA dehydratase; (3) PBG deaminase; (4) URO decarboxylase; (5) COPRO oxidase; (6) protoporphyrinogen oxidase; (7) ferrochelatase.

4,6-dioxoheptanoate (DOH) is a specific inhibitor of ALA dehydratase.

liver enzyme is the constitutive or housekeeping isoform and is subject to negative transcriptional regulation by heme. Transcription of the erythroid specific ALA synthase gene is not affected by heme [279]. A sequence similar to the IRE in ferritin and TfR mRNAs is present in the 5' UTR of the erythroid but not the housekeeping (liver) ALA synthase mRNA [206,207]. Low iron could stimulate the binding of a protein (like IRE-BP) and inhibit the translation of the ALA synthase mRNA. It remains to be determined whether ALA synthase is still made in the reticulocyte and whether its synthesis is subject to regulation by iron.

In addition to regulating its biosynthesis, heme regulates its own degradation by inducing transcription of the mRNA for heme oxygenase, the enzyme which breaks down heme to bilirubin (20-25 fold in mouse hepatoma and HL60 cells [165]). However, heme oxygenase is inhibited by heme in erythroid cells.

1.4.2 Cell growth and differentiation

Hemin increases the number of erythroid progenitor cells (BFU-E and CFU-E) in bone marrow and fetal liver cultures and promotes their differentiation [33,34,280]. Hemin also induces hemoglobin accumulation in the models described above (section 1.1.3). Heme may play a role in the terminal maturation of Friend cells, independent of its involvement in hemoglobin synthesis [281,282]. Hemin may also have a role in the differentiation of nonerythroid cells. The addition of hemin induced mouse 3T3 cells to differentiate into adipocytes [283] and increased neurite outgrowths by neurons [284]. The mechanisms underlying these effects are not known.

1.4.3 Protein translation

One of the best studied mechanisms of regulation by heme is that of protein translation. In reticulocyte lysates, hemin is required for the translation of endogenous and exogenous mRNA [285-289]. In the absence of heme, the heme-regulated inhibitor (HRI, eIF-2α kinase, or heme-controlled repressor [HCR]) accumulates [290.291]. HRI phosphorylates the α subunit of eIF-2 and inactivates the initiation factor [292]. When phosphorylated, eIF-2 binds tightly to the reversing factor (RF or guanosine nucleotide exchange factor), thereby sequestering it. Since the amount of reversing factor is limited, GTP cannot be exchanged for GDP and initiation of protein synthesis is inhibited [293]. Hemin inhibits the autophosphorylation of HRI as well as the activity of HRI after phosphorylation. Hemin can bind directly to purified HRI [294]. The effect of hemin on HRI is similar to thiol oxidation by diamide in that hemin promotes interdisulfide formation in both unphosphorylated HRI and phosphorylated HRI. HRI is activated by heme deficiency and sulfhydryl reagents (N-ethylmaleimide or o-iodosobenzoate) which prevent disulfide formation [295].

1.4.4 Protein degradation

While hemin is an inhibitor of ubiquitin-dependent proteolysis [296,297], it can stimulate the degradation of proteins in the presence of oxygen and a reducing agent [298]. Hemin catalyzes the production of hydroxyl free radicals (reduced oxygen species) from hydrogen peroxide by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$. Free radicals can cause oxidation of proteins, resulting in changes in structure, loss of activity, and increased degradation. Amino acids susceptible to oxidation are cysteine, methionine, histidine, proline, arginine, and lysine [299]. Oxidative damage is inhibited by antioxidants and free radical scavengers such as catalase, superoxide dismutase, and vitamin E.

Hemin may destabilize interactions between the cytoskeletal and peripheral membrane proteins underlying the erythrocyte membrane. Hemin alters the conformation of purified spectrin and decreases its resistance to proteolysis. Hemin also alters the conformation of protein 4.1 and weakens spectrin dimer-dimer, spectrin α-β, and spectrin-protein 4.1 interactions [300]. Chou and Fitch [301] observed that hemin causes lysis of erythrocytes by a mechanism not dependent on free radicals and lipid peroxidation. They suggested that heme impairs the cation gradient and causes a rapid loss of K^+ which leads to osmotic swelling and cell lysis. Sulfhydryl agents (glutathione, cysteine, β-mercaptoethanol) interact directly with hemin and prevent its effects [301,302].

1.5 Purpose of Work

The TfR is a useful tool in the study of a variety of problems: receptor-ligand interactions, clustering of receptors in coated pits, endocytosis, sorting, biosynthesis, regulation at the mRNA level, terminal differentiation of erythroid and leukemic cells. For our study, we adopted the TfR as a model for the biosynthesis and fate of transmembrane proteins during reticulocyte maturation.

Many cellular functions decrease during reticulocyte maturation such that they are absent or present in low amounts in the mature erythrocyte. Intracellular organelles disappear while the protein composition of the plasma membrane is altered during cell maturation. Reticulocytes still have the protein synthetic machinery and can make a variety of different proteins; however, the vast majority of protein synthesis is for hemoglobin which makes up 90% of the total protein in the reticulocyte. It has been known for some time that reticulocytes also synthesize a number of membrane-bound proteins [303-306], but until recently, probes to identify these membrane proteins, such as

antibodies, were not available. Also, our knowledge of membrane protein synthesis and processing subsequent to translation on ribosomes has expanded greatly over the past decade. Unlike cytoplasmic proteins (e.g. hemoglobin), membrane proteins appear to require translocation into organelles (ER, Golgi) and vesicular transport before being inserted into the cell membrane. The purpose of this thesis is to determine whether red cells at the reticulocyte stage have the capacity to synthesize and fully process membrane proteins. Since most of these organelles appear to have been lost by the reticulocyte stage, processing and translocation of newly made membrane proteins to the cell surface might not be possible. On the other hand, reticulocytes may contain all the necessary enzymes for post-translational processing although they are not organized into specific compartments. Using the TfR as an example of a membrane protein, the questions addressed are the following. (1) Does the sheep reticulocyte have the machinery to synthesize, glycosylate, and transport a transmembrane protein to the cell surface? (2) Is TfR expression in the reticulocyte regulated by iron availability as in other cell types? (3) What is the fate of newly synthesized TfRs? Is the fate of the new TfR different from that of preexisting TfRs which are externalized during reticulocyte maturation? (4) What is the mechanism of selective loss of membrane proteins during maturation? The effect of hemin on the level of TfRs (preexisting and newly synthesized) in the maturing reticulocyte is also examined. From the following study, it can be concluded that the maturing reticulocyte is unable to fully process a newly synthesized membrane protein. Hemin, which stimulates the loss of preexisting TfRs, could have a more general effect on the selective loss of membrane proteins during reticulocyte maturation.

Chapter 2 Materials and Methods

2.1 Materials

HL60 cells and GP4F cells were obtained from Caroline A. Enns, Oregon Health Sciences University, Portland, OR, and Judith White, University of California, San Francisco, respectively. Mouse anti-human TfR monoclonal antibody which recognizes the exofacial domain of the TfR originated from James Larrick, Cetus Corp., now at Genentech, Palo Alto, CA. A rabbit antibody made against twenty amino acids of the cytoplasmic domain (Ala_{8}-Arg_{27}) of the human TfR was a gift of Dr. Tamotsu Yoshimori, Osaka University, Japan (present address: Dept. of Physiology, Kansai Medical University, Japan). Both these antibodies recognize the sheep TfR. Anti-human band 3 was provided by Reinhart Reithmeier, University of Toronto. (Note: This antibody did not bind to sheep band 3 protein after the antigen was heated in the presence of β-mercaptoethanol.) Anti-human glycophorin A was obtained from Joel Chasis and Narla Mohandas, University of California, Berkeley. Anti-NSF was obtained from Duncan Wilson and James Rothman, Princeton University.

Endo H and Bio-Gel P6 (Bio-rad) were gifts of Prof. A. Herscovics, Dept. of Biochemistry, McGill University. Pyridoxal isonicotinoyl hydrazone (PIH; gift of Dr. P. Ponka, Dept. of Physiology, McGill University) was dissolved in 0.01 M HCl, diluted with culture medium, then neutralized with NaOH. FePIH was prepared by mixing equal volumes of 2 mM PIH and 1 mM $FeCl_3$:20 mM Na citrate and allowing the solution to stand for 30 min at room temperature prior to use [358]. Hemin and SIH were dissolved in 1 M NaOH, and diluted

with PBS, pH 7.4. A mM solution of hemin in 0.1 M NaOH gave an A_{610} of 4.6 [276].

[^{35}S]methionine (>1100 Ci/mmol), [2-^{14}C]glycine (50 mCi/mmol), D-[2-^{3}H]mannose (18.5 Ci/mmol), and UDP-D-[6-^{3}H]galactose (20 Ci/mmol) were purchased from Amersham, Oakville, Canada. Trans ^{35}S-Label ([^{35}S]methionine/cysteine), Na^{125}I, methionine-free MEM, and endo H were obtained from ICN Flow, Mississauga, Canada. [^{3}H]S-(p-nitrobenzyl)-6-thioinosine (NBMPR; 23 Ci/mmol) was obtained from Moravek Biochemicals, Brea, CA. [^{3}H]Palmitic acid (30 Ci/mmol), En^{3}Hance, Protosol, and Aquasol were from Dupont New England Nuclear, Boston, MA. S-MEM, D-MEM, RPMI 1640, fetal bovine serum, glutamine, non-essential amino acids, penicillin/streptomycin, trypsin/EDTA were from Gibco, Burlington, Canada. Dynabeads M-450 were purchased from P&S Biochemicals, Gaithersburg, MD. Protein A-Sepharose CL-4B, Sephadex G-25, CNBr-activated Sepharose 4B, Con A-Sepharose, and Percoll were from Pharmacia, Dorval, Canada. Lactoperoxidase, α-chymotrypsin, trypsin, soybean trypsin inhibitor, aprotinin, PMSF, human Tf, bovine albumin (Fraction V), luciferase (firefly lantern extract), α-methylmannoside, NBMPR, adenosine, hemin (equine), 4,6-dioxoheptanoic acid, and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO; 2-deoxy-D-glucose and trifluoromethanesulfonic acid (TFMS) were from Aldrich Chemical Co., Milwaukee, WI; Hepes and Pronase were from Calbiochem-Behring, La Jolla, CA; glucose oxidase, inosine, endoglycosidase F/N-glycosidase were from Boehringer-Mannheim, Dorval, Canada; heparin was from Organon Teknika, Canada. Deferoxamine was obtained from Ciba, Dorval, Canada. X-OMAT AR5 film was from Kodak Canada Ltd. Alkaline phosphatase-conjugated goat anti-rabbit IgG and substrates were from Biorad.

2.2 Cell Preparation and Culture

Isolation of sheep reticulocytes: Adult sheep were bled twice a week and maintained in an anemic state for ~6 weeks. Animals were injected with 2.5 ml of an iron-dextran solution (100 mg/ml) twice a week to prevent iron deficiency. Reticulocytes were enriched by differential centrifugation as described by Pan et al.[12], Benderoff et al.[68]. Whole blood was centrifuged and washed twice with 0.9% saline. The cells were transferred to 50-ml tubes and centrifuged at 1600*g* for 1 h. The top 20% of the cells including the buffy coat were transferred to 15-ml tubes and centrifuged for an additional hour. After the white cells at the top were removed with a Pasteur pipet, the red cells were fractionated starting from the top. The cells were washed three times in saline. Any contaminating white cells above the reticulocytes were removed carefully after each wash. Reticulocytes were counted on methylene blue stained smears. Those fractions containing 60-95% reticulocytes were used immediately after isolation or stored overnight in culture medium at 4°C prior to use. The reticulocytes from certain sheep were more susceptible to lysis after overnight storage and therefore 50 mM sucrose was included in the storage medium for these cells.

Preparation of avian reticulocytes: Fourteen day old fertilized eggs were cracked open over a sheet of plastic mesh, and the main blood vessel of the embryo was punctured, allowing the blood to be collected through the mesh. The blood cells were washed four times with 1% saline containing 0.25% albumin. The blood from 14 day chick embryos contain mainly reticulocytes of the definitive series [35].

HL60 cells: HL60 cells, a human promyelocytic cell line, were maintained between $2x10^5$ and $1.5x10^6$ cells/ml in RPMI 1640 containing 100 units/ml

penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 10% fetal bovine serum (complete RPMI). To prepare cells and conditioned medium for measuring protease activity, HL60 cultures were grown to 10^6 cells/ml. The cells were centrifuged and suspended in fresh medium, and the cell-free conditioned medium was concentrated by ultrafiltration (Amicon YM100).

Human blood: Human blood was derived from a hemochromatosis patient undergoing periodic phlebotomy and was discarded blood from the outpatient clinic. The heparinized blood was centrifuged and the cell-free plasma was either diluted 1:1 with saline and centrifuged at 135,000*g* for 90-120 min to obtain an exosome pellet or was incubated with iron-core beads crosslinked to sheep anti-mouse IgG and coated with anti-TfR MAb. The immobilized material was subjected to SDS-PAGE.

Iodination of the Reticulocyte Cell Surface: To obtain a quantitative measure of the TfR during reticulocyte maturation, red cells were iodinated using lactoperoxidase and glucose oxidase [307]. Before labelling, the cells were pre-incubated in MEM for 30-45 min to increase surface iodination sites [307]. The cells were suspended 50% in 2.5 mM PBS, pH 7.4, containing 10 mM glucose, 1 µM KI, and 50 µCi/ml $Na^{125}I$. After 2 min, 16 µg/ml lactoperoxidase (40 units/mg) and 1 µg/ml glucose oxidase (140 units/mg) were added and the mixture was shaken gently at room temperature for 15 min. The cells were washed with cold saline to remove the unincorporated ^{125}I and cultured as described below. The radioactivity recovered in the immunoprecipitates of the released TfR was compared to the amount of immunoprecipitable radioactivity lost from the cells.

Longterm incubation of red cells: Cells were transferred to a 1 L bottle containing Eagle's minimal essential medium (for suspension cultures) supplemented with 0.2 mM nonessential amino acids, 4 mM glutamine, 5 mM

adenosine, 10 mM inosine, 200 units/ml penicillin, and 200 µg/ml streptomycin (complete S-MEM). The bottle was capped with a rubber plug and gassed using a needle connected to a 95% O_2/5% CO_2 tank. The bottle was rotated slowly in a 37°C incubator.

Isolation of exosomes: After incubation, cells were centrifuged at 7700*g* for 8 min at 4°C. The supernatant was removed and re-centrifuged to remove any remaining cells. The cell-free supernatant from the second centrifugation was ultracentrifuged at 100,000*g* for 90 min to pellet the vesicles released during culture. Alternatively, the vesicles were collected by concentration of the cell-free supernatant with an Amicon ultrafiltration cell equipped with a YM100 membrane (at a flowrate of ~10 ml/h).

Trypsin treatment of cells: A 20% cell suspension in PBS was incubated with 0.1% trypsin on ice for 30 min, or at 37°C for 10 min, with gentle shaking. Soybean trypsin inhibitor was then added to achieve 0.5% final concentration. After 10 min on ice, the cells were washed several times with PBS.

2.3 Incorporation of Labelled Precursors

Measurement of heme synthesis: [2-^{14}C]Glycine incorporation into heme and protein was measured according to Ponka et al.[276]. A 5% cell suspension was incubated with 10 µCi/ml [2-^{14}C]glycine (50 mCi/mmol) for 1 h in MEM buffered with 20 mM NaHepes. Cells were washed in ice-cold PBS, pH 7.4, lysed in 10 volumes of cold distilled water, kept on ice for 20 min, and frozen overnight. After thawing, 1 ml ice-cold acidified acetone (3% concentrated HCl in acetone [v/v]) was added to a 50 µl aliquot of lysate to precipitate the protein. The precipitate was washed with acidified acetone, dissolved in 1 N NaOH, then re-precipitated with TCA and collected on Whatman GF/B filters. The filters

were dried and counted in scintillation fluid (5 g/L PPO, 0.05 g/L POPOP in toluene). The acetone-soluble heme was precipitated with the addition of 3 ml water. Seventeen ml of water were added and the heme was collected on a Millipore 0.22 μ nitrocellulose membrane.

Metabolic labelling of sheep reticulocytes: A 10-20% suspension of sheep reticulocytes was incubated in MEM supplemented with 0.2 mM nonessential amino acids, 4 mM glutamine, 200 units/ml penicillin, and 200 μg/ml streptomycin with (1) 70 μCi/ml [^{3}H]palmitate (specific activity: 30 Ci/mmol), (2) 50-200 μCi/ml L-[^{35}S]methionine (1-1.5 Ci/mmol), or (3) 1.0 mCi/ml D-[2-^{3}H]mannose (20 Ci/mmol). The duration of incubation is specified in the legends. The cell suspension was either incubated under a 95% O_2/5% CO_2 atmosphere or supplemented with 20 mM NaHepes, pH 7.4, and incubated under air. In either case, the cells were shaken gently in a 37°C water bath. For chase experiments, 5 volumes of medium containing 2 mM methionine were added. The labelled cells were washed with PBS, pH 7.4.

Because reticulocyte preparations may contain some nonerythroid cells and these might contribute to overall synthesis of the TfR, the contribution of the nonerythroid cells to isotopic incorporation was examined. For these experiments, isotonic NH_4Cl was used to lyse red cells after a period of [^{35}S]methionine incorporation. The labelled cells (50 μl) were suspended in 0.25 ml of 0.144 M NH_4Cl (containing 3 mM DTT) and 0.03 ml of NH_4HCO_3 [308]. After 5 min on ice the mixture was centrifuged over a 9.25% sucrose cushion at 1600*g*. White cells should remain intact and pellet through the sucrose while the red cell ghosts are recovered in the supernatant. Both the red cell lysate and white cell fractions were immunoprecipitated to measure ^{35}S incorporation into the TfR. Under the given conditions, all ^{35}S incorporation into the TfR was found to be due to reticulocytes.

Total protein synthesis: To determine the incorporation of ^{35}S into proteins, an equal volume of cold 10% TCA was added to an aliquot of lysate after incubation with [^{35}S]methionine. After at least 10 min on ice, the precipitate was collected by centrifugation at 12,000*g*, washed twice (by suspending the pellet with a glass rod), and heated for 5 min at 90°C in 5% TCA. The precipitate was then dissolved in 1 M NaOH. The samples were neutralized by the addition of 2 M acetic acid and counted in Aquasol. Alternatively, the filter paper disk method [309] was used. Ten µl of lysate was applied to a 1 cm^2 of Whatman 3 MM paper which had been presoaked with 50 µl of a 50X amino acid solution. The paper was soaked in ice-cold 10% TCA for at least 1 h, washed in 5% TCA, washed in ether/ethanol, dried and counted in scintillation fluid.

Metabolic labelling of avian reticulocytes: One ml avian reticulocytes were labelled in 5.0 ml D-MEM containing 50 U.S.P. units heparin, 0.25% bovine albumin, and 1.0 mCi D-[2-^{3}H]mannose for 4 h at 37°C. The cells were washed in 1% NaCl, 7.5 mM NaPi, 0.25% bovine albumin, pH 7.4, and lysed in 100 volumes of 5 mM NaPi, 4 mM $MgCl_2$, pH 7.4. Membranes were collected at 7700*g* and washed twice.

Metabolic labelling of HL60 cells: HL60 cells were washed with PBS and incubated in complete MEM (10^7 cells/ml) containing 10 µM methionine, 10% fetal bovine serum, 20 mM NaHepes, pH 7.4, and 20 µCi/ml [^{35}S]methionine for 1 h at 37°C. The labelled cells were cultured 12-38 h in complete RPMI under 95% O_2/5% CO_2.

2.4 Transferrin and NBMPR Binding to Cells and Vesicles

Preparation and iodination of Tf: Sheep Tf (iron-loaded) was isolated from plasma as follows [310, 217]. To 800 ml of plasma, 2 ml of 5 mM $FeCl_3$:20 mM sodium citrate and one-third volume of 0.01 M Tris-HCl, pH 8.8, were added,

and the mixture was stirred for one hour. This mixture was precipitated with 50% ammonium sulfate overnight at 4°C, and the soluble fraction was concentrated to 75 ml in an Amicon ultrafiltration cell (PM30 membrane), dialyzed against H_2O and lyophilized. Portions of the dried material were suspended in Tris-HCl and applied to a Sephadex G-25 column. Fractions containing Tf (by SDS-PAGE) were dialyzed and lyophilized. The protein was suspended in H_2O and applied to a DEAE-cellulose #52 column. Proteins were eluted with a 0-0.5 M NaCl gradient and fractions containing Tf were passed through a second anion exchange column. The purified Tf had an $A_{465\ nm}/A_{280\ nm}$ ratio of 0.046. Human Tf was iron-loaded (2 mol Fe^{3+}:1 mol Tf) with an $FeCl_3$:sodium citrate solution (20-fold molar excess citrate) containing 0.6 M $NaHCO_3$. After 3 h at room temperature, the solution was dialyzed overnight against PBS, pH 7.5.

Iodination was by the chloramine-T method [311]. Tf (2 mg) suspended in 40 µl of 50 mM PBS, pH 7.5, was added to 16 µl of PBS containing 3 µg KI and 1 mCi $Na^{125}I$. 0.1 mg (10 µl) of chloramine-T was added and the mixture was left at room temperature for 2 min. 0.5 mg (50 µl) of sodium metabisulfite and 0.4 mg (40 µl) of KI were added to stop the iodination. After 5 min on ice, ^{125}I-Tf was separated from free ^{125}I by filtration on Sephadex G-25 (PD10 column). Only fractions containing 100% TCA-precipitable ^{125}I were used.

Tf binding assay: Total ^{125}I-Tf binding to Triton X-100 solubilized cells and vesicles was measured at room temperature according to Klausner et al.[164] as modified by Adam et al.[312], Orr and Johnstone [98]. Cells (5 µl) or vesicles (from 50 µl cells) in 50 µl of PBS containing 0.025% bovine albumin (binding buffer) were solubilized by the addition of 50 µl of 1% Triton X-100 and incubated with 2 µg ^{125}I-Tf ($2x10^5$ cpm) in a final volume of 250 µl. Iron-loaded human Tf was added in 500-fold excess to measure nonspecific binding.

After 20 min at room temperature, an equal volume of 60% saturated ammonium sulfate, pH 7.0, was added and the mixture was put on ice for 10 min. The Tf-TfR complex was collected on Whatman GF/C filters and washed with 3 x 4 ml ice-cold 30% saturated ammonium sulfate containing 0.8% bovine albumin, pH 7.0. The dried filters were placed in gamma tubes and counted for 60 sec. Each sample was prepared in triplicate, two aliquots for total binding and one for nonspecific binding.

Binding of [^{3}H]NBMPR: Equilibrium binding of [^{3}H]NBMPR was measured as described by Johnstone et al [77]. Membranes or vesicles (100 µg protein) were incubated with 5 nM [^{3}H]NBMPR (23 Ci/mmol), 50 mM Tris, 1 mM EDTA, 50 µg soybean trypsin inhibitor, 6 µM PMSF, pH 7.1, in a final volume of 1.0 ml. Ten µM unlabelled NBMPR was added to measure nonspecific binding. After 30 min at room temperature, 50 µl of 33 mg/ml γ-globulins and 450 µl of 33% PEG 8000 in 50 mM Tris, 1 mM EDTA were added. The mixture was vortexed and allowed to sit at room temperature for 15 min. Four ml of 8% PEG was added and the mixture was filtered through Whatman GF/B filters. The filters were washed once with 4 ml of 8% PEG, dried, and counted in scintillation fluid.

2.5 Transferrin and Concanavalin A Sepharose Binding

Tf affinity column: Sheep Tf was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions. The column was loaded with iron by passing 1 ml of 5 mM $FeCl_3$:20 mM nitriloacetate in 0.6 M $NaHCO_3$ through the column and washed with PBS, pH 7.4, containing 0.2% Triton X-100. Solubilized membranes (see section 2.7 for solubilization protocol) were diluted to 1.0 ml in PBS (0.2% TX-100 final concentration) and mixed gently with 1 ml of Tf-Sepharose 4B for 30 min at room temperature.

The mixture was poured into a 3-ml syringe, and the column was washed with 0.15 M sodium citrate, pH 5.0, containing 0.2% TX-100 and 50 μg/ml desferrioxamine, as described by van Driel et al. [251]. TfR was eluted with a solution containing 1 M NaCl, 0.2% TX-100, 20 mM sodium phosphate, pH 7.4. The eluate was dialyzed overnight and lyophilized. The dried material was dissolved in H_2O and immunoprecipitated prior to analysis by SDS-PAGE (see below).

Con A-Sepharose binding: Solubilized membranes (0.2 ml) were mixed with 10 μl Con A-Sepharose in Con A buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 M NaCl, 0.5% Triton X-100, 20 mM Hepes-KOH, pH 7.4) for 15 h at 4°C. The Sepharose was collected by centrifugation at 4200*g* and washed three times in Con A buffer. The proteins were eluted by incubating the Sepharose for 15 min with 0.2 M and 0.5 M α-methylmannoside in Con A buffer. The eluates were immunoprecipitated for TfR as described below. The material remaining bound to Con A after the 0.5 M α-methylmannoside wash was heated in sample buffer and loaded directly onto the gel for electrophoresis.

2.6 Density Gradient Centrifugation

Percoll gradients: Cells were lysed in 10 volumes of 20 mM sodium phosphate, pH 7.5, containing 0.1 mM PMSF. After centrifuging the lysate at 800*g* for 10 min, the top two-thirds of the supernatant was removed, made iso-osmotic with 0.25 M sucrose, and loaded onto 20 ml of 20% (v/v) iso-osmotic Percoll (final density=1.055 g/ml). The gradient was formed by centrifugation at 40,000g_{max} for 1 h. Fractions (1 ml) were removed from the top and either immunoprecipitated for TfR or assayed for ER and Golgi marker enzymes.

Galactosyltransferase assay: To measure enzyme activity, 50 µl of the fraction was incubated with 50 µM (10^5 dpm) UDP-[^{3}H]galactose, 0.1 mg ovomucoid, 0.5% Triton X-100, 20 mM $MnCl_2$, 25 mM β-mercaptoethanol, 2 mM ATP, 50 mM Tris-HCl, pH 7.4, in a final volume of 100 µl. After 1 h at 37 °C, the reaction was stopped with 1 ml 10% TCA and left on ice for 30 min. The precipitated protein was collected on Whatman GF/C filters, washed with 5% TCA, with ethanol, then dried and counted in scintillation fluid.

Glucose-6-phosphatase: The method of Beaufay et al. [313] was used. Samples were incubated for 30 min at 37°C in 1 mM NaEDTA, 40 mM sodium glucose-6-phosphate, 20 mM histidine-HCl, pH 6.5, in a final volume of 0.25 ml. Proteins were removed by TCA precipitation and the supernatant was transferred to clean test tubes. Phosphate was determined with Molybdate reagent and reading the absorbance at 660 nm.

NADPH cytochrome c reductase: Samples were incubated at room temperature with 50 µM oxidized cytochrome C, 0.25 mM NaCN, 32 mM nicotinamide, 40 mM potassium phosphate, pH 7.4, in a final volume of 1 ml [313]. The reaction was initiated by the addition of 50 µl of 2 mM NADPH and the absorbance at 550 nm was followed. Cytochrome C (reduced) has an ε=29.5 $mM^{-1}cm^{-1}$.

2.7 Immunoprecipitation of the TfR

Preparation of monoclonal antibodies: The supernatant from hybridoma cultures was passed through a Protein A-Sepharose column which had been equilibrated in 50 mM PBS, pH 7.5. The IgG was eluted with 150 mM NaCl, 0.1 M glycine, pH 3.0, into tubes containing 500 mM PBS, pH 7.5, then dialyzed against water and lyophilized. The IgG was dissolved in PBS containing 10 mM

NaN_3. Non-immune IgG was purified from the ascites fluid of a mouse which was not producing immune IgG.

Reticulocytes: Red cells were lysed by osmotic shock [314] in 30 volumes of 5 mM sodium phosphate, pH 8.0, containing 1 mM EDTA and 20 μM PMSF (lysis buffer). After 10 min on ice, membranes were collected by centrifugation at 30,000*g* for 15 min, washed twice in lysis buffer and once in 20 mM sodium phosphate, pH 7.0. Membranes were solubilized, with stirring, for 30 min on ice, in at least 2.5 volumes of a solution containing 1% Triton X-100, 0.2 U/ml aprotinin, and 20 mM sodium phosphate, pH 7.0. After solubilization, an equal volume of PBS was added, and the extract was centrifuged at 27,000*g* for 30 min to remove undissolved material. The clear supernatant (containing the proteins from 0.1 ml cells) was incubated overnight at 4°C with 30-50 μg anti-TfR MAb, then 100 μl of a 20% suspension of Protein A-Sepharose CL-4B in PBS were added for 3 h at 4°C. The beads were collected by centrifugation at 12000*g* for 30-60 sec and washed four times in PBS prior to treatment with glycosidases or SDS sample buffer.

Exosomes: To retrieve the exosome-associated TfR released to the medium (or to the plasma), the cell-free medium was centrifuged at 100,000*g* (culture medium) or 130,000*g* (plasma) for 90-120 min to pellet any insoluble (membrane-associated) TfR. The pellet was solubilized with Triton X-100 and immunoprecipitated as outlined for the plasma membranes. Soluble TfR in the supernatant (PES) was also immunoprecipitated.

HL60 cells and medium: HL60 cells (~10^7 cells/ml) were solubilized with 1% Triton X-100 containing 0.1 mM PMSF and 0.2 U/ml aprotinin and the lysate was immunoprecipitated as for red cells (see above). The cell-free culture medium (with 0.1 mM PMSF added) was fractionated into an insoluble fraction (100,000*g* pellet) and soluble fraction (PES) by centrifugation. The PES was

concentrated 8-10X in an Amicon ultrafiltration cell (YM100 membrane) before immunoprecipitation.

2.8 Treatment of Immunoprecipitates with TFMS and Enzymes

Trifluoromethanesulfonic acid: After immunoprecipitation, the TfR (~10 µg) and IgG were eluted from the protein A-Sepharose beads by heating for 5 min at 100°C in 100 µl of 1% SDS in PBS. The eluted proteins were treated with 1% Triton X-100 (800 µl) containing 0.2 U/ml aprotinin and 100 µl of 100% (w/v) TCA. The precipitate was washed with acetone (-20°C) and dried in a speedvac. Treatment with TFMS was then carried out according to Edge et al. [315]. To the dried pellet, 5 µl of anisole was added. The TFMS vial was placed on dry ice and opened while thawing. Forty-five µl of the newly thawed TFMS was mixed with the pellet, N_2 was immediately blown over the mixture, and the capped tube was put on ice. After 2 h, 0.9 ml of ether (-70°C) was added and the tube was transferred to dry ice. This was followed by the dropwise addition of 100 µl of pyridine (-70°C). After 10 min at -70°C, the tube was centrifuged, uncapped, for 15 min at 4°C. The supernatant was discarded and the pellet was dried in a speedvac. The pellet was dissolved in 0.8 ml of 1% Triton X-100, precipitated with TCA and washed with acetone (-20°C).

Endo H: TfR bound to protein A-Sepharose was heated at 100°C for 3 min in 100 µl of 50 mM sodium citrate, pH 5.5, containing 0.02% SDS. Endo H (4 mIU) was added to the TfR-protein A-Sepharose suspension and the mixture was inverted at 37°C for 20-24 h. Fifty µl of 3X concentrated sample buffer was added, and after heating for 5 min at 100°C, the supernatant was applied to the electrophoresis gel.

Endoglycosidase F/N-glycosidase F: TfR, bound to protein A-Sepharose, was incubated with 0.2 units of enzyme in 20 mM sodium phosphate, pH 7.0, containing 20 mM EDTA for 18 h at 37°C. After the incubation, the beads were centrifuged and suspended in sample buffer.

2.9 Electrophoresis and Autoradiography

Electrophoresis: Sample buffer (2% SDS, 10% β-mercaptoethanol, 10% glycerol in 0.0675 M Tris-HCl, pH 6.8) was added to protein A-Sepharose to elute the bound immunoprecipitates. To prepare membrane suspensions for electrophoresis, one-half volume of 3X concentrated sample buffer was added. All samples were heated for 10 min at 100°C for 5-10 min. For nonreducing gels (where noted), β-mercaptoethanol was omitted from the sample buffer. Proteins were resolved on 5-15% polyacrylamide gradient gels (except where noted) using the Laemmli system [316]. Gels were stained with 0.2% Coomassie brilliant blue R (in 50% methanol, 7.5% acetic acid) and destained in 10% methanol, 7.5% acetic acid. Carbohydrates were detected with periodic acid-Schiff reagent [317,318] as follows. The gel was soaked in 10% methanol, 7.5% acetic acid overnight, then in 10% acetic acid to fix the gel and remove SDS. The gel was soaked in 0.5% periodic acid (2 h), 0.5% sodium arsenite in 5% acetic acid (30-60 min), 0.1% sodium arsenite in 5% acetic acid (2x 20 min), 5% acetic acid (10-20 min), 0.1% sodium metabisulfite in 0.01 N HCl (2x 5 min), Schiff reagent (overnight). Finally, the gel was washed in 0.1% sodium metabisulfite in 0.01 N HCl for several hours. To prepare Schiff reagent, 2.5 g basic fuchsin was dissolved in 500 ml H_2O, then 5 g sodium metabisulfite and 50 ml of 1 N HCl were added. The solution was stirred for several hours and decolourized with activated charcoal.

Peptide mapping: Cleveland mapping [319] was performed as follows. Following electrophoresis and staining, the 94 or 88 kDa (deglycosylated) TfR band was cut from the gel, washed 2 x 2 min in Buffer A, placed on a 12% polyacrylamide gel, and overlaid with 10 µg α-chymotrypsin (51 units/mg). The gel was run at 20 mA until the sample reached the bottom of the stacking gel, at which point the current was stopped for 30 min. The remainder of the gel was run at 30 mA.

Autoradiography and quantitation: Gels were dried under vacuum and exposed to Kodak X-Omat AR film at -80°C. To detect ^{3}H and ^{35}S labelled proteins, the destained gels were treated with En^{3}Hance for 1 h, rinsed in water, and soaked in 3% glycerol for 30 min before drying. To quantitate the radioactivity of ^{125}I-labelled proteins, bands were excised from the dried gel and counted in a gamma counter. Labelled bands from En^{3}Hance-treated and dried gels were excised and placed in scintillation vials containing 150 µl of water. The cellophane and paper backing were removed, and 400 µl of Protosol was added to cover the gel pieces. The vials were put in a 37°C incubator for 24 h. Ten ml of Aquasol were added and the digests were incubated for a further 24 h. The vials were equilibrated at room temperature before counting. To quantitate stained gels and autorads, an LKB Ultroscan densitometer equipped with a 2220 recording integrator was used. Varying amounts of standard proteins (albumin, IgG, or ^{35}S-TfR) in adjacent lanes were also scanned to assess the linear response of the densitometer. All data presented as a gel or autorad are representative of at least three experiments unless noted otherwise.

Immunoblotting: After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol as described by Towbin et al.[320]. The membranes were blocked for 30 min with 5% skim milk in TBS (10 mM Tris, 140 mM NaCl, pH 7.5), then incubated

with 0.5 μg/ml anti-human band 3 or anti-glycophorin A antibody overnight at 4°C. The membranes were washed in TBS, blocked, and incubated for 1 h with goat anti-rabbit IgG conjugated to alkaline phosphatase. The blots were developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitroblue tetrazolium chloride (NBT).

2.10 Bio-Gel P6 Chromatography

Glycopeptides were prepared and separated as described by Romero et al.[321]. Membranes (from 0.5 ml cells) were extracted successively with chloroform/methanol (2/1, v/v), three times with water, twice with methanol, and finally with chloroform/methanol/water (10/10/3). The protein residue was digested for 40 h at 50°C with 2 mg/ml Pronase in 10 mM Tris-HCl, pH 7.4, containing 1 mM sodium azide. More pronase was added every 12-15 h. The resulting glycopeptides were chromatographed on a column of Bio-Gel P6 (1 x 105 cm) in 0.1 M pyridine-acetate, pH 5.0, containing 0.5 mM sodium azide. The eluate was pooled, dried in a rotary evaporator at 40°C, suspended in 0.5 ml sodium citrate, pH 5.0, and treated with 20 mU endo H for 22 h at 37°C. The sample was heated 5 min at 95°C, then reapplied to the column. One millilitre fractions were collected and aliquots were counted either in Beckman Ready Caps (Beckman Instrumentals) or in Aquasol.

2.11 Cell Fusion

Polyethylene glycol-mediated cell fusion: PEG-mediated fusion was carried out using the method for the production of hybridoma cell lines [322]. 0.1 ml (10^9) labelled reticulocytes and 10^7 cultured cells were mixed in 1 ml of 45% PEG 8000 in PBS (w/w) for 1 min at 37°C. Nine ml of PBS (37°C) were added slowly, with stirring, and the cells were incubated for 30 min at 37°C. Unfused

reticulocytes were removed with 5.0 ml of isotonic NH_4Cl buffer (0.12 M NH_4Cl, 1 mM NH_4HCO_3).

GP4F/Reticulocyte Fusion: GP4F cells (2-5 x 10^5) were plated on 20x100 mm dishes 2-3 days before the experiment. Cells were washed twice in 5 ml of serum-free DMEM or PBS, then incubated with 5 ml of 5 µg/ml trypsin and 0.1 mg/ml neuraminidase in DMEM for 15 min at room temperature to activate the hemagglutinin precursor, HAO [323]. To stop the enzymes, 5 ml of DMEM containing 10% fetal bovine serum were added, followed by three washes in DMEM or PBS. Five ml of a 0.2 to 1% suspension of reticulocytes in PBS, pH 7.4, were added to the plates and left for 30 min at room temperature with gentle agitation every 10 min. Unbound reticulocytes were aspirated off and the plates were washed 3 times in PBS. To fuse the bound red cells, 10 ml of 10 mM MES, 10 mM Hepes, 20 mM NaH_2PO_4, 0.154 mM NaCl, pH 4.5 at 37°C (fusion buffer) were added. Control cells were treated with PBS, PH 7.3, instead of fusion buffer. After 5 min at 37°C, the fusion buffer was replaced with DMEM containing 10% fetal bovine serum, and the cells were incubated for 60 min at 37°C. The cells were washed twice in cold PBS. Bound but unfused reticulocytes were removed by a 5 min wash in 3 ml of ammonium chloride buffer (0.155 M NH_4Cl in 10 mM Tris, pH 7.4, mixed 10:1 [v/v] with 10 mM NH_4HCO_3 just before use). The cells were washed twice in cold PBS and harvested by either using a rubber spatula or 0.05% trypsin/EDTA. The cells were lysed with 10% TCA and the precipitated proteins were collected on Whatman GF/B filters. The precipitate was washed with 10% TCA, then with ethanol and counted in liquid scintillation fluid.

2.12 Entrapment of Liver Extracts into Reticulocytes

Preparation of Golgi-containing extracts: Mouse liver (6.5 g) was homogenized in 0.25 M sucrose, 10 mM NaHepes, pH 7.4, and centrifuged at 12,000*g* for 10 min. The supernatant (7.0 ml) was passed through 4 layers of cheesecloth and centrifuged at 27,000*g* for 15 min. The resulting supernatant contained 4.7-7.0 nmol/h/ml galactosyltransferase activity.

Entrapment by dialysis lysis and resealing: This method, taken directly from Magnani et al.[324], was used to entrap enzymes into erythrocytes. A dialysis bag (Spectra/por 2 molecular porous membrane tubing MW 12,000-14,000) containing a 30-50% hematocrit of reticulocytes and 0.5 ml liver extract were put into a 50-ml tube containing 20 mM glucose, 10 mM $NaHCO_3$, 10 mM NaPi, pH 7.4. The tube was placed on its side and shaken gently on an orbital shaker for 60 min at 4°C. The lysed cells were removed from the dialysis bag and placed in a tube. One-tenth of the lysate volume of 10X concentrated resealing solution (5 mM adenosine, 100 mM inosine, 100 mM sodium pyruvate, 100 mM glucose, 12% NaCl, 100 mM NaPi, pH 7.4) was added slowly, while mixing. The resealed ghosts were incubated for 20 min at 37°C and washed in PBS.

2.13 Other Methods

ATP assay: The cellular ATP concentration was determined by the luciferin-luciferase method [325]. Ten µl cells were centrifuged through 50 µl dibutylphthalate/900 µl PBS and the supernatant was removed. The cells were lysed with 110 µl of water and extracted with 80 µl of 10% TCA. After centrifugation for 2 min, 150 µl of the TCA supernatant was mixed with ATP buffer (3 ml final volume). The ATP buffer contained 19 parts buffer A (0.1 M $Na_2HAsO_4.7H_2O$, adjusted to pH 7.6 with H_2SO_4) and 1 part solution B (0.4 M $MgSO_4.7H_20$) and was mixed just before use. To 0.5 ml of luciferase in a scintillation vial, 0.2 ml of sample in ATP buffer was added, and the vial was

counted for 10 sec in a Packard liquid scintillation spectrometer with the coincidence switched off. An ATP standard curve (log counts vs. log concentration) was also prepared.

Lactate dehydrogenase: Lactate dehydrogenase was measured by the method of Nielands [326]. Twenty µl of a 1:30 lysate was incubated in 1.0 ml of 25 mM Tris-HCl, pH 9.0, containing 1 mM nicotinamide adenine dinucleotide (NAD) and 12.5 mM lactate. The change in absorbance at 340 nm was monitored at room temperature (ε=6.22 $mM^{-1}cm^{-1}$).

Extraction of glycophorins: Glycoproteins were extracted by the method of Hamaguchi and Cleve [327]. To 1 volume of membranes suspended in 20 mM sodium phosphate, pH 7.0, 9 volumes of chloroform/methanol (2/1, v/v) were added. The mixture was centrifuged at 1600*g* and the supernatant (aqueous layer) was dried in a speedvac, dissolved in sample buffer, and subjected to SDS-PAGE.

Protein determination: Protein was estimated by a modification of the Lowry procedure for the determination of proteins in membrane samples, using bovine albumin as a standard. This method includes 1% SDS and replaces the K,Na tartrate with Na tartrate [328].

Extraction of total RNA: Cells were lysed in guanidine isothiocyanate and centrifuged through CsCl at 180,000*g* for 21 h [329]. The pellet was dissolved in 0.3 M sodium acetate, pH 5.0, and precipitated with ethanol [329]. The OD_{260}/OD_{280} ratios were read (≥2.0) to determine purity.

Northern blot analysis: Total RNA was separated on 1.2% formaldehyde/agarose and transferred to nitrocellulose by capillary action. The filter was baked for 2 h in a vacuum oven and hybridized with a 2.5 kb (EcoRV/XbaI) fragment of the TfR cDNA clone, pCDTR-1 [257]. The ^{32}P-labelled probe was prepared using the Pharmacia oligolabelling kit.

RESULTS

Chapter 3 Effect of Hemin and Iron on Transferrin Receptor Expression

When reticulocytes are incubated in vitro, they mature into erythrocytes (as they do in vivo) by losing the characteristics associated with reticulocytes [12,68-82]. The TfR, which makes up 1-2% of the plasma membrane proteins, is a characteristic of the reticulocyte but not the erythrocyte [6]. As a result, the number of TfRs in reticulocytes will largely depend on the maturity of the cells and be subject to factors which influence the maturation process. Both in vitro and in vivo, a population of extracellular vesicles can be found that contain TfR as well as other activities of plasma membrane origin derived from reticulocytes [87,90,77]. To obtain a better understanding of the mechanism involved in this process, we have been studying factors that may influence the rate of reticulocyte maturation. New protein synthesis is not required for reticulocyte maturation [Adam and Johnstone, unpublished results], and although maturation can occur in a chemically defined medium, the presence of serum (2%) increases the rate at which TfR-containing vesicles are formed [91]. Moreover, both reduction of the temperature of incubation and lowering of cellular ATP levels reduce the loss of cellular TfR and the formation of TfR-bearing vesicles [91,98]. These observations suggested to us that components, other than proteins still synthesized by the reticulocyte, might play a role in the maturation process.

Heme is known to play an important role in many aspects of reticulocyte metabolism and its synthesis persists in peripheral reticulocytes [236]. Heme has been shown to stimulate globin synthesis as well as to inhibit ubiquitin-dependent proteolysis [285,286,296,297]. Heme itself, as well as iron, has been implicated in decreasing the expression of the TfR in cultured cell lines [196-199,330].

Heme is also an important negative regulator of its own formation by controlling iron uptake from transferrin in the reticulocyte [236,238]. For these reasons, we were prompted to examine the influence of heme on the level of TfRs in maturing reticulocytes. In this chapter, we show that heme, not iron, increases the loss of preexisting TfR. However, heme also stimulates de novo synthesis of TfR, but the newly formed TfR is segregated from the pool of preexisting TfR, and its turnover responds preferentially to nonheme iron.

3.1 Hemin and reticulocyte maturation

Hemin stimulates externalization of TfRs during reticulocyte maturation

Figure 10 and Table 2 show that the addition of hemin to the culture medium increases the rate at which the TfR is lost from the cells. TfR content in the cells was measured in three ways: 1) ^{125}I-Tf binding to reticulocyte lysates, 2) immunoprecipitation of the TfR from ^{125}I-surface labelled cells, and 3) scanning of Coomassie blue-stained gels of immunoprecipitates of total TfR. All three methods have previously been used to quantify TfR content in sheep reticulocytes [331,312]. Because there is variation from day to day (and sheep to sheep) in the absolute rates at which TfR is lost, it is not possible to compare precisely any two experiments carried out on different days with different cells. Therefore, the results are expressed as a percentage of their initial control values, and the averages are presented. It is evident that the same trends are seen with all three independent assays.

During maturation of reticulocytes, vesicles are formed that contain the TfR, and there is a reciprocal relationship between cellular and vesicular TfR content [331]. Therefore, the presence of hemin in the medium should increase vesicle-associated TfR. The results in Fig. 10 and Table 3 show that this prediction is correct. Both ^{125}I-Tf binding and the immunoprecipitates from

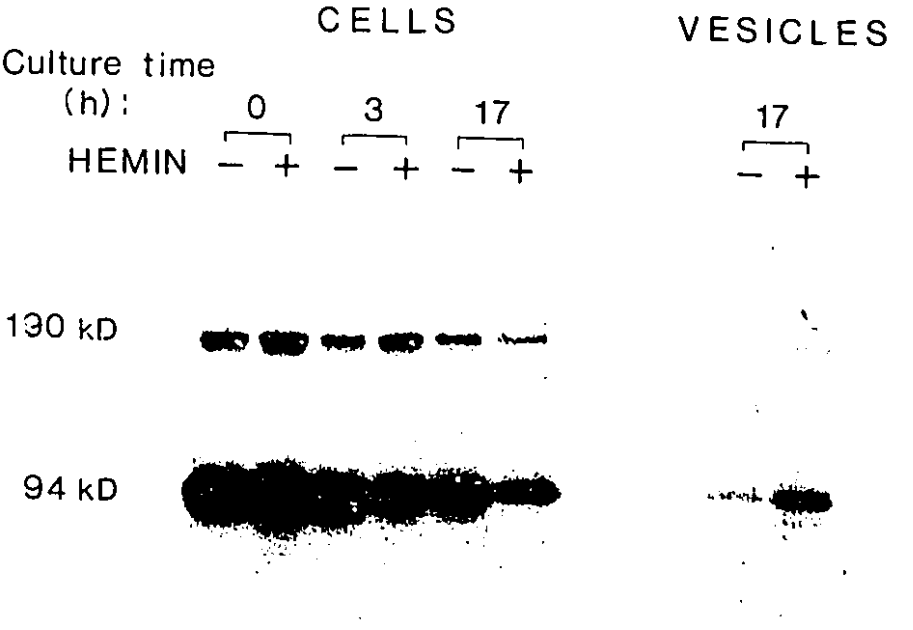


Fig. 10. Hemin stimulates externalization of ^{125}I-TfR

Reticulocytes were iodinated as described in Materials and Methods and suspended in culture medium containing 20 μM hemin (+) or an equivalent volume of PBS (-). After 0, 3, 17 h of culture, cells were centrifuged, washed and lysed. TfRs were immunoprecipitated from the cell membranes after solubilization. The vesicles were collected by concentrating the cell-free culture medium (after 17 h) in an Amicon ultrafiltration cell (YM100 membrane), and the vesicle TfRs were solubilized and immunoprecipitated.

Table 2. Effect of hemin on the loss of transferrin receptors during reticulocyte maturation

	A. ^{125}I-transferrin binding (% original binding)		B. Immunoprecipitation of ^{125}I-protein (% original activity)		C. Coomassie blue-stained gel (% original intensity)	
Culture time	- Hemin	+ Hemin	- Hemin	+ Hemin	- Hemin	+ Hemin
Short-term (1-5 h)	76 ± 9 (4)	50 ± 10 (4)	74 ± 16 (3)	39 (1)		
Long-term (8-17 h)	59 ± 9 (2)	33 ± 7 (2)	41 ± 6 (3)	27 ± 9 (3)	56 ± 20 (2)	27 ± 5 (2)

Reticulocytes were cultured without or with 20 μM hemin. At the indicated times, an aliquot of cells was used for ^{125}I-Tf binding (A) or immunoprecipitation (B,C) as described in Materials and Methods. To quantitate the loss of TfR protein in B, cells were iodinated before culturing. The immunoprecipitated ^{125}I-protein was separated by electrophoresis, and the excised bands were counted in a gamma counter. In C, the gel of nonlabelled immunoprecipitates was scanned with a densitometer. The values represent percent of initial TfR remaining after culture ± SD, where the number of determinations (n) = 3 or 4, or ±range, where n=2. ^{125}I-Tf binding has been corrected for nonspecific binding (<10% of total).

Table 3. Effect of hemin on the formation of vesicles containing the TfR

Method	- Hemin	+ Hemin
A. ^{125}I-transferrin binding to vesicles (cpm/vesicles from 10^9 cells)	43,600	131,500
B. ^{125}I-labelled receptor derived from ^{125}I-surface labelled cells (cpm/vesicles from 10^9 cells)	90	280
C. Coomassie blue stains of TfR immunoprecipitates from vesicles (relative density)	0.20	0.73

The cell-free medium after 18-24 h culture without or with 20 μM hemin was centrifuged at 100,000*g* or filtered under pressure. The TfR in the vesicles was measured by the three methods described in Table 2. Shown are typical results for each method, which was performed at least three times.

^{125}I-surface labelled cells are elevated in vesicles from cells cultured with hemin. Hemin has no direct effect on Tf or antibody binding (not shown). Replacement of hemin by FePIH or FeSiH [358,276], compounds which are known sources of iron for heme synthesis, does not appear to influence TfR loss (Fig. 11).

Hemin stimulates externalization of nucleoside transporters

A number of other membrane-bound cellular activities, including the nucleoside transporter, decrease during sheep reticulocyte maturation. The latter transporter is frequently assayed by equilibrium binding of the nucleoside analogue NBMPR [73]. To test whether heme has a similar effect on maturation-associated loss of the nucleoside transporter, we examined the effect of hemin on

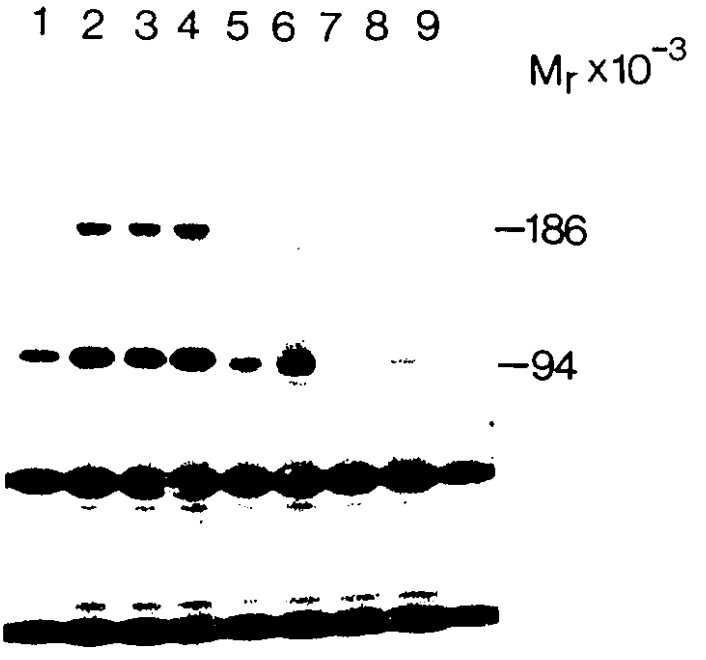


Fig. 11. Iron has no effect on externalization of preexisting TfRs

After incubation of reticulocytes for 18 h, with the additions below, plasma membranes (lanes 1-4) and exosomes (lanes 5-9) were harvested, immunoprecipitated, subjected to SDS-PAGE, and stained with Coomassie blue. Both dimer (186 kDa) and monomer (94 kDa) of the TfR are shown. The other two bands are characteristic of IgG. (1,6) 20 μM hemin; (2,7) 100 μM SIH; (3,8) 100 μM FeSIH; (4,9) 100 μM FeSIH + 1 mM DOH (inhibitor of heme biosynthesis); (5) no additions.

the loss of cellular NBMPR binding and its appearance in vesicles. The results show that greater NBMPR binding is recovered in the vesicles from hemin supplemented cultures and that cellular loss of binding is increased by hemin (Table 4). Lactate dehydrogenase levels do not change during maturation of sheep reticulocytes and the cellular levels are not influenced by hemin (activity = 1.2 ± 0.3 nmol/min/10^7 cells before and after culture ± heme).

Table 4. NBMPR binding to cell membranes and vesicles from reticulocytes cultured without and with 20 μM hemin

Culture time	Membranes (cpm/10^7cells)		Vesicles (cpm/10^7 cells)	
(h)	- Hemin	+ Hemin	- Hemin	+ Hemin
0	450 ± 70 (3)	405 ± 30 (2)		
17-22	362 ± 7 (3)	260 ± 20 (3)	30 ± 6 (3)	68 ± 15 (3)
43	275 ± 9 (2)	164 ± 8 (2)	40 ± 4 (2)	66 ± 3 (2)

Following culture, membranes were prepared from the cells, and vesicles were isolated from the medium by centrifugation. Washed membranes (~150 μg protein) or vesicles (~50 μg protein) were incubated with 5 nM [^{3}H]NBMPR (specific activity 50 dpm/fmol) for 30 min at room temperature in Tris-citrate buffer, pH 7.1, containing 1 mM EDTA. Nonspecific binding (≤15% of total binding) was measured in the presence of 10 μM unlabelled NBMPR. The values have been corrected for nonspecific binding. The means of two (2) or three (3) separate experiments, each done in duplicate, are shown, along with the range (2 experiments) or ±SD (three experiments).

It is unlikely that the effects of hemin on the protein content of the vesicle pellet are due to hemin's hemolytic effect on red cells [301]. If some lysis had occurred and contaminating plasma membranes accounted for the increased TfR in the 100,000*g* pellet, proteins characteristic of the plasma membrane would be detected in the pellet. The data (Fig. 12) show that with hemin there is an enrichment of the two major polypeptides characteristic of vesicles, the overall protein pattern being very different from that reported for the sheep plasma membrane [77]. Furthermore, control experiments have shown that most (~80%) of the plasma membranes released by cell lysis would have been retrieved by centrifugation at 7700*g* and thus could not account for the increased loss in cellular TfR seen with hemin. Direct measurements of cell lysis (loss of hemoglobin and lactate dehydrogenase) after culture with or without hemin also showed that overall lysis is under 10% and insufficient to account for the increased losses of Tf and NBMPR binding during incubation. Unlike other reports [301,332], hemin did not cause cell lysis in our culture system probably because sheep reticulocytes are less susceptible to hemolysis compared to erythrocytes and our culture medium contained cysteine and other compounds which are known to prevent cell lysis [301,302]. When reticulocytes were incubated with hemin in PBS (the buffer used by others [301,332]), we also observed significant cell lysis.

Differential loss of heme and globin synthetic capacities

Since reticulocytes undergo maturation in the absence of exogenous hemin, the cells either maintain a sufficient level of intracellular heme or heme is not absolutely required for maturation. A small pool of free heme is known to exist in reticulocytes [333]. This free heme may have a role during reticulocyte maturation. To determine whether sheep reticulocytes still synthesize heme, the

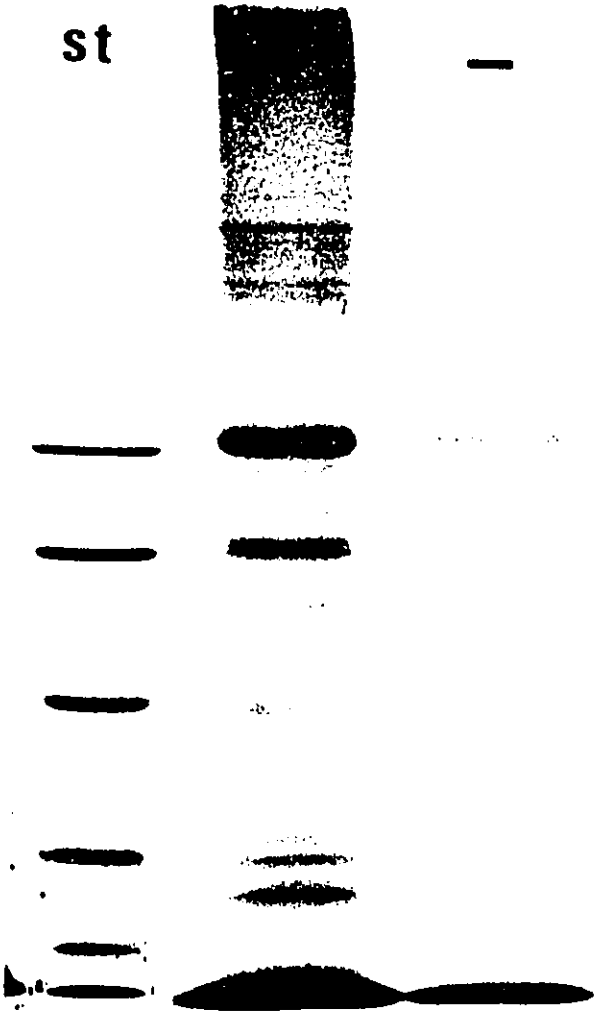


Fig. 12. Protein composition of vesicles derived from cells cultured with and without hemin

Reticulocytes (0.3 ml packed cells) were cultured with (+) or without (-) 20 μM hemin at 37°C. After 18 h, the cultures were centrifuged twice at 7700*g*. The cell-free medium was then centrifuged at 100,000*g*, and the resulting vesicle pellet was dissolved in SDS sample buffer for electrophoresis. Molecular weight markers (st) at left are 94,000, 67,000, 43,000, 30,000, 20,100 daltons.

incorporation of [2-^{14}C]glycine into heme was measured in cells after different times of incubation. After 2 h, the amount of heme synthesized is 65% of the initial level of synthesis while globin synthesis has fallen to ~20% (Table 5). Therefore, heme synthesis falls off less rapidly than globin synthesis, and the heme in excess of that bound to globin would exist as free heme and could influence the rate of maturation.

Table 5. Differential loss of heme and protein synthesizing capacity during reticulocyte maturation.

37°C Incubation	^{14}C - Heme		^{14}C - Protein	
(h)	cpm/10^7 cells	% Original	cpm/10^7 cells	% Original
0	15,000	100	62,300	100
0.5	16,700	111	41,300	66
1	12,600	84	19,800	32
2	9,800	65	9,400	15
4	5,900	39	6,900	11

Reticulocytes were cultured for the indicated times before labelling with [2-^{14}C]glycine for 1 hour. Heme and protein were separated in acidified acetone and processed as described in Materials and Methods. The values are from a typical experiment and are representative of four experiments.

3.2 Oxygen is required for maturation-associated loss of membrane functions

Since hemin has been implicated in the formation of oxygen free radicals and the oxidation of proteins [298,295], we asked whether the mechanism for the externalization of membrane proteins during reticulocyte maturation involves an oxidative modification catalyzed by hemin. If oxidation is required, depleting the culture medium of oxygen should inhibit the maturation process. Fig. 13 shows that cells cultured under 5% CO_2/95% N_2 do not release their TfRs in exosomes. When cells incubated anaerobically overnight are returned to an O_2 atmosphere, they release their TfRs, showing that the effect of O_2 deprivation is reversible (data not shown). Externalization of NBMPR binding sites is also inhibited by oxygen-depletion (Table 6).

Table 6. Effect of oxygen depletion on loss of NBMPR binding

	O_2	N_2
Original membranes	100	
Membranes after culture	60 ± 4	94 ± 12

Reticulocytes were cultured under 5% CO_2/95% O_2 or 5% CO_2/95% N_2 for 18 h. Cells were collected and membranes were prepared. Vesicles were harvested by centrifugation. NBMPR binding was measured as in Table 4. Results are expressed as % of original activity ± SD, where n=3.

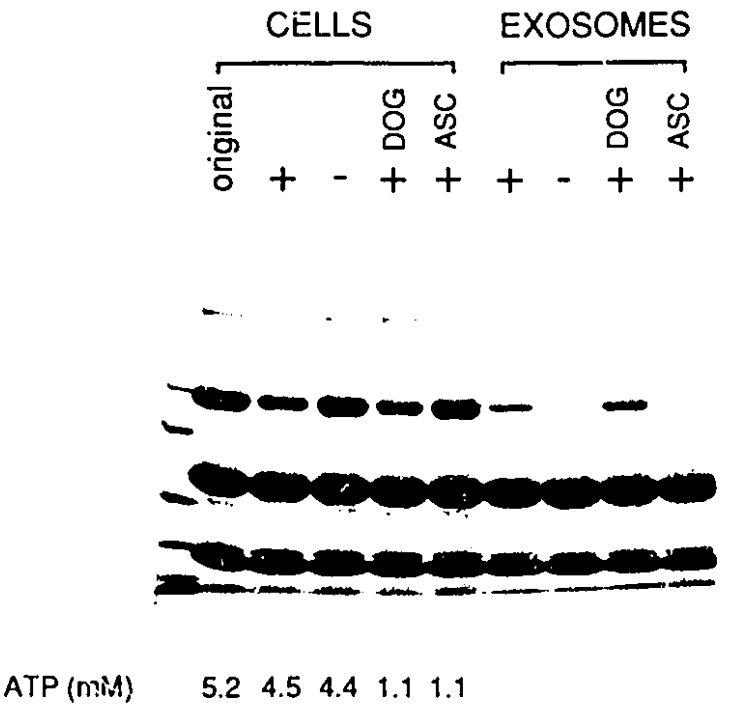


Fig. 13. Oxygen is required for TfR externalization

Reticulocytes were cultured for 18 h with (+) or without (-) O_2 and in the presence of 2 mM 2-deoxyglucose (DOG) or 1 mM ascorbate where indicated. For anaerobic cultures, the medium was gassed with 5% CO_2/95% N_2 before and after adding the reticulocytes. After culture, an aliquot of cells was extracted with TCA for ATP determination. The ATP values are given below each culture condition. The remainder of the cells were solubilized in Triton X-100 and used to immunoprecipitate TfRs. The cell-free culture medium was centrifuged at 100,000*g* and TfRs were immunoprecipitated from the exosomes. The gel was stained with Coomassie blue. Also shown are molecular weight markers (far left) and TfRs from reticulocytes before culture (original cells). The results with anaerobic cultures represent four experiments; the results with 2-deoxyglucose and ascorbate are representative of two experiments.

To show that the effect of oxygen-depletion is not due to a decrease in ATP, which is required for TfR externalization, the ATP concentration in the cells was measured after culture. There is no difference in the ATP level in cells cultured with or without O_2 (Fig. 13). Reticulocytes, like erythrocytes which lack mitochondria, can therefore maintain intracellular ATP through glycolysis. TfRs were externalized even when the cellular ATP concentration was lowered to less than 50% of the control with 2-deoxyglucose. This is in agreement with Pan and Johnstone [91], who found that a substantial decrease in ATP (>90%) must be achieved in order to inhibit the externalization of TfRs. A related ATP-dependent process, endocytosis of TfRs, was not inhibited until there was a large drop in the cellular ATP concentration [148].

A variety of antioxidants and free radical scavengers (vitamin E, ascorbate, γ-hydroxybutyrate) were tested for their ability to inhibit TfR externalization. Only ascorbate seems to have an effect similar to O_2 deprivation (Fig. 13).

3.3 Hemin stimulates TfR synthesis

In view of the effects of hemin on the loss of specific membrane proteins, particularly the TfR, we examined whether hemin influences *de novo* synthesis of the TfR in sheep cells. Reticulocytes from only one species (rabbit) have, to date, been shown to retain the capacity to synthesize the TfR [334].

In sheep cells, the addition of hemin has a pronounced stimulatory effect on ^{35}S incorporation into the TfR parallel to its effect on total protein formation. The increase in ^{35}S incorporation into the TfR is due to hemin and not to a change of cellular iron (Fig. 14). Whereas FePIH does increase *de novo* receptor formation (Fig. 14, lane 3), its effect is less than that of hemin (Fig. 14,

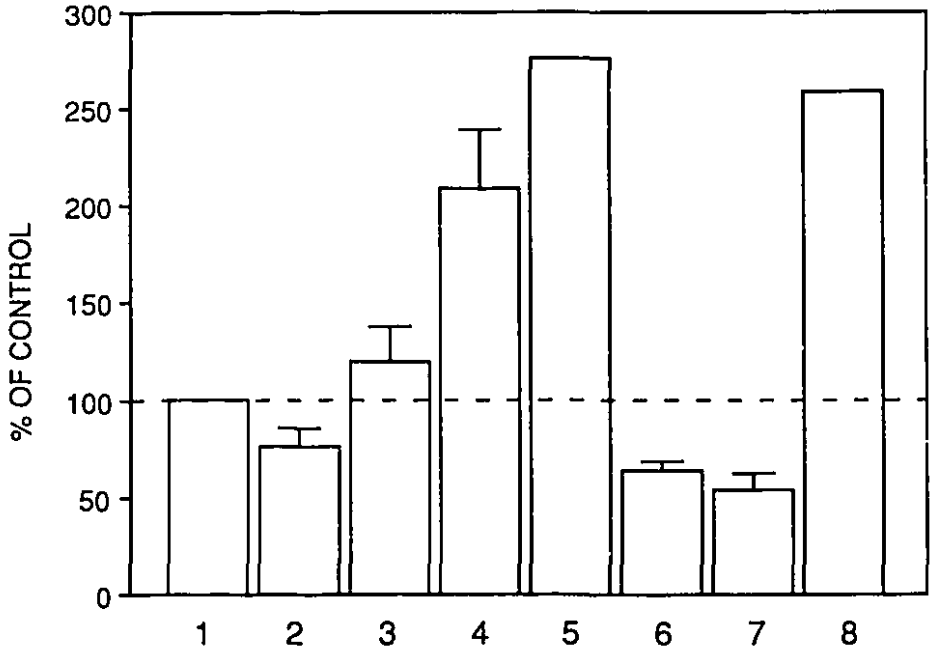


Fig. 14. Regulation of TfR synthesis by heme

Reticulocytes ($2x10^9$ cells) were incubated in 2.0 ml methionine-free media containing (1) no additions (control), (2) 1 mM PIH, (3) 1 mM FePIH, (4) 20 µM hemin, (5) 1 mM PIH plus 20 µM hemin, (6) 0.5 mM DOH, (7) 0.5 mM DOH plus 1 mM FePIH, or (8) 0.5 mM DOH plus 20 µM hemin. After 30 min, 20 µCi [^{35}S]methionine (1000 Ci/mmol) were added, and the cells were incubated for a further 2 h at 37°C. Receptor synthesis was compared by measuring the radioactivity in the 94 kDa (monomer) and 186 kDa (dimer) bands after electrophoresis of the immunoprecipitates. Synthesis is expressed as percent of control (1). The data are averages (±SD) of three separate determinations, except columns 5 and 8, which are single determinations.

lane 4) and can be blocked if heme formation is inhibited by 4,6-dioxoheptanoate (DOH; Fig. 14, lane 7). DOH alone also inhibits TfR synthesis (Fig. 14, lane 6), but the inhibition is overcome by hemin (Fig. 14, lane 8). Similar results were obtained when FePIH was replaced by sheep Tf (not shown). These results suggest that sheep reticulocytes do not synthesize enough heme to maintain maximal protein synthesis. In contrast, the addition of hemin to rabbit reticulocytes [334] did not stimulate either global protein synthesis or TfR synthesis when iron was available in the form of FeTf. The role of heme in stimulating TfR synthesis in rabbit reticulocytes was deduced from the inhibitory effect of DOH [334].

During incubation of sheep reticulocytes, the presence of hemin in the medium does not prevent the loss of the capacity to synthesize the TfR, although more ^{35}S incorporation is seen at any time if hemin is present (Fig. 15). The rate of decay of total protein synthesis as well as TfR synthesis in sheep cells is similar to that described by others for rabbit reticulocytes [285,51,334] if their data are expressed as in Fig. 15. It appears that incubated reticulocytes lose components of their protein synthetic machinery and this loss cannot be prevented by the addition of hemin. In contrast to the above studies, Schulman [335] found that rabbit reticulocytes after a 20 h incubation were capable of synthesizing protein at initial control levels when supplemented with exogenous hemin and concluded that the heme synthesizing capacity is lost before the protein synthetic capacity during reticulocyte maturation. This would mean that the initiation factor eIF-2 (the site of inhibition in heme-depleted medium) and other components of the protein synthetic machinery are present in 20 h incubated reticulocytes. The difference in these results could be due to an age difference in the rabbit reticulocytes used by Schulman [335] and sheep reticulocytes.

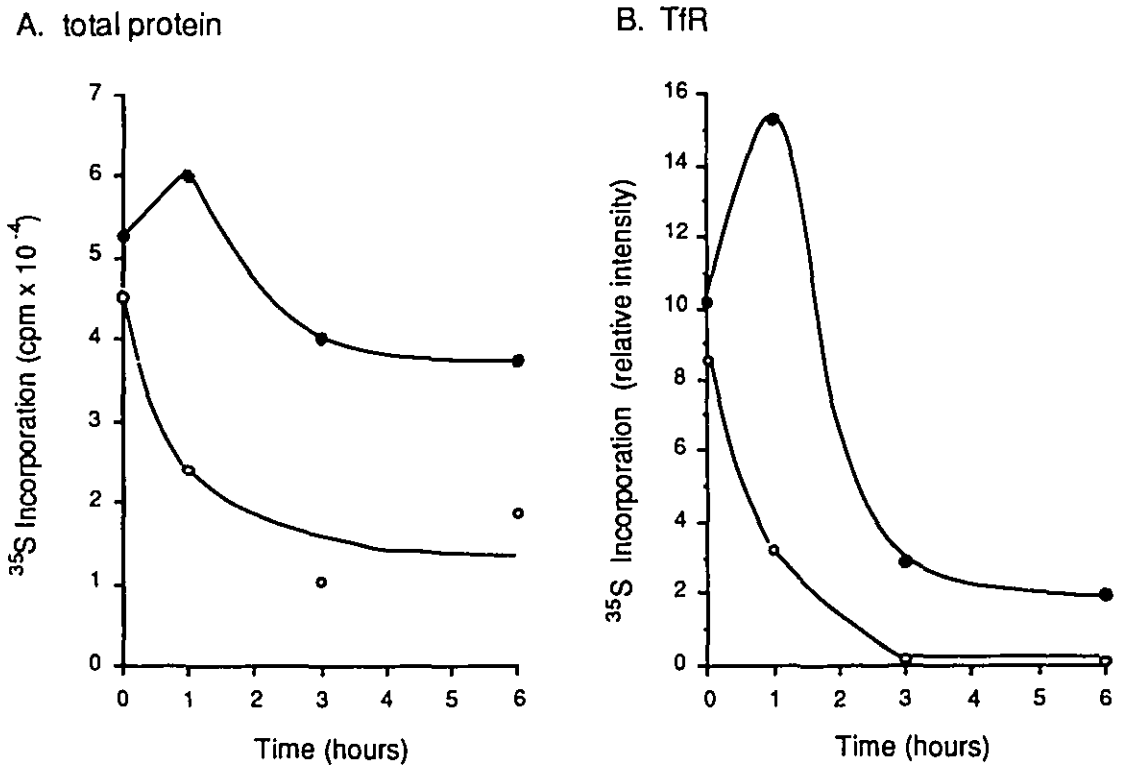


Fig. 15. Effect of hemin on the loss of total protein and TfR synthesis during in vitro incubation of sheep reticulocytes

Reticulocytes were preincubated for 30 min at 37°C without (o) or with (•) 10 μM hemin. At the indicated times, $2x10^9$ cells were labelled with [^{35}S]methionine for 90 min at 37°C, also in the absence (o) or presence (•) of hemin. Total protein synthesis (A) was measured by ^{35}S incorporation into TCA precipitates. TfR was immunoprecipitated and electrophoresed as described under Materials and Methods. The autoradiogram was scanned with a densitometer to measure receptor synthesis (B). Shown is a typical experiment, which was performed three times.

The rapid loss of the ability to synthesize TfRs may or may not be due to a depletion of TfR mRNA. To follow the rate of loss of TfR mRNA, Northern blot analysis was performed on total RNA isolated from reticulocytes before and after increasing periods of culture. A transcript of 5 kb was detected in fresh sheep reticulocytes using a human TfR cDNA probe [257], but the amount of message in the cell varied considerably after the reticulocytes were incubated at 37°C (data not shown). This was probably due to the very low level of TfR mRNAs and their increased susceptibility to degradation in incubated reticulocytes. The amount of total RNA isolated by the guanidine isothiocyanate method after increasing times of incubation agreed with previous A_{260} measurements of reticulocyte extracts [331], decreasing with a $t_{1/2}$ of 6-12 h during maturation.

3.4 Iron stimulates loss of newly synthesized TfRs

In contrast to preexisting TfR, the rate of loss of ^{35}S-labelled TfR from the cells is predominantly increased by iron. The data in Fig. 16 show that FePIH, even in the presence of DOH, is more effective than hemin in stimulating loss of ^{35}S-TfR during longterm incubation. Furthermore, the ^{35}S-TfR is not externalized (Fig. 17). It is evident from Fig. 17 that, after 18 h of incubation, more than 50% of the initial ^{35}S-TfR is still cell-associated. In contrast, earlier studies as well as the data in Table 2 and Fig. 17 indicate that the preexisting TfR is lost from the cell with a half-time of 6-12 h [87,331]. The different fates of the newly formed and preexisting TfRs and their differential responses to iron and hemin prompted us to determine what differences exist between these TfRs. The following chapter examines these differences.

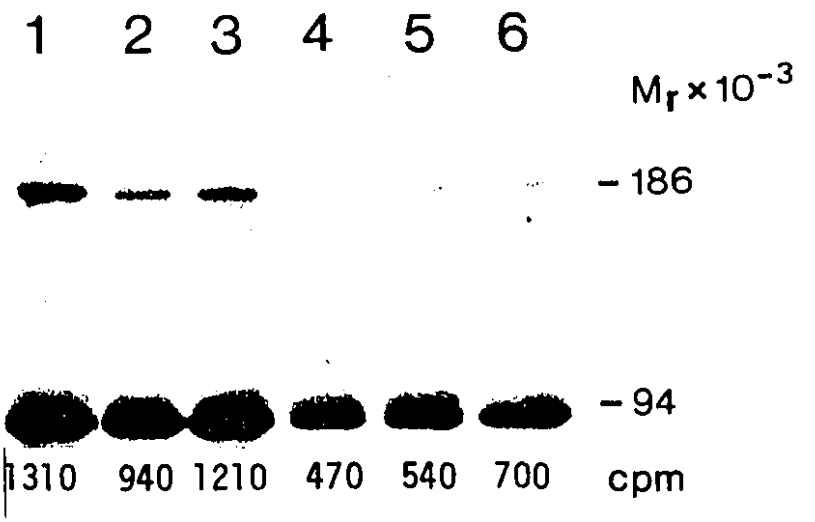


Fig. 16. Nonheme iron stimulates loss of ^{35}S-TfR

A 10% suspension of ~80% reticulocytes was labelled for 2 h with [^{35}S]methionine (lane 1), then chased for 24 h in media containing 2 mM unlabelled methionine and no further additions (lane 2), 0.5 mM PIH (lane 3), 0.5 mM FePIH (lane 4), 0.5 mM FePIH plus 1 mM DOH (lane 5), or 20 µM hemin (lane 6). Following immunoprecipitation and autoradiography, the gel bands corresponding to the TfR were excised, digested in Protosol, and counted. The resulting cpm are printed below the autoradiogram.

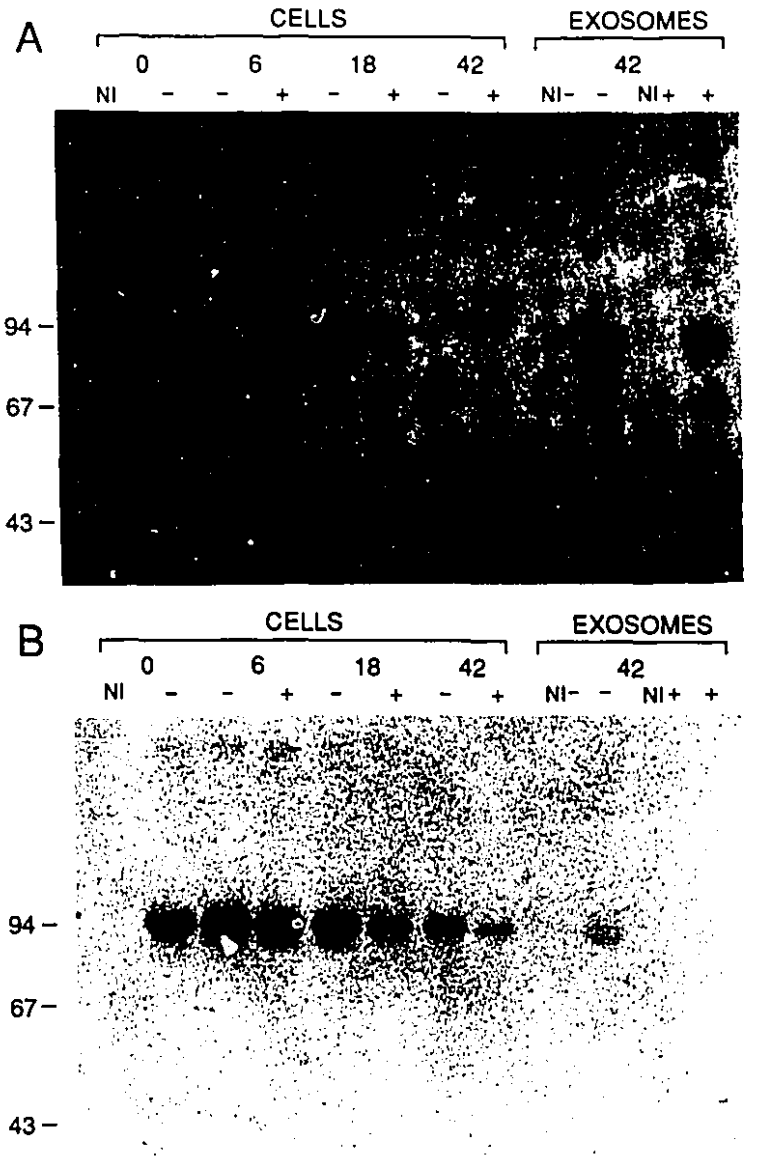


Fig. 17. Differential loss of newly synthesized and preexisting TfRs

Reticulocytes were labelled with [^{35}S]methionine and cultured without (-) or with hemin (+). Cells were harvested after 0, 6, 18, and 42 h. Exosomes (from twice the number of cells) were collected by centrifugation from the 42 h culture. TfRs were isolated using non-immune mouse IgG (NI) or anti-TfR antibody and detected by Coomassie blue staining (A) or autoradiography (B). Total TfRs are lost with a half-time of 6-12 h (A and ref. 87) and are partially recovered in the exosomes (A). ^{35}S-TfRs are lost with a halftime of ~24 h (B) and are not detected in the exosomes (B). The half-times were estimated by plotting the decrease in radioactivity versus culture time from > 4 experiments.

Summary

Hemin, but not iron, in the culture medium stimulates the maturation-associated loss of TfRs from sheep reticulocytes ($t_{1/2}$ for loss ~ 6 h) and their appearance in a population of externalized vesicles. A similar pattern is seen with NBMPR binding (a measure of the nucleoside transporter), where hemin increases the loss of binding activity from the cells during culture concomitant with an increase in nucleoside binding in the externalized vesicles. Sheep reticulocytes have retained the ability to synthesize TfRs, but ^{35}S-labelled TfRs are not detected in the released vesicles. Loss of ^{35}S-TfRs is slower ($t_{1/2}$ ~ 24 h) and is stimulated by iron rather than hemin. This difference in response of the native and ^{35}S-labelled TfR to hemin and iron supplements appears to be related to differences in the two TfRs.

Chapter 4 Incomplete Synthesis of Transferrin Receptors and Intracellular Localization

Although there is a net loss of TfRs, the mammalian reticulocyte continues to synthesize TfRs during the maturation period (Chapter 3 and ref. 334). The results in the previous chapter suggested that the newly synthesized TfR is processed differently from the preexisting TfR. Here, we examine the post-translational processing machinery of the maturing reticulocyte and its effect on the biosynthesis of the TfR.

4.1 Newly synthesized TfR is incompletely glycosylated

Newly synthesized and native TfRs comigrate after deglycosylation

The data in Fig. 18 show that the newly synthesized TfR does not comigrate with the native TfR on SDS gels but migrates as a polypeptide of 1~2 kDa less than the mature TfR. To highlight the difference in size between the preexisting TfR and the ^{35}S-TfR, the mobility of [^{3}H]palmitate-labelled, preexisting TfR was compared to the ^{35}S-labelled TfR on the same gel. The ^{3}H-acylated TfR comigrates with the Coomassie blue-stained TfR [261]. Pulse-chase experiments for up to 40 h failed to show the formation of ^{35}S-labelled TfR of mature size.

To assess whether the difference in size between labelled and preexisting TfR is due to glycosylation, the TfR was treated with TFMS, which leaves only one N-linked sugar attached to the polypeptide backbone [315]. After deglycosylation, the ^{35}S-labelled TfR comigrated with the Coomassie blue-stained deglycosylated, preexisting TfR (Fig. 19). Thus it appears that the peptide component of the newly synthesized TfR has the same mobility as the mature

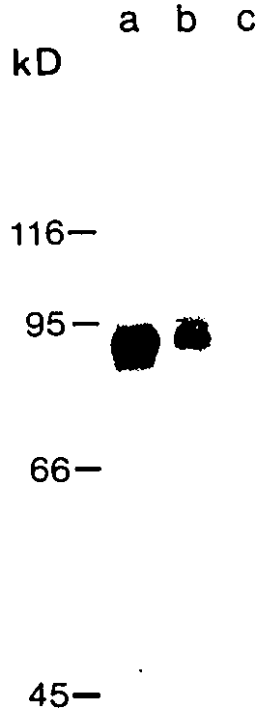


Fig. 18. The newly synthesized TfR does not comigrate with the preexisting TfR

One millilitre of a 20% reticulocyte suspension (>70% reticulocytes) was incubated for 2 h at 37°C with (a) 18 μCi [^{35}S]methionine (1 Ci/mmol), (b) 70 μCi [^{3}H]palmitic acid *plus* 18 μCi [^{35}S]methionine (100 mCi/mmol), or (c) 70 μCi [^{3}H]palmitic acid. TfR immunoprecipitates were prepared as described in Materials and Methods and separated on a 6-12% gradient gel.

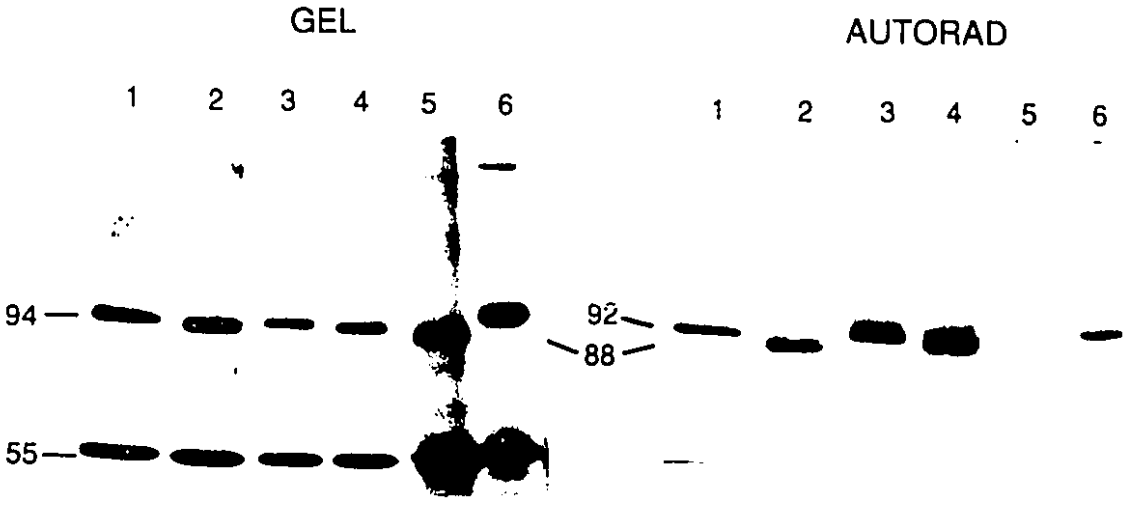


Fig. 19. Treatment of TfR with endoglycosidase H and TFMS

Reticulocytes were labelled with [^{35}S]methionine for 1 h, then processed immediately (lanes 1,2,5,6) or chased for 19 h (lanes 3,4). TfR immunoprecipitates (from 0.2 ml cells) were incubated without enzyme (lanes 1,3) or with 4 mU/100 µl endoglycosidase H (lanes 2,4) for 21 h at 37°C. For samples to be deglycosylated with TFMS, TfR was first eluted from the Sepharose beads with SDS, then treated with TFMS (lane 5) or untreated (lane 6) as described under Materials and Methods. The molecular weight estimates in kilodaltons are indicated. The IgG heavy chain migrates at 55 kDa.

TfR but that processing is incomplete.

To substantiate this conclusion we undertook Cleveland mapping of the TfR from [^{35}S]methionine labelled reticulocytes. It is evident in Fig. 20 that the peptide map generated by ^{35}S-TfR (lane 2) is different from that of the native TfR (lane 1). The difference in peptide maps is restricted to one or two peptides and the difference in peptide mobility disappears if the TfR is deglycosylated prior to mapping (compare lane 3 and lane 4 in Fig. 20). These data show that the original difference in TfR mobility as well as the mobility differences of the peptides were due to differences in the oligosaccharides attached to the polypeptide backbone. It is thus evident that the post-translational processing is deficient in these reticulocytes.

Newly synthesized TfRs contain high-mannose oligosaccharides

Human TfR contains both complex and high-mannose oligosaccharide chains [253,254]. To assess if sheep TfR also contains both types of oligosaccharides, we treated TfR immunoprecipitates with endo H which removes high-mannose and hybrid oligosaccharides [336] or TFMS (or endo F) which removes both high-mannose and complex oligosaccharides [315,337]. If the mature TfR contains both complex and high-mannose oligosaccharides, larger peptides will remain after endo H treatment than after endoglycosidase F or TFMS treatment. The results in Fig. 19A show that this is so, the TFMS product being smaller than the endo H product. However, with the ^{35}S-labelled TfR, cleavage with both endo H and TFMS yielded a product of the same size, ~88 kDa (Fig. 19B). Such a result is consistent with the conclusion that the newly synthesized TfR, unlike the native TfR, contains only high-mannose type oligosaccharides.

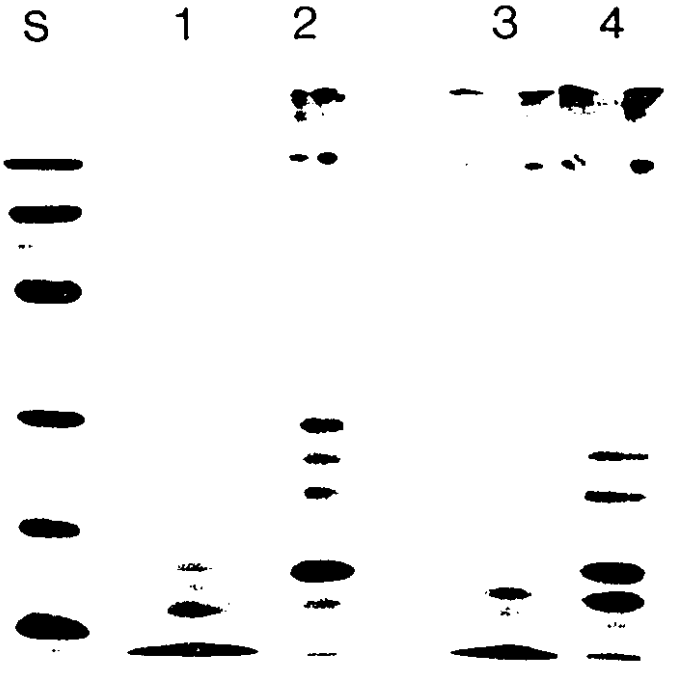


Fig. 20. Cleveland map of TfR before and after deglycosylation

TfR was isolated from reticulocytes that had been labelled with [^{35}S]methionine for 2 h. Half of the TfR (lanes 3 and 4) was treated with endoglycosidase F/N-glycosidase F for 18 h at 37°C before electrophoresis. Peptide mapping was performed as described under Materials and Methods using 10 μg α-chymotrypsin. Lane S, molecular weight markers (94, 67, 43, 30, 20, 14 kDa); lane 1, Coomassie blue stain of TfR peptide map; lane 2, autoradiogram of map shown in lane 1; lane 3, Coomassie blue stain of map of deglycosylated TfR; lane 4, autoradiogram of map shown in lane 3.

To judge whether the endo H-sensitive oligosaccharides on the ^{35}S-TfR are processed by the reticulocyte with time, the labelled cells were chased for 19 h before subjecting the TfR to endo H. After 19 h of chase, most of the ^{35}S-TfR was still more sensitive to endo H than the mature TfR (Fig. 19), indicating that high-mannose oligosaccharides on nascent TfRs are not efficiently processed by the maturing reticulocyte.

Newly synthesized TfR binds more tightly to Con A

To obtain additional experimental support for the absence of complex oligosaccharides in ^{35}S-TfR, studies with concanavalin A (Con A) were carried out. High-mannose oligosaccharides bind to Con A-Sepharose whereas biantennary complex oligosaccharides bind with a lower affinity and tri- and tetra-antennary complex oligosaccharides do not bind [338]. If ^{35}S-TfR has a higher number of high-mannose oligosaccharides than the native TfR, ^{35}S-TfR should be retained more avidly by Con A than mature TfR. Under our conditions, both the preexisting and the ^{35}S-TfR bound completely to the Con A column. A substantial fraction of the native TfR (30-50%) was eluted from the affinity column with 0.2 M α-methylmannoside. A much smaller fraction (≤3%) of the ^{35}S-TfR was removed under the same conditions (Fig. 21). Based on the amount of ^{35}S incorporated into TfR, the ^{35}S-TfR accounts for less than 0.1% of the total receptor. The relatively higher retention of ^{35}S-TfR by Con A, despite a much smaller absolute amount of ^{35}S-TfR suggests a tighter binding of ^{35}S-TfR due to the presence of a higher density of high-mannose chains in ^{35}S-TfR than in mature TfR. It is noteworthy that the small fraction of ^{35}S-TfR eluted with α-methylmannoside (Fig. 21, lane 3 of autorad) comigrates with the Coomassie blue stained native TfR. This is in contrast with the bulk of the ^{35}S-TfR which migrates at a lower molecular weight when compared to the native TfR.

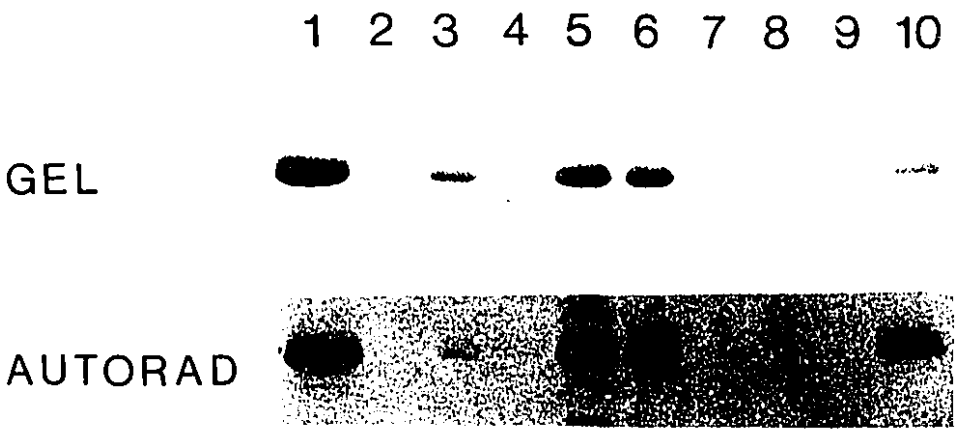


Fig. 21. Binding of TfR to concanavalin A-Sepharose 4B

Reticulocytes were labelled with [^{35}S]methionine for 1 h, then chased for 0 h (lanes 1-5) or 23 h (lanes 6-10). Triton X-100 extracts were mixed with concanavalin A-Sepharose and incubated overnight at 4°C. Lanes 1,6: total TfR in Triton X-100 extract; lanes 2,7: unbound TfR; lanes 3,8: TfR eluted with 0.2 M α-methylmannoside; lanes 4,9: TfR eluted with 0.5 M α-methylmannoside (after 0.2 M wash); lanes 5,10: protein eluted with SDS-ME sample buffer (following the α-methylmannoside washes). All fractions except those in lanes 5 and 10 were immunoprecipitated for TfR.

Since it is possible that formation of complex oligosaccharides is a slow process in maturing reticulocytes, and with time the TfR would be completed, we examined whether the ^{35}S-TfR could be chased into a form which bound Con A less avidly. The results in Fig. 21 show that there is no difference in the elution pattern of ^{35}S-TfR from a Con A column shortly after ^{35}S incorporation and after a chase of 23 h. These data are consistent with the conclusion that most of the newly formed TfR remains incompletely glycosylated and lacks complex oligosaccharides even after longterm incubation.

4.2 Sheep reticulocytes fail to process high-mannose oligosaccharides

If sheep reticulocytes largely fail to process mannose containing oligosaccharides on all newly formed glycoproteins, it should be possible to show that after proteolytic digestion of [^{3}H]mannose-labelled proteins, the end products contain only high-mannose residues. After labelling the reticulocytes with [^{3}H]mannose, the membranes were isolated and extracted with chloroform/methanol, and the residual proteins were digested with pronase. The resulting glycopeptides were separated on a column of Bio-Gel P6 (Fig. 22). Most of the ^{3}H-label was eluted in a single peak (K_{av} = 0.46). The eluate was treated with endo H and re-chromatographed on the same column. The ^{3}H-label eluted in a single peak, having an increased relative elution coefficient (K_{av} = 0.6). This result shows that the vast majority (≥90%) of the ^{3}H incorporated is in high-mannose oligosaccharides. If a substantial portion of the [^{3}H]mannose had been associated with glycopeptides containing complex oligosaccharides, then some fraction of the original ^{3}H-labelled glycopeptide should have been endo H resistant and eluted at K_{av} = 0.2-0.3. Such a result was obtained with reticulocytes from chick embryos. When total [^{3}H]mannose-labelled

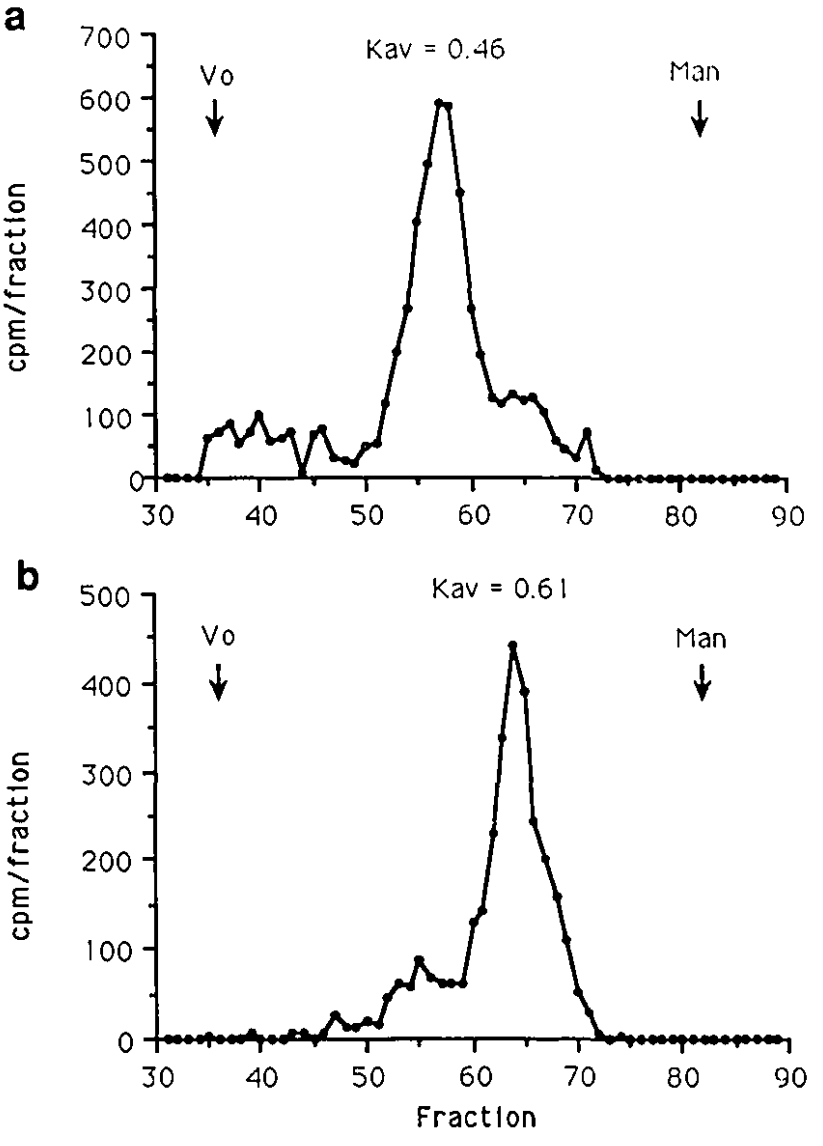


Fig. 22. Bio-Gel P6 chromatography of total [^{3}H]mannose-labelled glycopeptides from sheep reticulocytes

(a) Reticulocytes were labelled with [2-^{3}H]mannose for 4 h. Membrane proteins were digested with pronase and the resulting glycopeptides were separated on a column of Bio-Gel P6. (b) The eluate from the above column was pooled, concentrated, and treated with endoglycosidase H, then reloaded onto the column.

$$\text{Relative elution coefficient } (K_{av}) = \frac{V_e - V_o}{V_i - V_o}$$

where V_e = elution volume of oligosaccharide, V_i = elution volume of mannose, V_o = elution volume of bovine serum albumin.

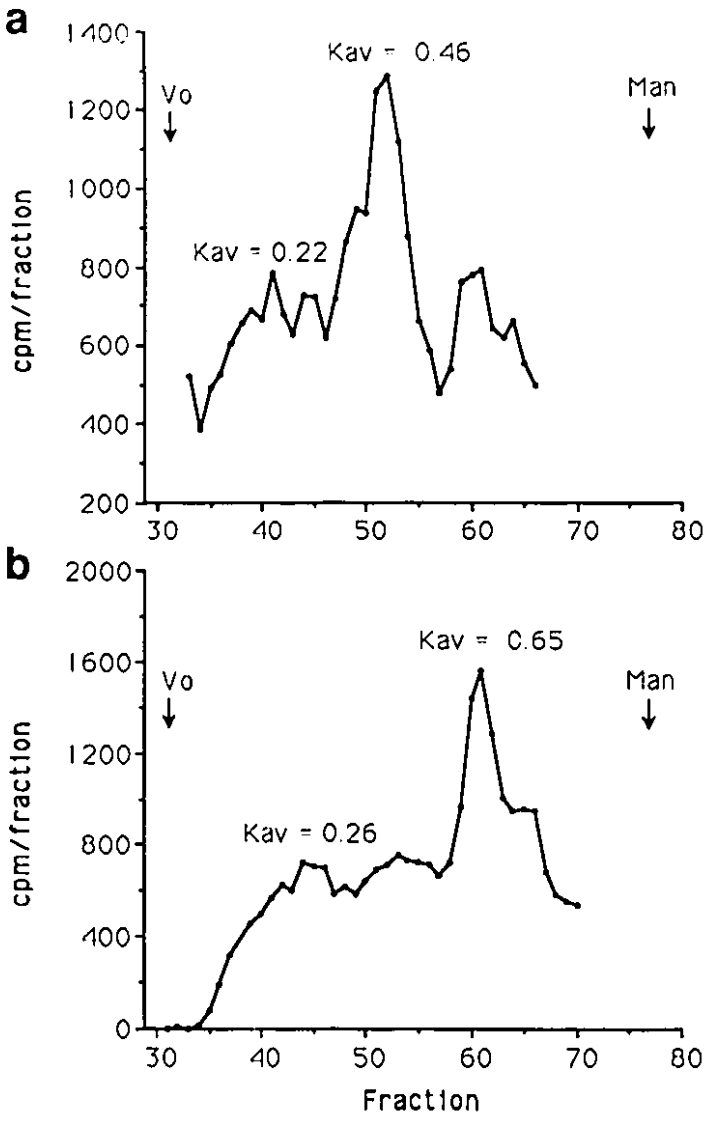


Fig. 23. Bio-Gel P6 chromatography of total [^{3}H]mannose-labelled glycopeptides from chick embryo reticulocytes

(a) Reticulocytes were labelled and the membrane proteins were digested with pronase as described under Materials and Methods. The resulting glycopeptides were chromatographed as in Fig. 22. (b) The eluate was pooled, concentrated, treated with endoglycosidase H, and reapplied to the column. K_{av} was calculated as in the legend to Fig. 22.

glycopeptides from avian reticulocytes were passed through the Bio-Gel P6 column, some of the ^{3}H-label eluted in a higher molecular weight fraction which was resistant to endo H (Fig. 23). Unlike sheep reticulocytes, these cells can process high-mannose oligosaccharides to complex oligosaccharides. There was insufficient mannose label in the TfR immunoprecipitates to assess directly whether the [^{3}H]mannose residues incorporated into the TfR are only in high-mannose oligosaccharides. In view of the data on total proteins, it is most likely to be so.

Additional experiments with sheep reticulocytes showed that the majority of the ^{3}H label from the mannose was incorporated into a series of peptides between 30 and 40 kDa (Fig. 24). [^{3}H]Mannose incorporation into these peptides was inhibited by cycloheximide, suggesting a requirement for de novo peptide synthesis. However, compared to the TfR, the 30-40 kDa peptides incorporated relatively little [^{35}S]methionine. The decrease in [^{3}H]mannose label during the chase period could be due to trimming of the high-mannose oligosaccharide chains on these proteins and/or a loss of these intensely glycosylated proteins whose presence is below the level of detection by [^{35}S]methionine labelling. These data show the continued synthesis in late stage sheep reticulocytes of highly glycosylated protein(s) whose identity has not yet been established.

4.3 Intracellular localization of newly synthesized TfR

Absence from the cell surface

Proteolysis of the cell surface with trypsin at 0°C reduces the cell-associated TfR (in Fig. 25A, compare lane 2 to lane 1 and lane 5 to lane 4) and releases 78 and 82 kDa fragments which are recognized by the anti-TfR antibody and are detectable by Coomassie blue staining (Fig. 25A, lanes 3 and 6) There is, however, no loss of ^{35}S from TfR after trypsin treatment (Fig. 25B, lanes 1-

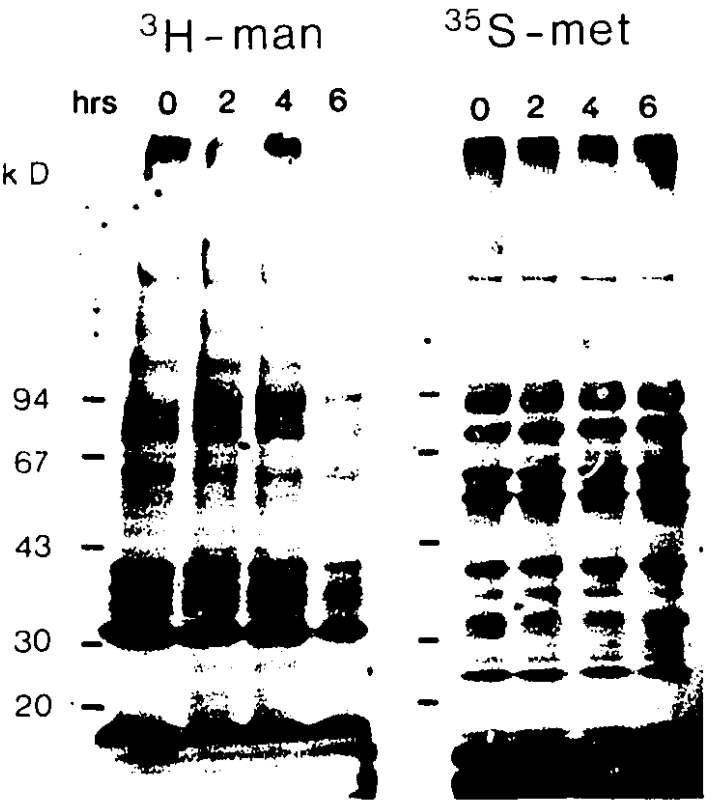


Fig. 24. Incorporation of [^{3}H]mannose into reticulocyte membranes

Left panel: A 30% suspension of reticulocytes was labelled with [2-^{3}H]mannose for 3 h. Ten volumes of medium containing 10 mM glucose were added and the cells were incubated for 0, 2, 4, 6 h as indicated. Membranes were prepared and dissolved in sample buffer for electrophoresis.

Right panel: Reticulocytes were labelled with [^{35}S]methionine as above, except that the chase medium contained 2 mM methionine.

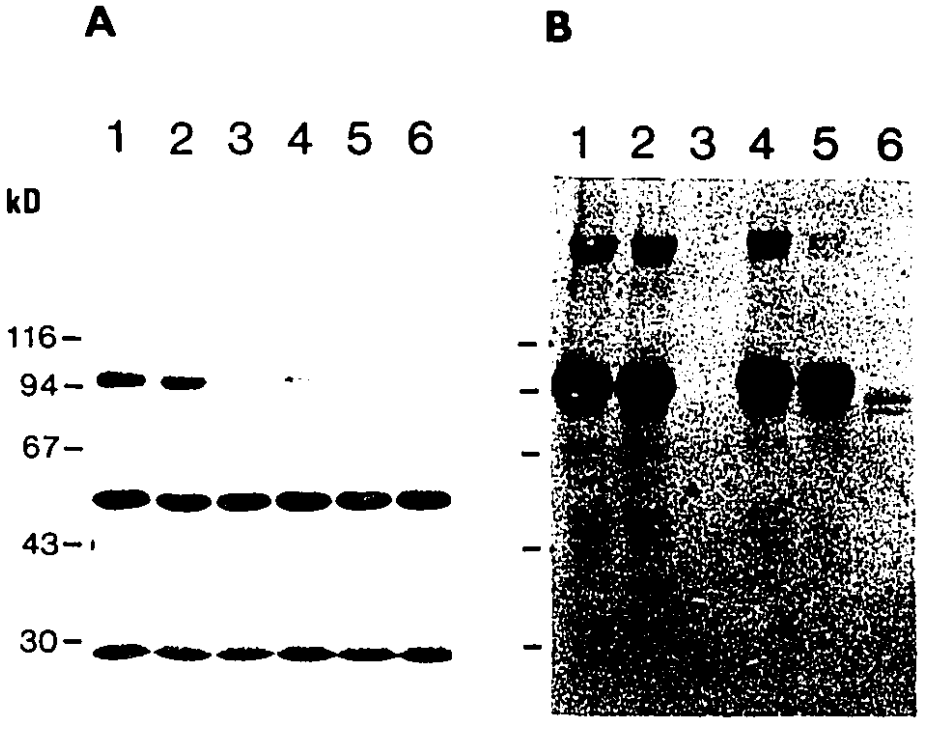


Fig. 25. Trypsin treatment of reticulocytes at 0°C

A 10% suspension of cells (~90% reticulocytes) was labelled with [^{35}S]methionine (25 μCi/ml) for 2 h at 37°C, then either trypsinized immediately at 0°C (lanes 1-3) or chased with unlabelled methionine (2 mM) for 28 h (lanes 4-6) before trypsin treatment. After digestion and addition of soybean trypsin inhibitor, the released fragments in the supernatant were immunoprecipitated with anti-TfR MAb, and the immunoprecipitates were subjected to gel electrophoresis. The cells were washed and lysed, and membranes were prepared. TfR was immunoprecipitated and subjected to gel electrophoresis. A: Coomassie blue-stained gel. Note the two characteristic IgG bands in addition to the TfR (94 kDa). B: Autoradiogram of A. Lanes 1,4: TfR from untreated cells; lanes 2,5: TfR from cells treated with trypsin; lanes 3,6: tryptic fragments released into medium from the cells shown in lanes 2 and 5, respectively.

3) Even after a chase with unlabelled methionine for 28 h, treatment with trypsin does not appear to reduce ^{35}S in cell-associated TfR (Fig. 25B, lanes 4 and 5) and the released (if any) ^{35}S-labelled tryptic polypeptides account for a maximum ~3% of the total ^{35}S incorporated into the TfR (Fig. 25B, lane 6) It appears that the small fraction of the newly formed TfR reaching the cell surface has acquired the mature size since the labelled and unlabelled peptides released by trypsin are identical in size.

Newly synthesized TfR does not recycle

To assess whether newly formed TfR is undergoing recycling, the cells were treated with trypsin at 37°C. At 0°C, only surface TfR is accessible to trypsin. At 37°C, trypsin should proteolyze the recycling TfR as it appears at the surface. Assuming that 15% of the total TfR is at the cell surface, and the recycling time is under 4 min [312], a 10 min treatment with trypsin at 37°C should remove ~40% of the recycling TfR. The results with the Coomassie blue-stained TfR indicated a loss of this order of magnitude (Fig. 26A). In contrast, an insignificant amount of ^{35}S-labelled TfR is lost (Fig. 26B) It cannot be argued that the ^{35}S-TfR is insensitive, rather than inaccessible, to trypsin, since addition of trypsin to isolated membranes or immunoprecipitates of the TfR resulted in digestion of ^{35}S-TfR(not shown).

When embryonic chick reticulocytes were labelled with [^{35}S]methionine and treated with trypsin in an identical manner, both ^{35}S-labelled and preexisting TfRs in the intact cell were susceptible to trypsin digestion (data not shown), implying that newly synthesized TfRs are transported to the cell surface in 13 day chicken reticulocytes.

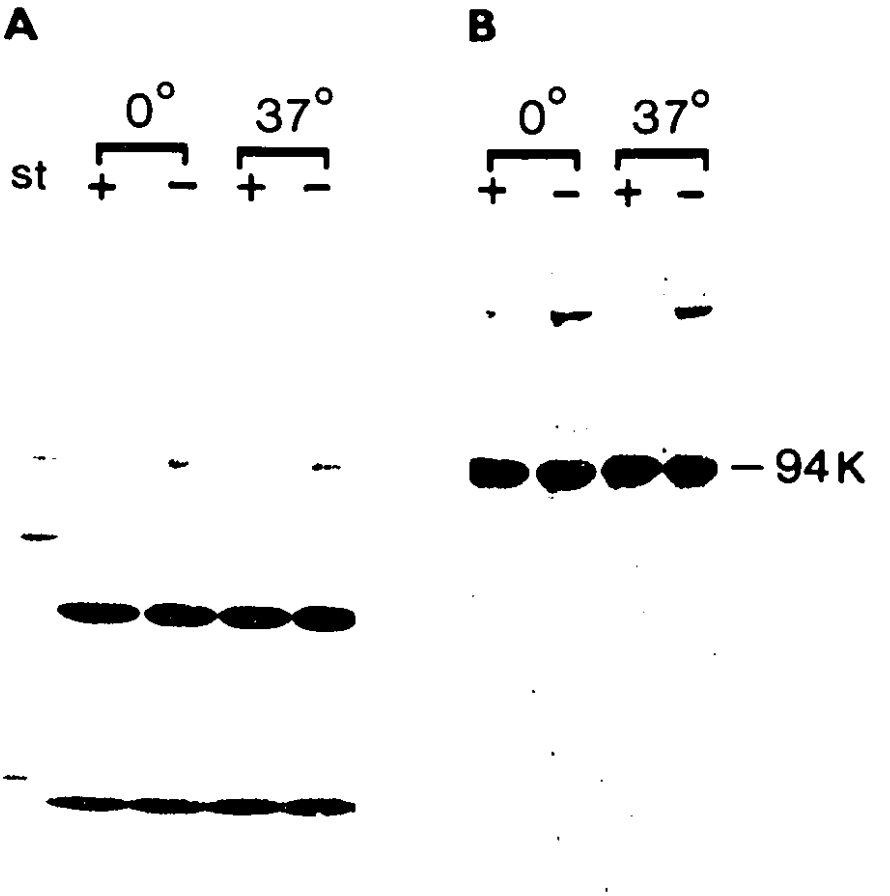


Fig. 26. The ^{35}S-TfR does not recycle

Cells were incubated with [^{35}S]methionine for 15 h, then treated with 0.1% trypsin at 0°C for 30 min or at 37°C for 10 min. The cells were washed, and immunoprecipitates were prepared and electrophoresed. A Coomassie blue-stained gel (A) and autoradiogram (B) of the TfR from control (-) and trypsin-treated (+) cells are shown. Molecular weight markers (st) are 94,000; 67,000; 43,000; 30,000; 20,100.

Separation of native and ^{35}S-TfRs using density gradient centrifugation

In the reticulocyte, TfRs are localized on the plasma membrane, in endosomes, and in multivesicular bodies. The surface TfR and the recycling TfR can be digested by treatment with trypsin at 37°C. The multivesicular body is the precursor from which exosomes are externalized. The presence of ^{35}S-TfR in multivesicular bodies may, therefore, be assessed by the appearance of ^{35}S-TfR in exosomes while the sensitivity to trypsin treatment at 37°C reflects the amount of TfR in endosomes and at the cell surface. The newly made ^{35}S-TfR does not appear to reach any of the above membrane compartments since ^{35}S-TfR is neither externalized in exosomes (Fig. 17) nor trypsin-sensitive at 37°C (Fig. 26). Since the ^{35}S-TfR lacks complex oligosaccharides, it is likely that the ^{35}S-TfR is retained in the ER or cis-Golgi membranes.

In attempting to separate internal membranes (ER, Golgi) from the membrane compartments containing preexisting TfR, reticulocyte membranes were subjected to density gradient centrifugation. Although we could not obtain a clean separation of these membrane compartments, the fraction containing the peak of ^{35}S-TfR was consistently of a higher density than that containing unlabelled TfR (Fig. 27). Galactosyltransferase, a Golgi enzyme, also peaked in the higher density membrane fractions and comigrated with ^{35}S-TfR, supporting the conclusion that the ^{35}S-TfR may be Golgi-associated or in membranes which comigrate with the Golgi. We could not assess whether the ER comigrates with the Golgi since very low levels of the ER markers (cytochrome c reductase, glucose-6-phosphatase) were present in the fractions.

It is unlikely that the absence of the complex sugars prevents the migration of the TfR to the plasma membrane and recycling pool, since it has been shown in other systems that incompletely glycosylated TfRs are transported to the cell

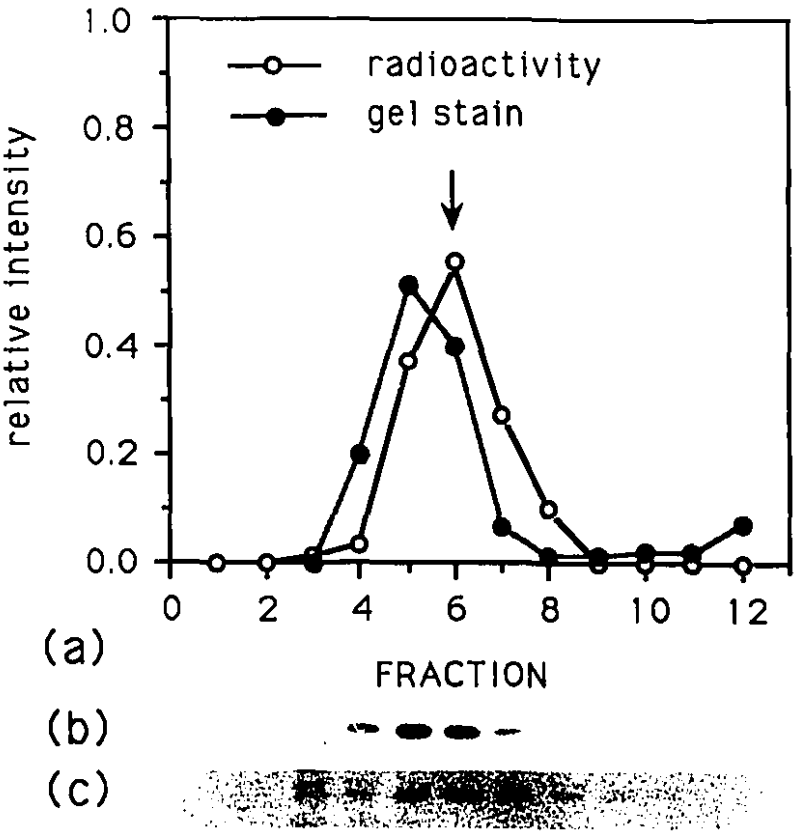


Fig. 27. Fractionation of membranes using a Percoll density gradient

The lysate from reticulocytes labelled 2 h with [^{35}S]methionine was applied to 20 ml of 20% (v/v) isoosmotic Percoll and centrifuged at 40,000g_{max} for 1 h. One-millilitre fractions were collected from the top and the TfR in each fraction was immunoprecipitated and separated by electrophoresis. The Coomassie blue-stained gel (b) and its autoradiogram (c) were scanned with a laser densitometer and the relative amounts of stained TfR (closed circles) and radioactive TfR (open circles) were plotted versus fraction number (a). The arrow indicates the peak of galactosyltransferase activity.

surface [166,264,268]. In K562 cells treated with deoxymannojirimycin (an inhibitor of Golgi mannosidase I), the newly formed TfR containing only trimmed, high-mannose oligosaccharides functions identically to the normal TfR and is transported to the cell surface within 3 h of synthesis [166]. This rate is similar to that of the transport of TfR in the control cells. Even with tunicamycin, where all glycosylation is prevented, some cell lines still export the TfR to the surface, albeit at a slower rate [253,267].

The ^{35}S-TfR, unlike native TfR, is not targeted to multivesicular bodies for externalization. This suggests that membrane proteins being targeted for externalization come from the recycling pool of TfRs and not from the biosynthetic pathway, i.e. ER or Golgi vesicles. The incompletely glycosylated TfR is retained intracellularly where it is slowly degraded by an iron-stimulated mechanism (Chapter 3).

4.4 Dimerization and transferrin binding of newly synthesized TfR

Work with viral proteins that are inserted into plasma membranes suggests that proper folding and oligomer formation are required for transport of proteins from the ER [115,116,121]. Using nonreducing gels, we determined that ~50% of the ^{35}S-labelled TfRs exist in dimeric form within 15 min of synthesis and that covalent dimerization is complete by 2 h (data not shown).

The newly synthesized TfR, moreover, is capable of binding transferrin (Fig. 28). When solubilized membranes containing ^{35}S-labelled TfR were absorbed to a transferrin affinity column, the same fraction of ^{35}S-TfR and total TfR was retained by this column. A similar procedure was used by Cox et al [334] to demonstrate the TfR binding capability of newly synthesized TfRs from

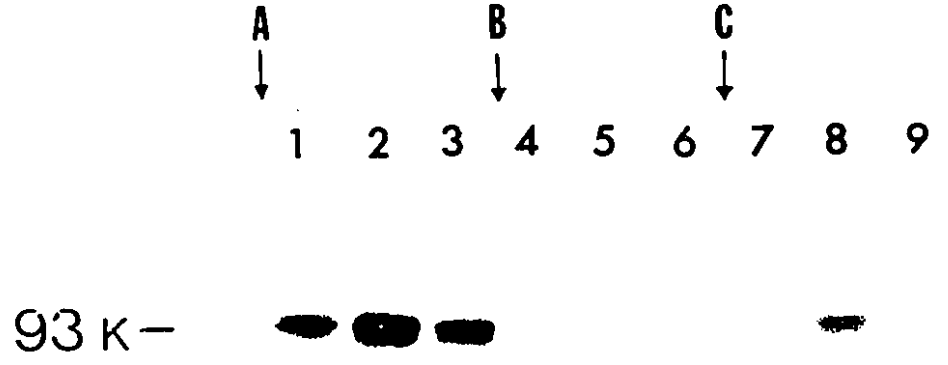


Fig. 28. Binding of newly synthesized TfR to Tf-Sepharose 4B

Cells were labelled with [^{35}S]methionine for 2 h at 37°C, and then the isolated membranes were solubilized in 1% Triton X-100. The TX-100 extract was centrifuged at 30,000*g* to remove insoluble material, and the supernatant was diluted with PBS to reduce the TX-100 concentration to 0.2% and mixed with 1 ml sheep Tf-Sepharose 4B and processed as described under Materials and Methods. The column was washed with PBS, pH 7.4, containing 0.2% TX-100. One millilitre fractions were collected (arrow A, lanes 1-3). Then the column was washed with 0.1 M citrate, pH 5.0, 0.2% TX-100, 50 µg/ml desferrioxamine (arrow B) to dissociate the iron from apotransferrin. Only the first three fractions of the extensive wash with desferrioxamine are shown (lanes 4-6). TfR was then eluted with 50 mM sodium phosphate, pH 7.0, 1 M NaCl, 0.2% TX-100 (arrow C, lanes 7-9). All fractions were neutralized with 1 M NaHepes, immunoprecipitated, and subjected to gel electrophoresis. The Coomassie blue-stained gel (not shown) is similar to the autoradiogram above.

rabbit reticulocytes. The data are consistent with reports by others that complex oligosaccharides on the TfR are not required for transferrin binding [267,268]. The ability to form dimers and bind transferrin suggests that the ^{35}S-TfR is folded properly and makes it unlikely that protein misfolding acts as a signal for ER retention. It is possible that another post-translational event such as O-linked glycosylation, phosphorylation or acylation is a prerequisite for transport to the Golgi and the ^{35}S-TfR has not acquired these modifications.

Many cytoplasmic enzymes are diminished or lost during red cell maturation [50]. The cytoplasmic protein(s) required for vesicle fusion from the ER to the Golgi, between Golgi cisternae and between endocytic vesicles, such as the NEM-sensitive factor (NSF) and SNAPs [124,125,339], may have been lost by the reticulocyte stage. Loss of NSF itself is unlikely since recycling of the TfR still takes place during this time. Using an antibody to NSF [123], we found that NSF is present in reticulocytes, as well as in mature red cells of the sheep (Fig. 29). Alternatively, intact Golgi and/or transport vesicles may be required for transport of newly synthesized proteins to the plasma membrane. EM sections of reticulocytes fail to show evidence of the usual Golgi stacks. Maturation may be analogous to the effect of brefeldin A, which blocks protein secretion in hepatocytes and intracellular transport of glycophorins in MEL cells [340,341]. Brefeldin A disperses the Golgi complex and induces a rapid redistribution of cis/medial-Golgi proteins to the ER [342-344]. The inability of the reticulocyte to complete the synthesis of membrane proteins may arise from a similar mechanism involving dispersal of the Golgi. The overall effect would be retention of newly formed glycoproteins in an intracellular compartment.

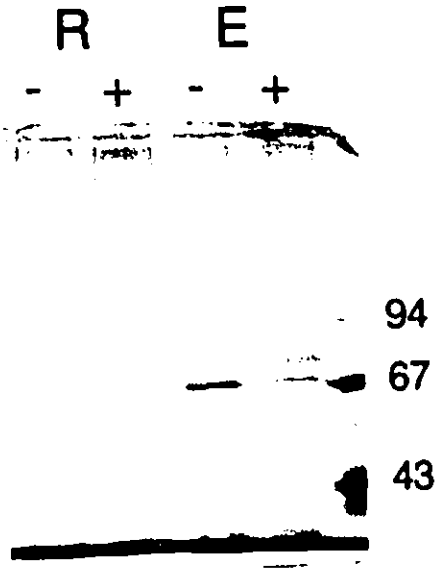


Fig. 29. Anti-NSF antibody reacts with a 76 kDa protein in sheep reticulocytes and erythrocytes

Reticulocytes (R) or erythrocytes (E) were lysed in 4 volumes of swelling buffer (20 mM Hepes-KOH, pH 7.4, 10 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 0.1 mM PMSF, 10 µg/ml leupeptin, and 1 µM pepstatin) and centrifuged at 12000*g* for 15 min. KCl was added to the supernatant to a final concentration of 0.1 M. 25 µl of magnetic beads (Dynabeads M-450) were coated with 0.75 µg anti-NSF (+) or mouse IgM (-) and incubated 4 h at 4°C with 50 µl cytosol. The beads were washed with 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris, pH 8.0. The proteins were eluted in sample buffer, separated on a 7.5% polyacrylamide gel, and visualized with silver stain.

4.5 Synthesis of other membrane proteins

Figure 30A shows that the pattern of [^{35}S]methionine-labelled proteins from the reticulocyte membrane (middle, lane 1) is different from that of total membrane proteins (left panel, lane 1), indicating that only a selected number of proteins are synthesized at the reticulocyte stage. Most noticeably absent in the ^{35}S-labelled membrane protein profile are the high molecular weight proteins, including spectrin, which are known to be synthesized early during erythroid cell differentiation [305]. Earlier work showed that rabbit reticulocytes still synthesize two major proteins (Mr ~55,000 and 36,000) which are localized on the cytoplasmic surface of the cell membrane. Since TfR and the anion transporter (band 3) migrate closely together on reducing gels, we used nonreducing gels to ascertain whether the anion transporter (a major protein of the erythrocyte membrane) is still being synthesized. Band 3 was detected by probing TfR-free Triton X-100 extracts of the plasma membrane with anti-band 3 antibody. After immunoprecipitation of the TfR, there is no ^{35}S-label at 94 kDa where band 3 is detected by immunoblotting (Fig. 30B). Therefore it appears that in sheep reticulocytes, as in reticulocytes of other species, band 3 is no longer synthesized.

To assess the extent of synthesis of glycophorins (highly glycosylated protein known to be present in red cell membranes), chloroform/methanol extracts of the membrane proteins were prepared. These extracts contain the majority of the PAS-positive glycoproteins (Lanes 1 and 2, PAS in Fig. 30A) but contain a minimal amount of ^{35}S (compare lanes 1 and 2, ^{35}S in Fig. 30A). These data support the conclusion that mature glycophorins are not synthesized de novo in sheep reticulocyte and is consistent with the TfR results. Only proteins with a very high carbohydrate content are extracted with chloroform/methanol and

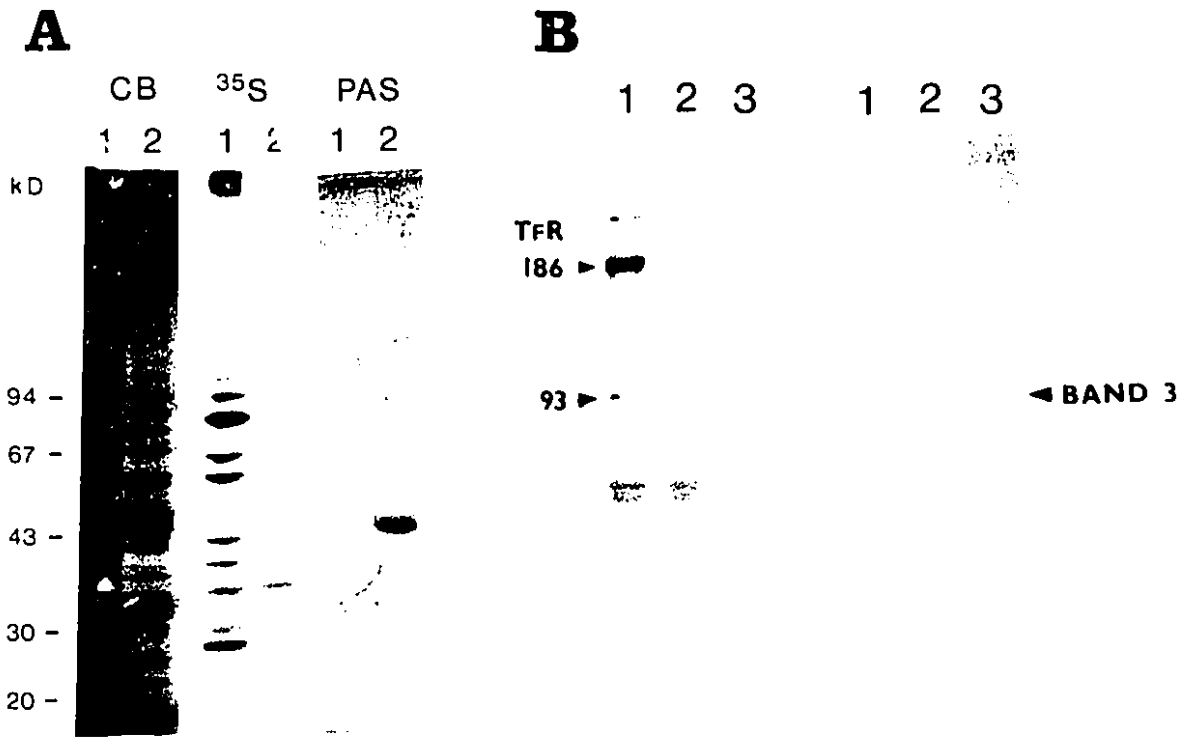


Fig. 30. Reticulocytes do not synthesize glycophorins nor band 3

(a) Detection of glycophorins in reticulocytes labelled with [^{35}S]methionine. Lane 1, total membranes from 40 µl of cells were loaded directly onto the gel; lane 2, membranes, equivalent to 200 µl of cells, were extracted with chloroform/methanol (2/1, v/v) and the glycoproteins in the aqueous layer were applied to the gel. The gel was either stained with Coomassie brilliant blue (CB) (left) or Schiff reagent (PAS)(right). The ^{35}S-labelled proteins were detected by autoradiography (middle). (b) Detection of band 3 in TfR-free extracts from reticulocytes labelled with [^{35}S]methionine. Electrophoresis was performed under nonreducing conditions by omitting β-mercaptoethanol from the sample buffer. Triton X-100 extracts were pretreated with nonimmune mouse IgG (lane 1) or anti-TfR antibody (lane 2). Lane 3 shows the TfR immunoprecipitate. Left, autoradiogram; right, Western blot probed with antibody to band 3.

detectable by PAS staining. If glycophorins are synthesized, they are unlikely to reach the Golgi apparatus which is the probable site of O-glycosylation for glycophorins [341,345], and therefore we would be unable to detect any ^{35}S-labelled polypeptides in the chloroform/methanol extracts.

A difference between human and sheep glycophorins should be mentioned. A peptide of 80 kDa reacting weakly with anti-human glycophorin A is detected in sheep reticulocytes; however, unlike human glycophorin A, the sheep protein is not extracted with chloroform/methanol indicating that it does not have the high sugar content necessary for partitioning into the chloroform/methanol layer. Since the polyclonal antibody reacts much more weakly with sheep membranes than with human red cell membranes, the 80 kDa sheep protein may in fact have only one epitope in common with human glycophorin A. The major sheep glycoprotein which is extracted with chloroform/methanol and stained with PAS is a 45 kDa protein (lane 2, PAS in Fig. 30A). This and other PAS-staining glycoproteins are not labelled with [^{35}S]methionine in sheep reticulocytes. Murine red cells also lack the major sialoglycoprotein of the human erythrocyte, and the major protein stained by PAS has a Mr of ~43 kDa [346].

Treatment of the reticulocyte surface with trypsin or chymotrypsin does not alter the mobility of the ^{35}S-labelled peptides, making it unlikely that any of these newly formed peptides are targeted to the plasma membrane (not shown). This is in agreement with others who have shown that the reticulocytes of rabbits and man synthesize several low molecular weight membrane proteins (≤90 kDa) which appear to be exposed only on the cytoplasmic surface of the plasma membrane [303,304,306,347]. One of these newly synthesized membrane proteins (43 kDa) comigrated with purified actin on a two-dimensional gel [347]. None of the other polypeptides were identified. We have studied the synthesis of a specific reticulocyte membrane protein and showed that the sheep reticulocyte

fails to insert new proteins into the plasma membrane. Contrary to our findings, Harris and Johnson showed that incorporation of [^{14}C]glucosamine into plasma membrane proteins of reticulocytes from phenylhydrazine-treated rabbits is sensitive to proteolytic digestion of the cell surface [348]. Cox et al [334] also noted that ^{35}S-labelled TfRs and ^{125}I-labelled TfRs of reticulocytes from phlebotomized rabbits comigrate during electrophoresis. Presumably rabbit reticulocytes are more immature and can synthesize and process membrane proteins to a later stage than reticulocytes obtained from phlebotomized sheep. Our experiments with avian reticulocytes showed that these cells may also be more immature and capable of final processing of membrane proteins.

4.6 Reconstitution of processing of newly synthesized sheep TfR

It appears that incompletely glycosylated proteins are held up in an intracellular compartment because the late stage sheep reticulocyte lacks the machinery to process newly synthesized membrane proteins. Reticulocytes may have lost the vesicles or proteins required to target newly synthesized membrane proteins from the ER to the Golgi apparatus, and to the plasma membrane. The ER and Golgi are among the many membrane functions which are lost during maturation of the reticulocyte into an erythrocyte. It may be possible to complete the processing of the reticulocyte TfR by introducing foreign microsomal and cytosolic components to the reticulocyte. Methods are available for loading red cells with proteins and fusion of red cells with cultured cells [349,350]. The two methods used were 1) fusion of reticulocytes with 3T3 fibroblasts expressing hemagglutinin [350] and 2) encapsulation of mouse liver cell extracts into reticulocytes by dialysis lysis and isotonic resealing [324]. These two sources of the protein processing machinery have the advantage that

the monoclonal antibody used to immunoprecipitate the sheep TfR does not recognize the mouse TfR. With positive results, an assay could be developed for studying factors required for the transport of nascent plasma membrane proteins through the internal membrane network en route to the surface membrane.

Fusion of reticulocytes and GP4F cells

To determine whether the processing machinery of another cell can complete the synthesis of the sheep TfR, we chose hybrid cell fusion. In initial experiments, reticulocytes were fused to YAC mouse lymphoma cells using polyethylene glycol (PEG). Unfused red cells were removed with isotonic ammonium chloride which selectively lyses red cells but not nucleated cells [308]. Cell fusion was monitored by the incorporation of radiolabelled reticulocyte proteins. Greater radioactivity was incorporated into the PEG-treated cells. Following this procedure, we could see that the pellet containing PEG-treated but not that containing untreated cells was red in colour, indicating that hemoglobin from the reticulocyte had been incorporated.

GP4F cells are mouse 3T3 fibroblasts expressing the hemagglutinin (HA) antigen of the influenza virus and can fuse with red cells with high efficiency [350]. Mild trypsin treatment of GP4F cells cleaves the hemagglutinin precursor HAO to active HA and prepares the cell for pH-dependent fusion with erythrocytes. Microscopically it was seen that sheep reticulocytes bound to GP4F cells. There was pH-dependent incorporation of ^{35}S-labelled proteins into GP4F cells (Table 7), with a reticulocyte/GP4F cell ratio varying from 1/1 to 1/10 (i.e. one reticulocyte fusing to one in ten GP4F cells, on average). However, the incorporated ^{35}S-labelled protein was not stable in the fused cell (Table 8), decreasing to 50% after 1 h of incubation, suggesting a half-life of about 1 h. The half-life of ^{35}S-labelled sheep hemoglobin in the intact

Table 7. pH dependent fusion of reticulocytes and GP4F cells

Active HA	pH	Incorporation of ^{35}S-proteins (cpm/ $2x10^6$ GP4F cells)	Protein recovered after fusion (mg)	Incorporation of ^{35}S-proteins (cpm/mg GP4F protein)
+	4.3	2660	0.14	19,000
+	4.5	1930	0.10	20,300
+	4.8	2910	0.26	11,200
+	7.3	1480	0.30	4,900
-	4.3	940	0.17	5,500
-	4.5	1100	0.22	5,000
-	4.8	1230	0.41	3,000
-	7.3	1420	0.43	3,300

Reticulocytes were labelled with [^{35}S]methionine (50,000 cpm incorporated/10^7 reticulocytes) and fused to 3T3 fibroblasts expressing hemagglutinin (HA) (GP4F cells) as described under Materials and Methods. The GP4F cells were either untreated (-) or treated with 5 µg/ml trypsin to activate HA (+). Only cells with active HA should fuse with reticulocytes when the pH is lowered and incorporate ^{35}S-proteins. Since the number of cells recovered after the fusion procedure was variable, the results are expressed per mg of total protein recovered as well as per original number of GP4F cells. This experiment was repeated once and similar results were obtained.

Table 8. Labelled reticulocyte proteins are not stable after cell fusion

pH	"Post-fusion" incubation (min)	Incorporation of ^{35}S-proteins (cpm/2x10^6 GP4F cells)
4.5	30	2860
4.5	60	1990
4.5	120	1010
7.3	60	600

Reticulocytes were labelled and fused to GP4F cells as in Table 7. Following exposure to pH 4.5 buffer, the cells were incubated in culture medium (pH 7.4, 37°C). The incubation time was varied from the standard 30 min to 120 min. The amount of radiolabelled protein incorporated was then determined. This experiment was repeated once with similar results.

reticulocyte is ~50 h. Hendil [349] reported that the degradation of radioactively labelled rabbit hemoglobin was increased 20-fold when introduced into hamster fibroblasts by virus-mediated fusion and that the half-life of the rabbit hemoglobin in the fibroblast was 28 h. However, human hemoglobin had an unchanged half-life of about 150 h in mouse and hamster fibroblasts [349]. Thus, there appears to be a difference in the stabilities of hemoglobins of different species.

More ^{35}S-labelled TfR was immunoprecipitated from the fused reticulocyte-GP4F cells than from control cells (maintained at pH 7.4). To determine whether the sheep TfR in the fused cells was transported to the cell surface following fusion, half of the cells were treated with trypsin and the other

half (untreated cells) were detached from the dishes with a scraper. ^{35}S-TfRs on the surface of fused cells would be digested by trypsin while unprocessed TfRs in fused cells would be protected from trypsin. These results showed that there was a considerable decrease (~50%) in the total radioactivity associated with trypsin-treated cells versus that of untreated cells, indicating that trypsin had access to intracellular proteins. From these experiments, it is not clear whether the reticulocyte-GP4F cells were leaky or whether a major portion of the reticulocyte proteins were adsorbed to the surface of GP4F cells. It is evident that trypsin sensitivity could not be used to measure the cell surface localization of the ^{35}S-labelled sheep TfR.

Encapsulation of mouse liver extracts by hypotonic lysis and resealing

While hybrid cell fusion could tell us whether the TfR that is translated by the reticulocyte can act as a substrate for foreign processing enzymes, we also sought to define the components of the processing pathway which are missing in the sheep reticulocyte. For this objective, the use of hypotonic lysis and resealing would serve to enclose specific functions into the ghosts. This method has been used to encapsulate drugs, hexokinase and other proteins into erythrocytes and reticulocytes [351]. It is not known whether membrane vesicles and membrane proteins can be inserted into the red cell this way.

We started with a crude liver extract for entrapment into the sheep reticulocyte. If the sheep TfR changes its size and/or is targeted to the surface of the resealed ghost, then the crude extract could be fractionated with the goal of identifying the factor which is missing in the reticulocyte. We chose the mouse liver cell as the source for microsomes and cytosol because it is easily obtained and known to contain the machinery for glycoprotein synthesis. The marker we

used to monitor entrapment of Golgi-associated proteins was the enzyme galactosyltransferase (GT). There was a significant increase of GT in the resealed ghost following hypotonic lysis and resealing of erythrocytes (0.7 nmol/h/ml cells), but the change in GT activity in reticulocytes upon entrapment was not readily quantifiable because the endogenous GT activity in reticulocytes was 4 times greater (2.5 nmol/h/ml cells). When the liver extract was added to erythrocytes or reticulocytes in an isotonic solution, there was no increase of GT. A difference in response of erythrocytes and reticulocytes to the lysis/resealing method was apparent from the recovered volume of resealed ghosts. The yield of resealed reticulocytes was 80% while only 10% of the erythrocyte packed cell volume could be recovered as resealed ghosts following dialysis lysis and resealing. This suggests that reticulocytes do not lyse as well as erythrocytes which may make them poor acceptors of exogenous enzymes.

Although glycosyltransferases are membrane-bound proteins found in the Golgi, active soluble forms have been found [377]. The encapsulated GT in these reticulocytes was probably membrane-bound since the activity was found in the pellet after centrifugation of the liver extract at 100,000*g*.

Following encapsulation and incubation for 1 h at 37°C to allow processing to take place, the resealed cells were treated with trypsin to determine whether any of the ^{35}S-TfR was transported to the cell surface. The liver extract had no effect on either the size or the protease accessibility of the ^{35}S-TfR. The Coomassie blue stained preexisting TfR was digested by trypsin treatment at 37°C whereas the newly synthesized ^{35}S-TfR was still intracellular and not cleaved by trypsin. Therefore, the liver extract components entrapped by this procedure were unable to process the TfR that was incompletely synthesized by the sheep reticulocyte. Further studies are needed to assess whether better

success is attainable by encapsulating a more concentrated (purified) extract, e.g. microsomes.

Summary

Sheep reticulocytes synthesize an incompletely glycosylated TfR which is not transported to the plasma membrane. The ^{35}S-TfR is 1~2 kDa smaller than the native TfR and fails to acquire a mature size even when chased for 24 h. Deglycosylation obliterates the size difference. There is preferential binding of the ^{35}S-TfR to Con A-Sepharose, indicating the existence of a higher density of mannose sugars on the ^{35}S-labelled TfR. Moreover, when total [^{3}H]mannose-labelled glycopeptides from reticulocytes are separated on a column of Bio-Gel P6, the [^{3}H]mannose associates with endo H-sensitive high-mannose oligosaccharides, but not with complex oligosaccharides. After Percoll density gradient centrifugation, the ^{35}S-TfR peaks in a fraction which has separated from the bulk of the native TfR. These results and the observation that the ^{35}S-TfR of intact cells is inaccessible to exogenous trypsin at both 0°C and 37°C support the conclusion that the ^{35}S-TfR is retained in a nonrecycling compartment. Nevertheless, the ^{35}S-TfR binds Tf and forms intersubunit disulfide bonds. The transmembrane glycoproteins, band 3 and glycophorins, are not synthesized in the sheep reticulocyte. It appears that the reticulocyte, at this stage of red cell development, has lost the vesicles and/or proteins which are required to transport proteins from their site of translation to the cell surface.

Chapter 5 The Origin of a Soluble Truncated Transferrin Receptor

Truncated TfRs have been found in the circulation of man and have been associated with iron-deficiency anemia and red cell production [101,103]. The mechanism for the release of soluble truncated TfR is not known. Because of the potential clinical importance of measuring serum TfR levels, there is interest in determining the source of truncated TfRs. The majority of the circulating non-cellular TfR was identified as a cleaved receptor representing the exofacial domain of ~85 kDa on nonreducing gels [102]. The cleavage occurred distal to the pair of disulfide bonds (Cys_{89} and Cys_{98} [256]) between residues Arg_{100} and Leu_{101} [102].

In contrast to the human samples, the sera from anemic sheep and rats contain vesicular TfRs of native size (~94 kDa on reducing gels) [77,97]. These results argue for a mechanism of release which does not involve cleavage of the receptor. Since the human material cited above originated from patients in the clinic, processing of the released receptor may have been ongoing leading to the formation of breakdown products.

The fact that plasma from seven different species of anemic animals show the presence of exosomes argues strongly that exosome formation is associated with reticulocyte maturation [97,99,100]. A population of multivesicular bodies which appear to be the precursors of the circulating exosomes has been identified inside immature red cells of several species [90,97,99,352]. Based on this common behaviour of reticulocytes, it has been proposed that exosome formation is a major route for the elimination of specific membrane proteins during red cell maturation. However, given the incomplete recovery of the TfR in exosomes, there may be other means for the loss of TfR from the red cells during maturation.

To understand the differences between the sheep and human systems we undertook to examine, in a quantitative and time-dependent manner, the formation of externalized TfR under controlled conditions. Experiments were done to detect both native and truncated receptor with isolated sheep reticulocytes incubated in vitro. As well, cultured human cells were examined.

5.1 Recovery of the TfR

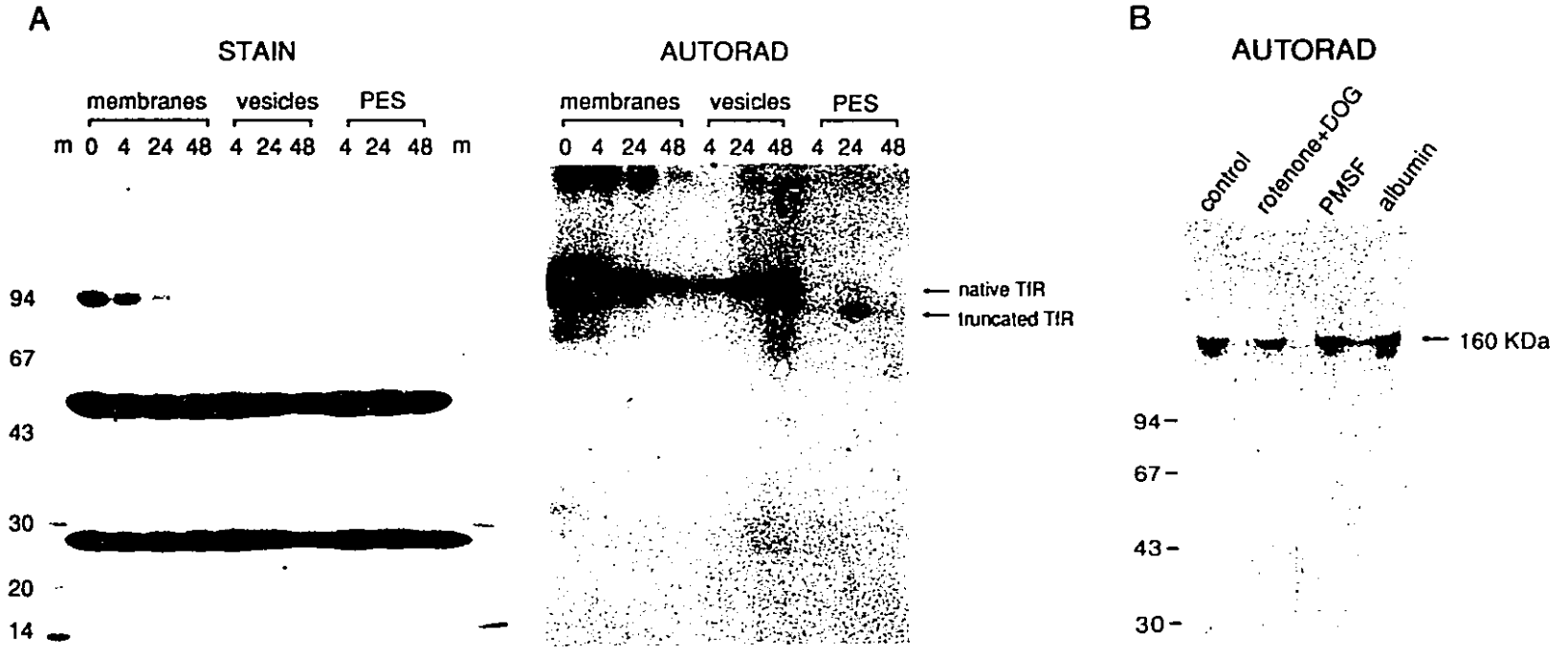
The quantitative recovery in exosomes of the TfR lost during reticulocyte maturation has been poor (~30%), particularly using transferrin binding capacity as a measure. These experiments suffered from several problems which may have compromised the extent of recovery in vesicles. Most of the data originated from experiments where the earliest sample was obtained after overnight incubation. Since released transferrin binding activity may be unstable during longterm incubation at 37°C as it is in storage, a low recovery is not unexpected. Secondly, degradation could result in the release of material which would escape detection in our standard experimental protocol, thereby also contributing to poor recovery. By using ^{125}I surface labelled cells, followed by measuring the level of radioactivity in the immunoprecipitates (i.e. measuring receptor directly), the fractional recovery improved somewhat and 35-40% of the ^{125}I-TfR lost from the cells could be recovered in exosomes from sheep reticulocytes. However, since the recovery in exosomes of another membrane protein, the nucleoside transporter, was ~80% [99], the data prompted us to conclude that although exosome formation could be a major mechanism for the loss of membrane-bound proteins during sheep reticulocyte maturation, additional unknown factors contributed to the poor recovery of the TfR.

The possibility was considered that receptor proteolysis and the release of the exofacial domain of the TfR (which recognizes both transferrin and the anti-TfR MAb) may contribute to the poor recovery of the TfR. Using ^{125}I surface-labelled sheep reticulocytes cultured in vitro, we tested for the presence of proteolytic cleavage products of the TfR in the medium. Both reducing and non-reducing gels were used to recover the four potential forms of the receptor (intact dimer, intact monomer, cleaved dimer, cleaved monomer). The following fractions were immunoprecipitated: (a) cell membranes from the initial population of reticulocytes, (b) cell membranes after incubation of the cells for 4 to 48 h at 37°C, (c) exosomes (the 100,000*g* pellet obtained by centrifugation of the cell-free culture medium), (d) the cell-free, exosome-free supernatant (post-exosome supernatant [PES]).

The pattern shown in Fig. 31A is characteristic of that obtained with reducing gels. The stained immunoprecipitates and autoradiograms from the cell membranes showed residual dimer (~190 kDa) and monomer (~94 kDa). The majority of the label was recovered in the intact monomer. Some dimer is frequently found despite the presence of mercaptoethanol. In the exosome fraction, the labelled peptide pattern was similar to that in the cells with the majority of the label in the 94 kDa polypeptide. The labelled peptide pattern in the PES was different, with the radioactivity appearing in a band ~80 kDa, characteristic of a cleaved, monomeric form of the receptor. A similar size peptide is obtained by trypsin digestion of the reticulocyte surface followed by immunoprecipitation of the cell-free medium with anti-TfR MAb (Chapter 4). It is evident that in immunoprecipitates from the plasma membrane of cultured cells, an intact receptor (dimer or monomer) was the principle product. In the medium, the majority of the receptor was intact and found in the exosomes. Of the total lost receptor, ~25% was present as a truncated form (~80 kDa)

Fig. 31. Loss of TfR from sheep reticulocytes during in vitro maturation and recovery in the culture medium

Reticulocytes were labelled with ^{125}I and cultured for 4, 24, or 48 h. TfR was immunoprecipitated from cellular membranes, vesicles (exosomes) or the post-exosome supernatant (PES) as described under Materials and Methods and then electrophoresed on reducing (A) or non-reducing (B) gels. In B, only the TfR immunoprecipitates from the PES are shown. ^{125}I-labelled reticulocytes were cultured in the presence of 1 μg/ml rotenone plus 2 mM 2-deoxyglucose (DOG), 10 μM PMSF plus 0.5 U/ml aprotinin, 0.85% albumin. m, molecular weight markers ($M_r \times 10^{-3}$) are shown.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truncated TfR
B
AUTORAD
control
rotenone+DOG
PMSF
albumin
160 KDa
94
67
43
30

representing the exofacial domain in the post-exosome supernatant (PES) (Fig. 31A).

On occasion we have also detected an intact receptor (94 kDa) in the PES. On those occasions we found that re-centrifugation of the medium for an additional thirty minutes at 130,000*g* clears the supernatant of all the intact receptor, suggesting that there may be a small population of less dense or smaller exosomes requiring additional force to bring them down. For this reason, in all subsequent experiments, only the peptide at ~80 kDa (under reducing conditions) was regarded as "soluble" released receptor. Any 94 kDa peptide in the PES was attributed to contaminating vesicles. In 8-10 experiments, the ~80 kDa peptide was always a relatively small fraction of the total ^{125}I-TfR released indicating that only a small percentage of the externalized receptor is cleaved.

In contrast to reports with cleaved human receptor [102,353,354], the released soluble peptide migrated as a peptide of ~160 kDa on non-reducing gels (Fig. 31B). Upon reduction, monomers were obtained (Fig. 31A). The present results suggest that the sheep receptor may have a disulfide bridge more distal to the plasma membrane than the human receptor. It is known that not all species have disulfide bridges with an identical disposition to those of the human receptor (e.g. the chicken receptor lacks one cysteine [260]). At present, we cannot distinguish whether an additional bridge exists or whether the cysteine residues of the sheep TfR are situated downstream of the protease cleavage site.

5.2 Time of formation of cleaved TfR

We were interested in determining whether the release of truncated TfR preceded or followed the formation of exosome-associated TfR. The results in Fig. 31 and Table 9 show that at 3-4 h of incubation, uncleaved receptor was

Table 9. Improved recovery of ^{125}I-TfR from reticulocytes incubated with albumin

Incubation time	3 hrs				24 hrs			
Albumin	0%	0.5%	1%	2%	0%	0.5%	1%	2%
	cpm				cpm			
Membrane	36,150	45,000	49,180	48,670	20,400	36,700	39,000	39,320
Exosomes	2,460	2,950	2,400	3,020	11,500	14,900	14,900	18,800
PES	130	460	660	730	2,200	2,800	2,800	2,720
Recovery (%)	77	95	Single expt.		67	100		
			Average of five expts.		51	85		
			Range (n=5)		(32-66)	(57-100)		

Sheep reticulocytes surface labelled with ^{125}I were incubated at 37°C. Samples were taken at 0, 3, and 24 h of incubation. Cells were harvested, plasma membranes isolated, solubilized and immunoprecipitated. The 100,000*g* pellet (exosomes) from the cell-free incubation medium and the post-exosome supernatant (PES) were also collected and immunoprecipitated. All immunoprecipitates were subjected to SDS-PAGE. All samples had control non-immune immunoprecipitates. After radioautography, the specific ^{125}I labelled bands were cut from the gel and counted. The radioactivity in the immunoprecipitate of membranes at time 0= 50,600 cpm. Complete details from a single experiment, representative of five experiments, are shown. In addition, the pooled data of average recovery from five experiments after 24 h of incubation are also presented. All values have been corrected for their respective nonimmune controls (less than 5% of corrected values). All values are based on recovery from 10^8 cells.

detected in exosomes but little was found in the PES in the truncated form. At 24 h of incubation, when over 50% of the receptor was lost from the cells, both exosome-bound and PES soluble (~80 kDa) receptor were present. The majority of the radioactivity was associated with the exosomes. The radioactivity in the soluble receptor was never found to exceed the radioactivity in exosome associated receptor (8 experiments).

5.3 Proteolysis and release of truncated TfR

To assess whether the soluble TfR arose from the action of unspecified proteases, albumin was added to the medium to provide an alternate substrate for the putative protease. The presence of albumin in the incubation medium improved recovery of ^{125}I-TfR so that after overnight incubation, the majority of the lost radioactivity was accounted for. However, the amount of soluble, truncated TfR was not affected (Table 9). The increased recovery was due to two factors: (a) the amount of ^{125}I-TfR recovered in the cell membranes after an overnight culture was increased by a factor of nearly two, and the amount of label recovered from exosomes was increased. In the experiment reported in Table 9, with 0.5% albumin, virtually all of the original 50,600 cpm in the membranes could be accounted for in the sum of the residual receptor radioactivity in the membranes, the exosomes and the PES compared with 77% (at 3 h) and 67% recovery (at 24 h) without albumin. A composite of five different experiments summarizes these observations (Table 9).

Attempts to assess directly whether the exosomes could give rise to the truncated TfR were not successful. When exosomes were reincubated in fresh or conditioned medium, the exosomes disintegrated and no antibody-detectable,

soluble TfR was found. These data should be contrasted with those of Chitambar et al.[355] where reincubation of rat exosomes gave rise to intact, soluble TfR.

Attempts to decrease the formation of truncated receptor from sheep reticulocytes by adding a variety of proteolytic inhibitors including PMSF, aprotinin, trypsin inhibitor, leupeptin, and pepstatin were not effective in a consistent manner.

If the formation of truncated TfR is a natural process in sheep, the presence of a truncated receptor in the plasma of anemic sheep should be detectable. We were unable, however, with our procedures, to detect truncated, circulating TfR in the 100,000*g* supernatant of plasma from phlebotomized sheep. The exosome pellet from an equivalent volume of plasma had readily detectable, full length TfR.

5.4 Human cleaved TfR: HL60 cells release a truncated TfR

To assess the presence of intact and/or cleaved TfR in the human circulation, plasma from patients with hemochromatosis was incubated with iron core beads coated with an antibody to the human TfR. The retained material was then subjected to SDS-PAGE. An intact TfR, as well as a lower molecular weight peptide of ~80 kDa, was obtained (not shown).

Our studies on TfR synthesis with HL60 cells showed the presence of an ~80 kDa ^{35}S-labelled peptide in the 100,000*g* supernatant which immunoprecipitated with anti-TfR MAb. Although HL60 cells release membranous components into the medium, no labelled receptor was associated with the high speed pellet from the incubation medium of these cells (Fig. 32). However, native size, ^{35}S-TfR was still present in the cells after a 38 h chase with unlabelled methionine. These data on the release of a truncated receptor are in

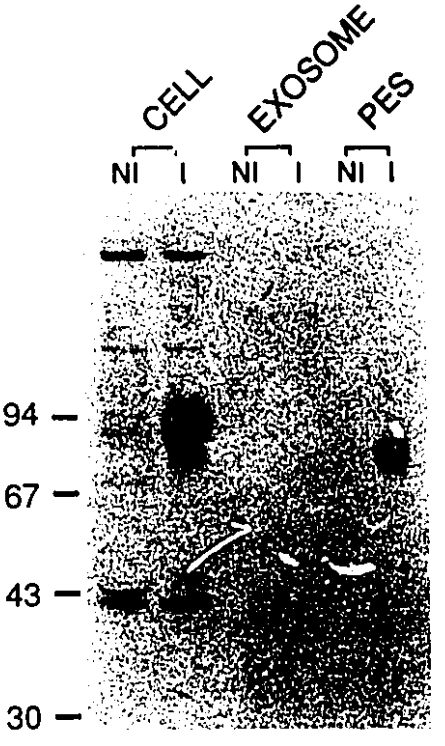

Fig. 32. HL60 cells release a soluble truncated TfR

Cells were labelled with [^{35}S]methionine and cultured for 21 h. The cells, the 100,000*g* pellet (exosome) and supernatant (PES) were immunoprecipitated with a non-immune mouse IgG (NI) or anti-TfR MAb (I). Shown is an autoradiograph after a 3 day exposure. Molecular weight markers (M_r x 10^{-3}) are indicated.

accord with studies done by Chitambar and Zivkovic [353] who also found a soluble ~80 kDa fragment in the medium.

We considered the possibility that HL60 cells possess a cell surface protease (or secrete a protease) which might cleave the extracellular domain of the TfR from non-cellular but membrane-bound forms of the TfR. To test for the putative protease activity, ^{35}S-labelled TfR immunoprecipitates (bound to Protein A-Sepharose) were incubated with either a suspension of HL60 cells or with the cell-free conditioned medium from an HL60 culture. Figure 33 shows that HL60 cells cleaved the ^{35}S-TfR, releasing a peptide of ~80 kDa.

5.5 Origin of the cleaved TfR: Detection of the cytoplasmic domain of the TfR in vesicles but not cells

If TfR is cleaved from the red cell surface and/or the exosomes, the transmembrane and cytoplasmic domains of the TfR may be retained in the originating membranes. Recent studies have suggested that in HeLa cells, the membrane-bound, cytoplasmic domain of the TfR is recycled like an intact TfR [356]. Using an antibody against the cytoplasmic domain of the TfR (anti-cdTfR), we probed sheep erythrocyte membranes, sheep reticulocyte membranes and sheep exosomes for the presence of the N-terminal (cytoplasmic) domain of the TfR. The results show that anti-cdTfR recognizes native (94 kDa and 190 kDa) TfR in sheep reticulocyte membranes (Fig. 34). No other reactive peptides were seen. In exosomes, however, the presence of two lower molecular weight antibody reacting peptides were evident in addition to the native form of the TfR. The faster migrating peptide (~17 kDa) corresponds approximately to the expected size of the human N-terminal domain (~15 kDa) if cleavage had occurred at (or near) the exofacial domain at residue 130 (a tryptic cleavage

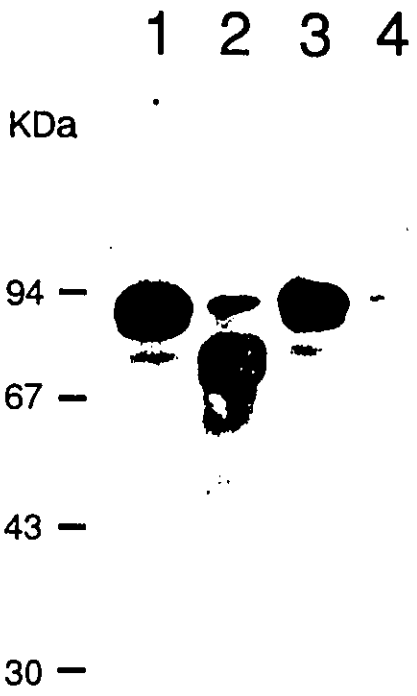

Fig. 33. A cellular protease cleaves ^{35}S-TfR to produce an 80 kDa fragment which is recognized by anti-TfR MAb

HL60 cells were labelled with [^{35}S]methionine for 1 h, then chased for 1 h with the addition of five volumes of complete MEM containing 10% fetal bovine serum and 1 mM methionine. The cells were solubilized as described under Materials and Methods and 1 ml of supernatant (containing ^{35}S-TfR from 5 x 10^6 cells) was incubated with 20 µg anti-TfR MAb (lanes 1-3) or non-immune mouse IgG (lane 4) for 2 h, then with 20 µl Protein A-Sepharose for 1 h, both at room temperature. The Sepharose was washed with PBS, then incubated for 12 h at 37°C with 1 ml of RPMI 1640 medium (lane 1), medium containing 10^7 HL-60 cells (lane 2) or 10X concentrated conditioned medium (lane 3). Following the incubation, the Sepharose was allowed to settle and the cell suspension and medium were removed. The Sepharose was washed with PBS, and the ^{35}S-TfR eluted with SDS sample buffer for electrophoresis.

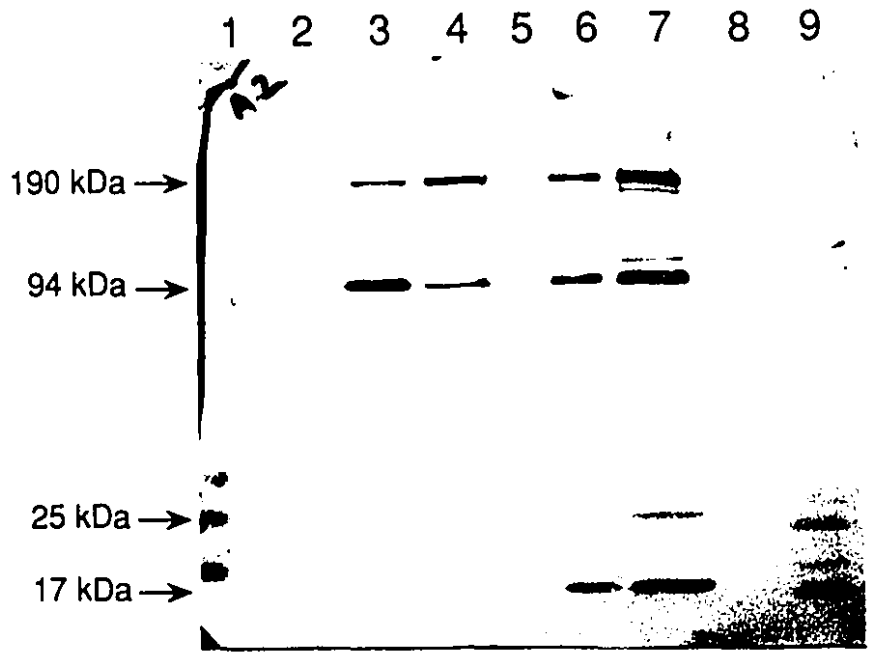

Fig. 34. Detection of the cytoplasmic domain of the TfR by immunoblotting

Lane 1, prestained molecular weight markers of 106,000; 80,000; 49,500; 32,500; 27,500; 18,500; lane 2, sheep erythrocyte membranes, lane 3, sheep reticulocyte membranes before culture; lane 4, sheep reticulocyte membranes after culture; lanes 5-7, sheep exosomes after culture of 10, 20, and 50 µl cells, respectively; lane 8, human membranes after culture; lane 9, human exosomes. The intact TfR and cytoplasmic domains are indicated by arrows.

site). The fact that a second peptide of roughly double the size was also present suggests that there may be a second cleavage site (or that the latter peptide is the residual dimer of the ~17 kDa component). Further study is required to address this question. In exosomes from a patient with hemochromatosis, anti-cdTfR also recognized two peptides of ~17 kDa and ~25 kDa (lane 9, Fig. 34). In the cell membranes from the reticulocytes of the same individual, no cleavage products were detectable.

5.6 Recycling of a cleaved TfR in sheep reticulocytes

Although we could not detect the cytoplasmic domain of the cleaved TfR in fresh or incubated sheep reticulocytes, the possibility exists that any cytoplasmic component would be rapidly removed from the cells, but not the exosomes which may not contain the necessary enzymes. For this reason, we generated a cleavage product by treatment of the sheep reticulocytes with trypsin prior to incubation. The results showed that the cleaved, cell-associated fragment generated by trypsin was recognized by anti-cdTfR. This domain was retained in the membranes after overnight incubation (Fig. 35). The exosomes generated from these cells also contained the cleaved cytoplasmic domain of the TfR. It is evident that in exosomes from trypsin-treated cells, there is a substantial increase in the amount of cleaved cytoplasmic domain. Thus, if the cleaved, soluble TfR were generated directly from reticulocytes during normal maturation, the residual cytoplasmic domain would likely be detected in the cell membranes after overnight incubation. These data are consistent with the conclusion that cleavage of the TfR from exosomes results in the formation of truncated circulating TfRs.

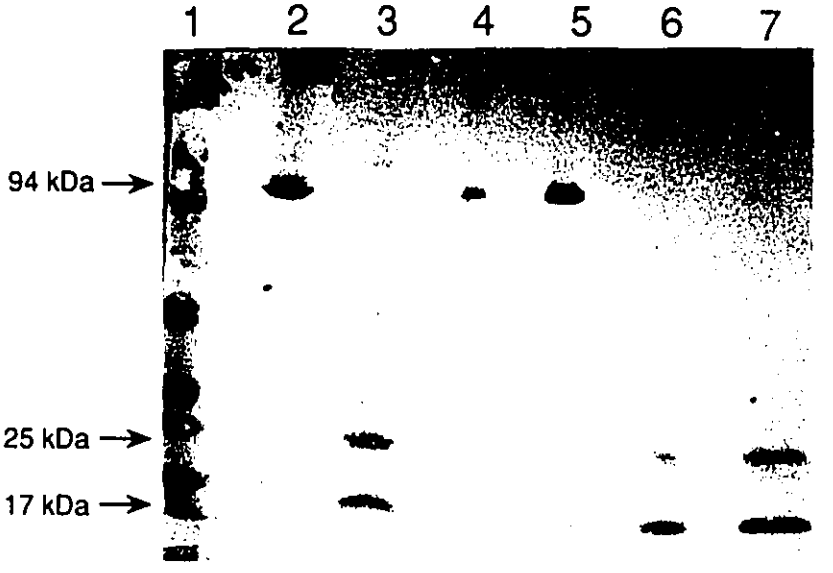

Fig. 35. Recycling of a trypsin generated cytoplasmic domain in sheep reticulocytes

Reticulocytes were treated with 0.1% trypsin in PBS for 10 min at 37°C. The cells were washed with PBS containing 0.2% soybean trypsin inhibitor, then cultured for 16 h. The cell membranes and exosomes were subjected to SDS-PAGE, blotted and probed with anti-cdTfR. Lane 1, prestained molecular weight markers, as in Fig. 34; lane 2, untreated reticulocytes (20 µl) after culture; lane 3, trypsin-treated reticulocytes (20 µl) after culture; lanes 4-5, exosomes from culture of 20 µl or 50 µl untreated reticulocytes, respectively; lanes 6-7, exosomes from culture of 20 µl or 50 µl trypsin-treated reticulocytes, respectively. Arrows indicate the position of the TfR.

Summary

Elevated levels of serum TfR are clinical indicators of iron deficiency and increased erythropoiesis. In man, most of the circulating TfRs are truncated, representing the extracellular domain of ~80 kDa. Although exosome formation is a mechanism for the shedding of TfRs by maturing reticulocytes of several species, a recovery of less than 50% suggested that other mechanisms could account for TfR release, such as cleavage from the cell surface. In this chapter, we presented evidence to indicate that in sheep reticulocytes (and possibly in human reticulocytes), a minor portion of the TfR is cleaved from exosomes during or after their release from the maturing reticulocyte. The 17 kDa cytoplasmic and transmembrane domains following cleavage remain associated with exosomes but are absent from the cells making direct cleavage from the cells unlikely. It therefore appears that exosome formation is the major route for shedding TfRs and that the circulating, extracellular domain is a secondary product from the circulating exosomes.

Chapter 6 General Discussion

During reticulocyte maturation, many cellular and membrane functions are lost [6,10-12,50,62,68-82]. Nearly a decade ago, it was shown that TfR-containing vesicles are exocytosed during in vitro culture of sheep reticulocytes [87]. This temperature- and ATP-dependent process could be the means by which the reticulocyte loses specific membrane proteins, such as the TfR, during maturation. How this process is controlled is largely unknown. The question arose as to whether the reticulocyte synthesizes new TfRs during this time and whether synthesis is regulated by heme or iron. The aim of this thesis was to study the factors influencing the synthesis and externalization of TfRs in the maturing reticulocyte.

The ability of peripheral reticulocytes to synthesize proteins has been known for decades [357,285,286,335]. Equally well known is the fact that this ability is lost during reticulocyte maturation so that the protein synthetic machinery is absent in erythrocytes [335,51]. Ninety percent of the protein synthesized by reticulocytes is hemoglobin; however, the syntheses of nonhemoglobin proteins and membrane proteins have also been shown in rabbit and mice reticulocytes [52,289,303-305,347]. Protein synthesis in reticulocytes is dependent on heme [285-289] which prevents the formation of an inhibitor of translation initiation [290,291]. Hemin must be supplied to reticulocyte lysates for in vitro protein synthesis since cell-free lysates do not synthesize heme. Unlike cell-free lysates, intact reticulocytes are capable of heme synthesis and can maintain protein synthesis for a longer period without exogenous hemin.

We have shown that intact sheep reticulocytes synthesize a membrane-bound receptor for transferrin which is under heme regulation. When heme synthesis was inhibited with 4,6-dioxoheptanoate (DOH), TfR synthesis was also

inhibited. Cox [334] also showed that DOH inhibited TfR synthesis and that synthesis could be restored by the addition of exogenous hemin. Heme synthesis in rabbit reticulocytes was increased by FeSIH (which is a better iron donor than FeTf), but FeSIH had no effect on protein synthesis [276], suggesting that rabbit reticulocytes are already synthesizing proteins at a maximum level. Therefore, increasing intracellular heme either through increased synthesis (by adding FeSIH) or by adding hemin to the medium does not increase overall protein synthesis. With sheep reticulocytes, however, exogenous hemin increased TfR synthesis (as well as total protein synthesis) above the level observed in media supplemented with FeTf or FePIH. This suggests that sheep reticulocytes, unlike rabbit reticulocytes, do not synthesize sufficient endogenous heme to maintain maximal protein synthesis.

Recently, it was shown that TfR synthesis in cultured cell lines is regulated post-transcriptionally by iron [200-203]. The intracellular iron concentration influences the binding of a cytoplasmic protein (IRE-BP) to the TfR mRNA and hence the stability of the mRNA [200,201]. Iron decreases TfR mRNA stability and hence TfR synthesis. The regulation of TfR synthesis by this mechanism was not apparent in sheep reticulocytes. Iron stimulated TfR synthesis but this was probably mediated through heme synthesis since DOH blocked the effect of iron (delivered as FeTf or FePIH). All the iron taken up by reticulocytes is incorporated into heme [240] with little or no free iron accumulating to destabilize the TfR mRNA. Even in the presence of DOH, which is known to increase iron uptake by reticulocytes [240,237,241], the nonheme iron accumulates in the mitochondria and not in the cytosol where mRNA is regulated. DOH inhibited TfR synthesis by limiting heme availability, which is required for the translation of mRNAs. The addition of an iron chelator, PIH, inhibited TfR synthesis probably by making iron unavailable for heme synthesis.

These results emphasize the importance of maintaining heme levels for TfR synthesis in reticulocytes and suggest that the regulation of mRNA by iron is a secondary factor. Cells such as uninduced K562 cells synthesize very little heme and must have a "defence mechanism" to prevent the accumulation of free iron.

Alternatively, the TfR mRNA in reticulocytes may not respond to iron as it does in nonerythroid or K562 cells. Red cell precursors must take up large amounts of iron for heme synthesis for the production of hemoglobin, yet these cells must continue to synthesize TfRs in order to meet the demand for iron. Therefore, the IRE mechanism for regulating TfR synthesis in other cells may be modified or absent in reticulocytes. Macrophages are also adapted for increased iron uptake (for iron storage) and their TfRs are positively regulated by iron [359]. The mechanism for this positive regulation is not known. It would be interesting to directly measure the effect of iron and iron chelators on the level of TfR mRNA in reticulocytes. One should determine if the IRE-BP is present in erythroid cells and what effect iron has on the binding of IRE-BP to both erythroid and nonerythroid TfR mRNA. A recent publication noted that IRE-BPs are present in chick embryonic erythroid cells but that TfR mRNA stability is not regulated by iron levels in these cells [360].

The ability of the sheep reticulocyte to synthesize TfR and hemoglobin is lost after a few hours of culture, even in the presence of hemin. Schulman [335], however, showed that rabbit reticulocytes could synthesize protein at initial rates after a 20 h incubation period if supplemented with exogenous hemin, suggesting that the heme synthesizing capacity was lost more rapidly than the protein synthesizing capacity in these cells. Heme deficiency was considered to limit protein synthesis after longterm incubation. We measured heme synthesis in sheep reticulocytes by following [2-^{14}C]glycine incorporation into heme after culturing cells for up to 6 h. The ability to synthesize heme was not lost as

rapidly as the ability to synthesize proteins. This suggests that some factor(s) other than heme limits protein synthesis in the maturing sheep reticulocyte. In our experiments, hemin was present throughout the incubation period whereas Schulman added hemin only during the labelling period. Hemin may have other effects on reticulocytes such as stimulating the loss of components of the protein synthetic machinery (in addition to the loss of membrane proteins [see below]) during reticulocyte maturation. If this were so, we would see a rapid decline in protein synthesis even though eIF-2α is available.

It has been shown in erythroid cells that heme synthesis is limited by the rate of uptake of iron from Tf [275,276]. The rate of iron uptake by cells is proportional to the number of surface TfRs and their rate of recycling. In sheep reticulocytes, the ability to take up iron is lost with a $t_{1/2}$ of about 4 h [312]. Assuming that the enzymes of the heme biosynthetic pathway are stable during this time and iron uptake is rate-limiting, we can also expect heme synthesis to decrease with a $t_{1/2}$ of 4 h. Our results show that the heme synthetic capacity falls at this rate. A parallel decline of heme synthesis and iron uptake may be necessary so that every iron molecule which is transported into the cell is incorporated into heme rather than accumulating as free iron which would be toxic to the cell. This is consistent with studies showing a tight coupling of iron uptake and heme synthesis [240]. Since protein synthesis is lost more rapidly ($t_{1/2}$ ~1 h), not all of the newly synthesized heme will be bound to globin. This may increase the intracellular pool of free heme which in turn could inhibit iron uptake and stimulate the selective loss of proteins (such as the TfR) during reticulocyte maturation (discussed below).

In early reticulocytes, when iron uptake is high, maximal heme and globin synthesis is achieved. As TfR expression decreases with cell maturation, iron uptake also decreases accompanied by a decrease in heme synthesis. As the heme

supply falls, initiation of protein synthesis is inhibited. Components of the protein synthetic machinery and mRNA also decline in amount during red cell maturation and contribute to the shutdown of protein synthesis.

The only other study on TfR synthesis in reticulocytes [334] did not report any unusual features about the newly synthesized TfR. We have, however, detected the formation of an incomplete TfR. Our conclusion is based on four criteria. 1) The newly formed TfR migrated as a smaller polypeptide on SDS gels. The difference in mobility appeared to be due to incomplete post-translational processing, since removal of the majority of carbohydrates with TFMS or endoglycosidase F resulted in the formation of comigrating labelled and unlabelled polypeptides. Further studies showed that the newly synthesized TfR lacked complex-type oligosaccharides. 2) When reticulocytes were exposed to trypsin at 0°C or 37°C, there was little or no apparent digestion of the ^{35}S-TfR in contrast with digestion of the preexisting TfR. These data suggest that the newly formed TfR was not delivered to the cell surface. After density gradient centrifugation of a reticulocyte lysate, fractions containing the peak of ^{35}S-TfR also contained galactosyltransferase, a Golgi-associated enzyme, and separated from the peak of native TfR. The lack of complex oligosaccharides on the ^{35}S-TfR and the accumulation of high-mannose glycopeptides suggested an ER or cis-Golgi localization. 3) The half-time for the loss of newly formed TfR from the cell was ~24 h, two to three times longer than that for native preexisting TfR. No evidence was found for the presence of ^{35}S-TfR in the exosomes released to the medium during maturation of reticulocytes. These exosomes contained the preexisting TfR. 4) Further confirmation that the newly formed TfR existed in a pool separated from the majority of the cellular TfR stems from the difference in response of the preexisting TfR and the ^{35}S-TfR to iron and hemin supplements in the culture medium. Whereas hemin, not iron, stimulated the loss

from the cell of unlabelled native TfR, iron (as FePIH) was more effective than hemin in causing the loss of the ^{35}S-TfR. Thus immature red cells may have two mechanisms for regulating TfR protein levels, both of which are influenced, directly or indirectly, by the supply of iron.

Since we were unable to identify any of the other newly synthesized membrane proteins, we do not know with certainty whether all newly made membrane proteins are incompletely processed or whether the newly made TfR is selectively held up intracellularly. However, our experiments with [^{3}H]mannose-labelled glycoproteins and protease treatment of the surface of cells labelled with [^{35}S]methionine suggest that the sheep reticulocyte has lost the machinery to fully process newly synthesized membrane proteins.

While the reticulocyte must synthesize hemoglobin and lipoxygenase [52], it does not need to make functional TfRs which have no role in the mature cell. The majority of the proteins of the erythrocyte membrane (spectrin, band 3, glycophorin) are synthesized before the reticulocyte stage [304, our results]. Only a small fraction of the total TfR (less than 0.1%) is synthesized at the reticulocyte stage in the sheep. The physiological significance of this late stage synthesis is not clear but it may be related to the fact that the TfR, unlike band 3 or glycophorin, is shed during red cell development so that its synthesis continues until the need for iron disappears.

The biosynthetic pathway of the TfR and other membrane proteins requires the coordinated actions of many membrane-bound and cytosolic proteins. While previous studies showed that the reticulocyte has the components necessary for the synthesis of soluble proteins, they did not clearly show whether any oligosaccharide processing and/or targeting to the cell surface take(s) place in the reticulocyte. Ultrastructural and biochemical methods have been used to detect the presence of ER and Golgi in reticulocytes. Electron micrographs have

revealed ER and Golgi-like membranes in reticulocytes which may be the sites of post-translational processing [2,361], and the ER marker enzyme, NADH-cytochrome c reductase, is present although the level is 1% of that in the liver [50]. Reticulocytes, but not erythrocytes, can also synthesize dolichol phosphate oligosaccharides and transfer the sugar chains to endogenous proteins [362,363]. Our studies on TfR synthesis in the reticulocyte showed for the first time, that a specific protein is inserted into the microsomal membrane, acquires high-mannose N-linked oligosaccharides, but is not transported to the cell surface. Therefore, the loss of Golgi-associated functions precedes the loss in capacity for protein translation during reticulocyte maturation such that a pool of incompletely processed polypeptides accumulate in an intracellular compartment.

These results also indicate that in sheep reticulocytes, the biosynthetic pathway has been separated from the endocytic pathway. Cell surface TfRs may either recycle between the plasma membrane and endocytic vesicles or enter a nonrecycling pool involving multivesicular bodies (MVBs). The MVB is the site where TfRs and other membrane proteins are packaged into vesicles for externalization. When the MVB fuses with the plasma membrane, these vesicles are released into the culture medium (in vitro) or plasma (in vivo). Newly synthesized TfRs, on the other hand, do not enter the pathway to the cell surface and are not externalized during reticulocyte maturation. It is of interest to add that ER and Golgi marker enzymes were not detectable in exosomes, as would be expected if the biosynthetic pathway does not have direct access to MVBs.

Aside from its role in protein synthesis, hemin has been recognized as an important regulator of red cell function for many years at the level of gene transcription, commitment to terminal differentiation and protein degradation [49,296,297,364,365]. In HeLa cells, heme has also been implicated as a down-regulator of TfR synthesis [330], but other investigators have concluded that free

iron, not heme, down-regulates the expression of the TfR at the post-transcriptional level in a variety of cells [199-203]. In Chapter 3, we showed that externalization of TfRs was stimulated by the addition of hemin. Earlier work had shown that serum increases externalization of the TfR from sheep reticulocytes [91], an effect that may have been mediated, in part, by hemin in serum or heme synthesis upon iron delivery. However, heme is found in the serum bound to hemopexin and although heme-hemopexin enters several different cell types by receptor-mediated endocytosis [165,366], it is not known whether reticulocytes have a receptor for heme-hemopexin. In vitro, hemin, being hydrophobic, is likely to enter the cell to some extent by diffusing through the membrane.

The effect of hemin on the externalization of TfRs may be part of a more general effect on membrane differentiation during reticulocyte maturation. NBMPR binding is also known to diminish during maturation of sheep reticulocytes, and the loss of this function was shown to be increased by hemin concomitant with an increase in vesicle formation and increased TfR and NBMPR binding in these vesicles. Thus, in addition to its already diverse roles in red cell metabolism, heme may be an important regulator of reticulocyte maturation. Hemin had no effect on lactate dehydrogenase, whose concentration in the red cell is not affected by maturation.

To assess how heme could be involved in reticulocyte maturation, we will first consider the known affects of hemin on red cell proteins. Hemin can disrupt skeletal protein-protein interactions in erythrocytes, especially those between protein 4.1 and glycophorin A [300]. It is unlikely that the stimulation of membrane protein loss during culture is due to a disruption of skeletal protein-protein interactions in the reticulocyte membrane since glycophorin A

and band 3, proteins which interact with the cytoskeleton [368-371], are not lost during reticulocyte maturation.

Both diamide, an oxidant of thiol groups, and hemin promote the formation of disulfide bonds in the heme-controlled repressor and prevent its activation in vitro [295]. A direct association between the iron of heme and the thiol of glutathione has been suggested [302]. Loss of heme from hemoglobin is induced by oxidation of the sulfhydryls [372]. These observations suggest that there may be an association between heme and the free sulfhydryls of proteins.

Hemin may also affect proteins indirectly by catalyzing the production of free radicals which cause oxidation. In reticulocytes, oxidatively damaged proteins can be selectively degraded [298,373]. Thorburn and Beutler [375] have proposed that oxidative damage may selectively mark hexokinase for proteolysis. The decline in hexokinase activity was enhanced when the cells were incubated with a free radical generating system (Fe^{2+}/ascorbate). Although the addition of hemin is not required for reticulocyte maturation, oxygen seemed to be required and this was not due to ATP-depletion.

From our results and the known effects of heme and oxidation described above, we may speculate on the mechanism for the release of membrane proteins during reticulocyte maturation. It has already been proposed that a structural change in the TfR accompanies the shedding of TfRs [92]. The oxidation of an amino acid(s) in the cytoplasmic domain of membrane proteins may lead to unfolding of the cytoplasmic domain. A 2 h exposure of reticulocytes to 43°C (heat shock) is known to stimulate loss of TfRs and nucleoside transporters [99]. Both heat shock and hemin may catalyze protein unfolding. These unfolded domains interact more strongly with the 70 kDa protein which is known to bind unfolded proteins [95]. The alteration in the cytoplasmic domain may explain why the exosomal TfR, unlike the cellular TfR, is not a substrate for protein

kinase C [92]. TfRs associated with the 70 kDa protein are segregated into a nonrecycling pool where multivesicular bodies are formed by budding of vesicles from the endosomal membrane into the lumen of the endosome. The altered TfR may or may not play an active part, i.e. induce the formation of exosomes, during reticulocyte maturation. When the MVB fuses with the plasma membrane, the exosomes containing TfR and intravesicular 70 kDa protein are released into the medium. Heme is known to inhibit iron uptake [236,237]. It is possible that the decreased iron uptake is partly due to routing of internalized TfRs to MVBs so that they no longer undergo recycling.

An alternate hypothesis could be envisioned where TfR-containing endocytic vesicles are constitutively sequestered into multivesicular bodies because of the need to decrease the cell's surface area. The reticulocyte eliminates membranes by budding vesicles into the lumen of the endosome and then releasing these vesicles as the endosome fuses with the plasma membrane. Intracellular proteins such as hemoglobin and the 70 kDa protein may also become trapped in these vesicles. In this situation, all recycling membrane proteins would be externalized. With the formation of multivesicular bodies, the recycling pathway is eliminated. Such a mechanism, however, cannot explain the selective nature of the elimination of membrane proteins during reticulocyte maturation. Membrane proteins are externalized at different rates, for example, the nucleoside transporter is lost more slowly ($t_{1/2}$ ~24 h) than the TfR ($t_{1/2}$ ~6-12 h) [99]. The same membrane protein may be lost to different degrees by different species, e.g. glucose transporter is lost completely by pig but not human red cells [76]. This therefore is the salient feature of the first hypothesis: membrane proteins are removed *selectively* from the recycling pathway. We suggest that a structural motif in the membrane protein promotes the clustering of these proteins in the buds during exosome formation. The degree to which

membrane proteins are lost in different species may be due to species differences in the structure of the protein or interactions which form between the membrane protein and species-specific cellular proteins. Other membrane proteins like band 3 and glycophorin are not lost during reticulocyte maturation. These proteins are anchored at the cell surface by forming associations with components of the membrane skeleton (ankyrin, protein 4.1 [368-371]). The cytoplasmic domain of band 3 can also associate with a number of cytoplasmic proteins [374]. These interactions restrict the mobility of band 3 and glycophorin in the plasma membrane as well as protecting their cytoplasmic domains from undergoing changes in folding which mark them for externalization.

To carry these speculations further, we would show that externalized membrane proteins have undergone some kind of alteration in their cytoplasmic domains and determine whether additional components are involved in targeting and releasing these membrane proteins. It is interesting to note that the cytoplasmic domains of TfRs that have so far been cloned are conserved. The 61 amino acid residues from the N-terminus of the hamster TfR is identical to that of the human TfR except for one amino acid (Tyr_{20} -> Cys_{20}) [376]. The cytoplasmic domains of the human and mouse TfRs are 93% identical [257-259]. Since the TfRs of all species are lost during reticulocyte maturation and exosome formation has been observed in seven different species [87,90,97,99,352], these TfRs may undergo a common change during the course of maturation which signals their removal from the recycling pathway. The extracellular domains, in contrast, are not as well conserved between species [260]. This may explain why the Tf of one species has different affinities for receptors of other species. For example, the sheep TfR does not bind well to human Tf. This model agrees well with the result showing that the extracellular domain of the sheep TfR can be

removed enzymatically with no effect on the subsequent externalization of the remaining cytoplasmic and transmembrane domains (Chapter 5). This result demonstrates that the N-terminal domains and not the ectodomain carry the information for selective externalization.

The fact that plasma from several different species of anemic animals show the presence of exosomes argues strongly that exosome formation is associated with reticulocyte maturation. A population of multivesicular bodies inside immature red cells of several species which appear to be the precursors of the circulating exosomes have also been identified. Based on this common behaviour of reticulocytes, it has been proposed that exosome formation is a major route for the elimination of specific membrane proteins during red cell maturation. However, given the incomplete recovery of the TfR in exosomes, there could be other means for the loss of TfR from the red cells during maturation, such as proteolysis and direct cleavage from the cell surface.

In Chapter 5, it was shown that the addition of albumin to the culture medium significantly improved the recovery of the TfR in cells and exosomes, suggesting that some proteolysis takes place during overnight culture. When the culture supernatant following centrifugation of exosomes (PES) was examined, a soluble truncated TfR of 160 kDa (80 kDa on reduced gels) was detected. Recent work with blood from anemic patients has shown the presence of a truncated form of the TfR in the circulation [101,103,104]. The level of truncated TfR is proportional to the degree of iron deficiency and the reticulocyte level. The existence of such a form of receptor in the circulation raises the question of its origin. Is shedding of TfR by cleavage from the cell surface a major mechanism for TfR loss from the red cell surface during maturation? Or is the cleaved TfR a consequence of exosome formation followed by cleavage from the surface of exosomes? Alternatively, is a soluble truncated TfR synthesized and secreted?

The last possibility can be eliminated in the sheep system since very little de novo synthesis of TfR occurs. Moreover, the small amount of newly synthesized TfR is not detected in the medium. Therefore, the soluble TfR does not originate from direct synthesis followed by secretion.

There is strong evidence to suggest that exosomes released during reticulocyte maturation are the major source of the soluble truncated TfRs found in the circulation. Exosomes formed during in vitro maturation of sheep reticulocytes showed the presence of both intact TfR and the 17 kDa cytoplasmic domain of the TfR. A cleaved cytoplasmic domain was absent in cultured reticulocytes making direct cleavage from the cell surface unlikely. The cleaved cytoplasmic domain was stable in the reticulocyte and remained cell-associated for 16 h as shown by the stability of the 17 kDa cytoplasmic domain generated artificially with trypsin. Therefore, its presence in the cell membrane would have been detected in the maturing reticulocyte. Furthermore, intact exosomal TfR was found in the medium (after 3-4 h) before the appearance of truncated TfRs (~24 h).

HL60 cells also release soluble 80 kDa TfRs to the medium during culture. We have shown that intact HL60 cells contain a surface protease which can cleave Protein A-bound TfR to produce an 80 kDa fragment recognized by an antibody to the ectodomain. The activity of this protease in HL60 cultures may be sufficiently high to cleave any non-cell-associated TfR that is released into the medium. This may explain why intact TfRs are not detected in the cell-free culture medium of HL60 cells. Similarly in sheep reticulocytes, a cell surface protease may cleave some non-cell-associated TfR. The activity of this protease in sheep reticulocytes, however, may be much lower, and therefore most of the TfR that is released from sheep reticulocytes is intact and associated with exosomes. Since no cleaved cytoplasmic domain was detected in the sheep

reticulocyte, the TfR on the cell surface is probably not accessible to the surface protease. These results suggest that the truncated TfRs are cleaved from non-cellular sources and that the major route for shedding TfRs during maturation is via exosome formation.

The process of exosome formation and externalization may not be limited to the maturing reticulocyte. Furthermore, externalization may require factors similar to and perhaps even identical to those involved in the widespread phenomenon of endocytosis (e.g. clathrin uncoating ATPase). Future investigations would focus on the molecular mechanism of targeting proteins for externalization and the potential of such a mechanism for the loss of membranes (proteins and lipids) during the earlier stages of erythroid cell differentiation and during the terminal differentiation of nonerythroid cells (e.g. HL60 cells).

In conclusion, with sheep reticulocytes, we have shown two distinct effects of hemin on the TfR: 1) increased de novo synthesis and 2) an increase in the loss of preexisting TfR. Neither effect is duplicated by iron presented to the cell as FeTf or FePIH when heme formation is blocked. Heme may play a dual role in maintaining the level of reticulocyte TfR. One may speculate that, during the early stages of red cell development when protein synthesis is high, the primary effect of heme will be to increase the level of the TfR by a general effect on protein synthesis. As the cell ages and overall synthesis of protein falls, the effect of heme on TfR loss will become the dominant effect. Based on our data, we are able to propose a mechanism for the selective externalization of plasma membrane proteins during reticulocyte maturation. This mechanism of selective loss by exosome formation appears to be the major route for eliminating native TfRs and other membrane proteins from the maturing reticulocyte.

References

1. Hemmaplardh, D., and Morgan, E.H. (1977) The role of endocytosis in transferrin uptake by reticulocytes and bone marrow cells. *Br. J. Haematol.* **36**, 85-96.
2. Sullivan, A.L., Grasso, J.A., and Weintraub, L.R. (1976) Micropinocytosis of transferrin by developing red cells: an electron-microscopic study utilizing ferritin-conjugated transferrin and ferritin-conjugated antibodies to transferrin. *Blood* **47**, 133-143.
3. Iacopetta, B.J., and Morgan, E.H. (1983) The kinetics of transferrin endocytosis and iron uptake from transferrin in rabbit reticulocytes. *J. Biol. Chem.* **258**, 9108-9115.
4. Choe, H.-R., Moseley, S.T., Glass, J., and Nunez, M.-T. (1987) Rabbit reticulocyte coated vesicles carrying the transferrin-transferrin receptor complex: purification and partial characterization. *Blood* **70**, 1035-1039.
5. Bar-Zvi, D., Mosley, S.T., and Branton, D. (1988) In vivo phosphorylation of clathrin-coated vesicle proteins from rat reticulocytes. *J. Biol. Chem.* **263**, 4408-4415.
6. Jandl, J.H., and Katz, J.H. (1963) The cell to plasma transferrin cycle. *J. Clin. Invest.* **42**, 314-326.
7. Appleton, T.C., Morgan, E., and Baker, E. (1971) A morphological study of transferrin uptake by reticulocytes. In: The Regulation of Erythropoiesis and Hemoglobin Synthesis. (T. Trávnicek and J. Neuwirt, eds.) Universita Karlova, Praha, pp. 310-315.
8. Nunez, M.T., Glass, J., Fischer, S., Lavidor, L.M., Lenk, E.M., and Robinson, S.H. (1977) Transferrin receptors in developing murine erythroid cells. *Br. J. Haematol.* **36**, 519-526.
9. Verhoef, N.J., and Noordeloos, P.J. (1977) Binding of transferrin and uptake of iron by rat erythroid cells in vitro. *Clin. Sci. Mol. Med.* **52**, 87-96.
10. van Bockxmeer, F.M., and Morgan, E.H. (1979) Transferrin receptors during rabbit reticulocyte maturation. *Biochim. Biophys. Acta* **584**, 76-83.
11. Frazier, J.L., Caskey, J.H., Yoffe, M., and Seligman, P.A. (1982) Studies of the transferrin receptor on both human reticulocytes and nucleated

human cells in culture: comparison of factors regulating receptor density. *J. Clin. Invest.* **69**, 853-865.

12. Pan, B.-T., Blostein, R., Johnstone, R.M. (1983) Loss of the transferrin receptor during the maturation of sheep reticulocytes in vitro: an immunological approach. *Biochem. J.* **210**, 37-47.
13. Till, J.E., and McCulloch, E.A. (1980) Hemopoietic stem cell differentiation. *Biochim. Biophys. Acta* **605**, 431-459.
14. Koury, M.J., Bondurant, M.C., and Atkinson, J.B. (1987) Erythropoietin control of terminal erythroid differentiation: maintenance of cell viability, production of hemoglobin, and development of the erythrocyte membrane. *Blood Cells* **13**, 217-226.
15. Telen, M.J. (1990) Expression of human erythrocyte membrane protein antigens during erythroid differentiation. In *Blood Cell Biochemistry, Vol. 1: Erythroid Cells* (J.R. Harris, ed.) Plenum Press, New York, pp. 27-43.
16. Fauser, A.A., and Messner, H.A. (1979) Proliferative state of human pluripotent hemopoietic progenitors (CFU-GEMM) in normal individuals and under regenerative conditions after bone marrow transplantation. *Blood* **54**, 1197-1200.
17. Nakahata, T., and Ogawa, M. (1982) Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. *Proc. Natl. Acad. Sci. USA* **79**, 3843-3847.
18. Stephenson, J.R., Axelrad, A.A., McLeod, D.L., Shreeve, M.M. (1971) Induction of colonies of hemoglobin synthesizing cells by erythropoietin in vitro. *Proc. Natl. Acad. Sci. USA* **68**, 1542-1546
19. Tepperman, A.D., Curtis, J.E., and McCulloch, E.A. (1974) Erythropoietic colonies in cultures of human marrow. *Blood* **44**, 659-669.
20. Eaves, A.C., and Eaves, C.J., (1984) Erythropoiesis in culture. *Clinics in Haematology* **13**, 371-391.
21. Pain, B., Woods, C.M., Saez, J., Flickinger, T., Raines, M., Peyrol, S., Moscovici, C., Moscovici, M.G., Kung, H.J., Jurdic, P., Lazarides, E., and Samarut, J. (1991) EGF-R as a hemopoietic growth factor receptor: the c-erbB product is present in chicken erythrocytic progenitors and controls their self-renewal. *Cell* **65**, 37-46.

22. Sorba, S., Grasso, J.A., and Dainiak, N. (1991) Transferrin binding to and uptake by purified human colony-forming units-erythroid (CFU-Es). *Blood* **78**, 89a.

23. Skutelsky, E., and Danon, D. (1967) An electron microscopic study of nuclear elimination from the late erythroblast. *J. Cell Biol.* **33**, 625-635.

24. Chasis, J.A., Prenant, M., Leung, A., and Mohandas, N. (1989) Membrane assembly and remodeling during reticulocyte maturation. *Blood* **74**, 1112-1120.

25. Zweig, S.E., Tokuyasu, K.T., and Singer, S.J. (1981) Member-associated changes during erythropoiesis: on the mechanism of maturation of reticulocytes to erythrocytes. *J. Supramol. Struct. Cell. Biochem.* **17**, 163-181.

26. Skutelsky, E. and Farquhar, M.G. (1976) Variations in distribution of Con A receptor sites and anionic groups during red cell differentiation in the rat. *J. Cell Biol* **71**, 218-231.

27. Gieduschek, J.B., and Singer, S.J. (1979) Molecular changes in the membrane of mouse erythroid cells accompanying differentiation. *Cell* **16**, 149-163.

28. Wraith, D.C., and Chesterton, C.J. (1982) Cell-surface remodelling during mammalian erythropoiesis. *Biochem. J.* **208**, 239-242.

29. Patel, V.P., and Lodish, H.F. (1984) Loss of adhesion of murine erythroleukemia cells to fibronectin during erythroid differentiation. *Science* **224**, 996-998.

30. Waugh, R.E. (1991) Reticulocyte rigidity and passage through endothelial-like pores. *Blood* **78**, 3037-3042.

31. Ngai, J., and Lazarides, E. (1989) Intermediate filament expression in erythroid differentiation and morphogenesis. In: *Red Blood Cell Membranes: structure, function, clinical implications.* (P. Agre and J.C. Parker, eds) Marcel Dekker, New York, pp. 47-75.

32. Monette, F.C., and Sigounas, G. (1988) Growth of murine multipotent stem cells in a simple "serum-free" culture system: role of interleukin-3, erythropoietin, and hemin. *Exp. Hematol.* **16**, 250-255.

33. Ibrahim, N.G., Linton, J.D., Levere, R.D. (1982) The role of haem biosynthetic and degradative enzymes in erythroid colony development: the effect of haemin. *Br. J. Haematol.* **50**, 17-28.

34. Monette, R.F., Holden, S.A., Sheehy, M.J., (1984) Specificity of hemin action in vivo at early stages of hematopoietic cell differentiation. *Exp. Hematol.* **12**, 782-787.

35. Bruns, G.A.P., and Ingram, V.M. (1973) The erythroid cells and haemoglobins of the chick embryo. *Philos. Trans. R. Soc. Lond. (Biol.)* **266**, 225-305.

36. Sawyer, S.T., and Krantz, S.B. (1986) Transferrin receptor number, synthesis, and endocytosis during erythropoietin-induced maturation of Friend virus-infected erythroid cells. *J. Biol. Chem.* **26**, 9187-9195.

37. Gahmberg, C.G., Jokinen, M., Andersson, L.C. (1979) Expression of the major red cell sialoglycoprotein, glycophorin A, in human leukemic cell line K562. *J. Biol. Chem.* **254**, 7442-7448.

38. Sassa, S. (1988) Heme stimulation of cellular growth and differentiation. *Seminars in Hematol.* **25**, 312-320.

39. Dean, A., Erard, F., Schneider, A.B., and Schechter, A.N. (1981) Induction of hemoglobin accumulation in human K562 cells by hemin is reversible. *Science* **212**, 459-461.

40. Tsiftsoglou, A.S., Wong, W., Tsamadou, A.I., and Robinson, S.H. (1991) Cooperative effects of hemin and anthracyclines in promoting terminal erythroid maturation in K562 human erythroleukemia cells. *Exp. Hematol.* **19**, 928-933.

41. Hooper, W.C., Pruckler, J., Jackson, D., and Evatt, B.L. (1991) The synergistic effect of hemin and transforming growth factor-beta on hemoglobin accumulation in HEL erythroleukemia cells. *Leukemia Res.* **15**, 753-758.

42. Friend, C., Scher, W., Holland, J.G., and Sato, T. (1971) Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethylsulfoxide. *Proc. Natl. Acad. Sci. USA* **68**, 378-382.

43. Sassa, S. (1976) Sequential induction of heme pathway enzymes during erythroid differentiation of mouse Friend leukemia virus-infected cells. *J. Exp. Med.* **143**, 305-315.

44. Hu, H.Y., Gardner, J., Aisen, P., and Skoultchi, A.I. (1977) Inducibility of transferrin receptors on Friend erythroleukemic cells. *Science* **197**, 559-561.

45. Glass, J., Nunez, M., Fischer, S., and Robinson, S. (1978) Transferrin receptors, iron transport and ferritin metabolism in Friend erythroleukemia cells. *Biochim. Biophys. Acta* **542**, 154-162.
46. Patel, V.P., and Lodish, H.F. (1987) A fibronectin matrix is required for differentiation of murine erythroleukemia cells into reticulocytes. *J. Cell Biol.* **105**, 3105-3118.
47. Volloch, V., and Housman, D. (1982) Terminal differentiation of murine erythroleukemia cells: physical stabilization of end stage cells. *J. Cell. Biol.* **93**, 390-394.
48. Tsiftsoglou, A.S., Barrnett, R.J., and Sartorelli, A.C. (1979) Enucleation of differentiated murine erythroleukemia cells in culture. *Proc. Natl. Acad. Sci. USA* **76**, 6381-6385.
49. Ross, J., and Sautner, D., (1976) Induction of globin mRNA accumulation by hemin in cultured erythroleukemia cells. *Cell* **8**, 513-520.
50. Rapoport, S.M. (1986) *The Reticulocyte.* CRC Press, Boca Raton, FL.
51. Höhne, M., Bayer, D., Prehn, S., Schewe, T., and Rapoport, S.M. (1983) In vitro maturation of rabbit reticulocytes III. Response of lipoxygenase. *Biomed. Biochim. Acta* **42**, 1129-1134.
52. Thiele, B.J., Belkner, J., Andree, H., Rapoport, T.A., and Rapoport, S.M. (1979) Synthesis of non-globin proteins in rabbit-erythroid cells: synthesis of a lipoxygenase in reticulocytes. *Eur. J. Biochem.* **96**, 563-569.
53. Gronowicz, G., Swift, H., and Steck, T.L. (1984) Maturation of the reticulocyte in vitro. *J. Cell Sci.* **71**, 177-197.
54. Noble, N.A. (1989) Extrusion of partially degraded mitochondria during reticulocyte maturation. In *The Red Cell: Seventh Ann Arbor Conference.* Alan R. Liss. pp. 275-290.
55. Papayannopoulou, T., and Finch, C.A. (1975) Radioiron measurements of red cell maturation. *Blood Cells* **1**, 535-546.
56. Johnstone, R.M., Adam, M., and Pan, B.T. (1984) The fate of the transferrin receptor during maturation of sheep reticulocytes in vitro. *Can. J. Biochem. Cell Biol.* **62**, 1246-1254.
57. Freedman, M.L., Geraghty, M., and Rosman, J. (1974) Hemin control of globin synthesis: isolation of a hemin-reversible translational repressor from human mature erythrocytes. *J. Biol. Chem.* **249**, 7290-7294.

58. Jansen,G., Koenderman, L., Rijksen, G., Cats, B.P., and Staal, G.E.J. (1985) Characterisics of hexokinase, pyruvate kinase, and glucose-6-phosphate dehydrogenase during adult and neonatal reticulocyte maturation. *Am.J.Hematol.***20**, 203-215.

59. Magnani, M., Stocchi, V., Chiarantini, L., Serafini, G., Dachà, M., Fornaini, G. (1986) Rabbit red blood cell hexokinase: decay mechanism during reticulocyte maturation. *J. Biol. Chem.* **261**, 8327-8333.

60. Stocchi, V., Magnani, M., Piccoli, G., and Fornaini, G. (1988) Hexokinase microheterogenity in rabbit red blood cells and its behavior during reticulocyte maturation. *Mol. Cell. Biochem.* **79**, 133-136.

61. Thorburn, D.R., and Beutler, E. (1991) The loss of enzyme activity from erythroid cells during maturation. *Adv. Exp. Med. Biol.* **307**, 15-27.

62. Weigensberg, A.M., and Blostein, R. (1983) Energy depletion retards the loss of membrane transport during reticulocyte maturation. *Proc. Natl. Acad. Sci. USA* 80, 4978-4982.

63. Hershko, A., and Ciechanover, A. (1986) The ubiquitin pathway for the degradation of intracellular proteins. *Progr. Nucl. Acid Res. Mol. Biol.* **33**, 19-56.

64. Waxman, L., Fagan. J.M., and Goldberg, A.L. (1987) Demonstration of two distinct high molecular weight proteases in rabbit reticulocytes, one of which degrades ubiquitin conjugates. *J. Biol. Chem.* **262**, 2452-2457.

65. Fagan, J.M., Waxman, L., and Goldberg, A.L. (1987) Skeletal muscle and liver contain a soluble ATP+ubiquitin-dependent proteolytic system. *Biochem. J.* **243**, 335-343.

66. Speiser, S., and Etlinger, J.D. (1982) Loss of ATP-dependent proteolysis with maturation of reticulocytes and erythrocytes. *J. Biol. Chem.* **257**, 14122-14127.

67. Raviv, O., Heller, H., and Hershko, A. (1987) Alterations in components of the ubiquitin-protein ligase system following maturation of reticulocytes to erythrocytes. *Biochem. Biophys. Res. Commun.* **145**, 658-665.

68. Benderoff, S., Johnstone, R.M., and Blostein, R. (1978) Electrogenic sodium-dependent glycine transport in sheep reticulocytes. *Can. J. Biochem.* **56**, 545-551.

69. Blostein, R., and Grafova, E. (1987) Characteristics of membrane transport losses during reticulocyte maturation. *Biochem. Cell Biol.* **65**, 869-875.

70. Benderoff, S., Blostein, R., and Johnstone, R.M. (1978) Changes in amino acid transport during red cell maturation. *Memb. Biochem.* **1**, 89-106.

71. Antonioli, J.A., and Christensen, H.N. (1969) Differences in schedules of regression of transport systems during reticulocyte maturation. *J. Biol. Chem.* **244**, 1505-1509.

72. Tucker, E.M., and Young, J.D. (1980) Biochemical changes during reticulocyte maturation in culture: a comparison of genetically different sheep erythrocytes. *Biochem. J.* **192**, 33-39.

73. Jarvis, S.M., and Young, J.D (1982) Nucleoside translocation in sheep reticulocytes and fetal erythrocytes: A proposed model for the nucleoside transporter. *J. Physiol.* **324**, 47-66.

74. Thomopoulos, P., Berthellier, M., and Laudat, M.H. (1978) Loss of insulin receptors on maturation of reticulocytes. *Biochem. Biophys. Res. Commun.* **85**, 1460-1465.

75. Im, J.H., Zeidler, R.B., Rackley, C.E., and Kim, H.D. (1984) Developmental changes in insulin receptors of pig red blood cells. *Arch. Biochem. Biophys.* **232**, 26-37.

76. Zeidler, R.B., and Kim, H.D. (1982) Pig reticulocytes IV. In vitro maturation of naturally occurring reticulocytes with permeability loss to glucose. *J. Cell. Physiol.* **112**, 360-366.

77. Johnstone, R.M., Adam, M., Hammond, J.R., Orr, L., and Turbide, C. (1987) Vesicle formation during reticulocyte maturation: association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* **262**, 9412-9420.

78. Inaba, M., and Maede, Y. (1986) Na,K-ATPase in dog red cells: immunological identification and maturation associated degradation by the proteolytic system. *J. Biol. Chem.* **261**, 16099-16105.

79. Limbird, L.E., Gill, D.M., Stadel, J.M., Hickey, A.R., and Lefkowitz, R.J. (1980) Loss of β-adrenergic receptor-guanine nucleotide regulatory protein interactions accompanies decline in catecholamine responsiveness of adenylate cyclase in maturing rat erythrocytes. *J. Biol. Chem.* **255**, 1854-1861.

80. Montandon, J.-B., and Porzig, H. (1984) Changes in beta-adrenoceptor binding properties and receptor-cyclase coupling during in vitro maturation of rat reticulocytes. *J. Receptor Res.* **4**, 91-102.

81. Patel, V.P., Ciechanover, A., Platt, O., and Lodish, H.F. (1985) Mammalian reticulocytes lose adhesion to fibronectin during maturation to erythrocytes. *Proc. Natl. Acad. Sci. USA* **82**, 440-444.

82. Rennie, C.M., Thompson, S., Parker, A.C., and Maddy, A. (1979) Human erythrocyte fractionation in "Percoll" density gradients. *Clin. Chim. Acta* **98**, 119-125.

83. Watts, R.P., Brendel, K., Luthra, M., and Kim, H.D. (1979) Inosine as a possible energy source for pig red blood cells. *Life Sci.* **25**, 1577-1582.

84. Come, S.E., Shohet, S.B., and Robinson, S.H. (1974) Surface remodeling vs. whole-cell hemolysis of reticulocytes produced with erythroid stimulation or iron deficiency anemia. *Blood* **44**, 817-830.

85. Gasko, O., and Danon, D. (1974) Endocytosis and exocytosis in membrane remodelling during reticulocyte maturation. *Br. J. Haematol.* **28**, 463-470.

86. Zaman, Z., Heynen, M.-J., Verwilghen, R.L. (1980) Studies on the mechanism of transferrin iron uptake by rate reticulocytes. *Biochim. Biophys. Acta* **632**, 553-561.

87. Pan, B.-T., and Johnstone, R.M. (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* **33**, 967-977.

88. Pan, B.-T., Teng, K., Wu, C., Adam, M.,and Johnstone, R.M. (1985) Electron microscopic evidence for the externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* **101**, 942-948.

89. Harding, C., Heuser, J., and Stahl, P. (1983) Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* **97**, 329-339.

90. Harding, C., Heuser, J., and Stahl, P. (1984) Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *Eur. J. Cell Biol.* **35**, 256-263.

91. Pan, B.-T., and Johnstone, R.M. (1984) Selective externalization of the transferrin receptor by sheep reticulocytes in vitro: response to ligands and inhibitors of endocytosis. *J. Biol. Chem.* **259**, 9776-9782.

92. Adam, M.A., and Johnstone, R.M. (1987) Protein kinase C does not phosphorylate the externalized form of the transferrin receptor. *Biochem J.* **242**, 151-161.

93. Davis, J.Q., Dansereau, D., Johnstone, R.M., and Bennett, V. (1986) Selective externalization of an ATP-binding protein structurally related to the clathrin-uncoating ATPase/heat shock protein in vesicles containing terminal transferrin receptors during reticulocyte maturation. *J. Biol. Chem.* **261**, 15368-15371.

94. Schlossman, D.M., Schmid, S.L., Braell, W.A., and Rothman, J.E. (1984) An enzyme that removes clathrin coats: purification of an uncoating ATPase. *J. Cell Biol.* **99**, 723-733.

95. Pelham, H.R.B.(1986) Speculations on the functions of the major heat shock and glucose-related proteins. *Cell* **46**, 959-961.

96. Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A., and Shekman, R. (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332**, 800-805.

97. Johnstone, R.M., Bianchini, A., and Teng, K. (1989) Reticulocyte maturation and exosome release: transferrin receptor containing exosomes show multiple plasma membrane functions. *Blood* **74**, 1844-1851.

98. Orr, L., Adam, M., and Johnstone, R.M. (1987) Externalization of membrane-bound activities during sheep reticulocyte maturation is temperature and ATP-dependent. *Biochem. Cell Biol.* **65**, 1080-1090.

99. Johnstone, R.M., Mathew, A., Mason, A.B., and Teng, K. (1991) Exosome formation during maturation of mammalian and avian reticulocytes: evidence that exosome release is a major route for externalization of obsolete membrane proteins. *J. Cell. Physiol.* **147**, 27-36.

100. Johnstone, R.M. (1992) Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. *Biochem. Cell Biol.* **70**, 179-190.

101. Flowers, CH, Skikne, BS, Covell, AM, Cook, JD (1989) The clinical measurement of serum transferrin receptor. *J. Lab. Clin. Med.* **114**, 368-377.

102. Shih, Y.J., Baynes, R.D., Hudson, B.G., Flowers, C.H., Skikne, B.S., and Cook, J.D. (1990) Serum transferrin receptor is a truncated form of the tissue receptor. *J. Biol. Chem.* **265**, 19077-19081.

103. Kohgo, Y., Niitsu, Y., Kondo, H., Kato, J., Tsushima, N., Sasaki, K., Hirayama, M., Numata, T., Nishisato, T., and Urushizaki, I. (1987) Serum transferrin receptor as a new index of erythropoiesis. *Blood* **70**, 1955-1958.

104. Skikne, B.S., Flowers, C.H., and Cook, J.D. (1990) Serum transferrin receptor: a quantitative measure of tissue iron deficiency. *Blood* **75**, 1870-1876.

105. Walter, P. , and Lingappa, V.R. (1986) Mechanism of protein translocation across the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* **2**, 499-516.

106. Wiedmann, M., Kurzchalia, T.V., Hartmann, E., and Rapoport, T.A. (1987) A signal sequence receptor in the endoplasmic reticulum membrane. *Nature* **328**, 830-833.

107. Zimmermann, R., Sagstetter, M., Lewis, M., and Pelham, H. (1988) Seventy-kilodalton heat shock proteins and additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. *EMBO J.* **7**, 2875-2880.

108. Singer, S.J., Maher, P.A., Yaffe, M.P. (1987) On the transfer of integral proteins into membranes. *Proc. Natl. Acad. Sci. USA* **84**, 1960-1964.

109. Shaw, A.S., Rottier, P.J.M., and Rose, J.K. (1988) Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **85**,7592-7596.

110. Mueckler, M., and Lodish, H.F. (1986) The human glucose transporter can insert posttranslationally into microsomes. *Cell* **44**, 629-637.

111. Kornfeld, R., and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**, 631-664.

112. Olson, T.S., and Lane, M.D. (1989) A common mechanism for posttranslational activation of plasma membrane receptors? *FASEB J.* **3**, 1618-1624.

113. Neefjes, J.J., Hengeveld, Tol, O., and Ploegh, H.L. (1990) Intracellular interactions of transferrin and its receptor during biosynthesis. *J. Cell Biol.* **111**, 1383-1392.

114. Peters, B.P., Hartle, R.J., Krzesicki, R.F., Kroll, T.G., Perini, F., Balun, J.E., Goldstein, J., and Ruddon, R.W. (1985) The biosynthesis, processing, and secretion of laminin by human choriocarcinoma cells. *J. Biol. Chem.* **260**, 14732-14742.

115. Gething, M.J., McCammon, K., and Sambrook, J. (1986) Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* **46**, 939-950.

116. Copeland, C.S., Doms, R.W., Bolzau, E.M., Webster, R.G., and Helenius, A. (1986) Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* **103**, 1179-1191.

117. Copeland, C.S., Zimmer, K.-P., Wagner, K.R., Healey, G.A., Mellman, I., and Helenius, A. (1988) Folding, trimerization, and transport are sequential events in the biogenesis of influenza virus hemagglutinin. *Cell* **53**, 197-209.

118. Mains, P.E., and Sibley, C.H. (1983) The requirement of light chain for the surface deposition of the heavy chain of immunoglobulin M. *J. Biol. Chem.* **258**, 5027-5033.

119. Bole, D.G., Hendershot, L.M., Kearney, J.F. (1986) Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* **102**, 1558-1566.

120. Kreis, T.E., and Lodish, H.F. (1986) Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. *Cell* **46**, 929-937.

121. Doms, R.W., Keller, D.S., Helenius, A., and Balch, W.E. (1987) Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitus virus G protein trimers. *J. Cell Biol.* **105**, 1957-1969.

122. Malhotra, V., Serafini, T., Orci, L., Shepherd, J.C., and Rothman, J.E. (1989) Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell* **58**, 329-336.

123. Block, M.R., Glick, B.S., Wilcox, C.A., Wieland, F.T., and Rothman, J.E. (1988) Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc. Natl. Acad. Sci. USA* **85**, 7852-7856.

124. Beckers, C.J.M., Block, M.R., Glick, B.S., Rothman, J.E., and Balch, W.E. (1989) Vesicular transport between the endoplasmic membrane and the Golgi stack requires the NEM-sensitive fusion protein. *Nature* **339**, 397-398.

125. Weidman, P.J., Melançon, P., Block, M.R., and Rothman, J.E. (1989) Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. *J. Cell Biol.* **108**, 1589-1596.

126. Goud, B., Zahraoui, A., Tavitian, A., and Saraste, J. (1990) Small GTP-binding protein associated with Golgi cisternae. *Nature* **345**, 553-556.

127. Mack, D., Kluxen, B., and Kruppa, J. (1989) Accessibility to proteases of the cytoplasmic G protein domain of vesicular stomatitis virus is increased during intracellular transport. *J. Cell Biol.* **109**, 2057-2065.

128. Dahms, N.M., Lobel, P., and Kornfeld, S. (1989) Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J. Biol. Chem.* **264**, 12115-12118.

129. Munro, S., and Pelham, H.R.B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907.

130. Dean, N., and Pelham, H.R.B. (1990) Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.* **111**, 369-377.

131. Vaux, D., Tooze, J., and Fuller, S. (1990) Identification by anti-idotype antibodies of an intracellular membrane protein that recognizes a mammalian endoplasmic reticulocyte retention signal. *Nature* **345**, 495-502.

132. Semenza, J. C., Hardwick, K.G., Dean, N., and Pelham, H.R.B. (1990) ERD2, a gene required from the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* **61**, 1349-1357.

133. Nilsson, T., Lucocq, J.M., Mackay, D. and Warren, G. (1991) The membrane spanning domain of β-1,4-galactosyltransferase specifies trans Golgi localization. *EMBO J.* **10**, 3567-3575.

134. Teasdale, R.D., D'Agostaro, G., Gleeson, P.A. (1992) The signal for Golgi retention of bovine β1,4-galactosyltransferase is in the transmembrane domain. *J. Biol. Chem.*. **267**, 4084-4096.

135. Chanat, E., and Huttner, W.B. (1991) Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. *J. Cell Biol.* **115**, 1505-1519.

136. Bomsel, M., and Mostov, K. (1991) Sorting of plasma membrane proteins in epithelial cells. *Curr. Opin. Cell Biol.* **3**, 647-653.

137. Vogel, L.K., Spiess, M., Sjöström, H., and Norén, O. (1992) Evidence for an apical sorting signal on the ectodomain of human aminopeptidase N. *J. Biol. Chem.* **267**, 2794-2797.

138. Lippincott-Schwartz, J., Bonifacino, J.S., Yuan, L.C., and Klausner, R.D. (1988) Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. *Cell* **54**, 209-220.

139. Marshall, S. (1985) Kinetics of insulin receptor internalization and recycling in adipocytes. *J. Biol. Chem.* **260**, 4136-4144.

140. Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W., and Schneider, W.J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system.*Annu. Rev. Cell Biol.* **1**, 1-39.

141. Watts, C. (1985) Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. *J. Cell Biol.* **100**, 633-6[illegible].

142. Ajioka, RS, Kaplan J (1986) Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors. *Proc. Natl. Acad. Sci. USA* **83**, 6445-6449.

143. Stein, B.S., and Sussman, H.H. (1986) Demonstration of two distinct transferrin receptor recycling pathways and transferrin-independent receptor internalization in K562 cells*J. Biol. Chem.* **261**,10319-10331.

144. Pearse, B.M.F., and Robinson, M.S. (1990) Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* **6**, 151-171.

145. Iacopetta, B.J., Rothenberger, S., and Kühn, L.C. (1988) A role for the cytoplasmic domain in transferrin receptor sorting and coated pit formation during endocytosis. *Cell* **54**, 485-489.

146. Miller, K., Shipman, M., Trowbridge, I.S., and Hopkins, C.R. (1991) Transferrin receptors promote the formation of clathrin lattices. *Cell* **65**, 621-632.

147. Nuñez, M.T., and Glass, J. (1983) The transferrin cycle and iron uptake in rabbit reticulocytes: pulse studies using ^{59}Fe, ^{125}I-labeled transferrin. *J. Biol. Chem.* **258**, 9676-9680.

148. Schmid, S.L., and Carter, L.L. (1990) ATP is required for receptor-mediated endocytosis of intact cells. *J. Cell Biol.* **111**, 2307-2318.

149. Chen, W.-J., Goldstein, J.L., and Brown, M.S. (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* **265**, 3116-3123.

150. Canfield, W.M., Johnson, K.F., Ye, R.D., Gregory, W., and Kornfeld, S. (1991) Localization of the signal for rapid internalization of the bovine cation-independent mannose-6-phosphate/IGF-II receptor to amino acids 24-29 of the cytoplasmic tail. *J. Biol. Chem.* **266**, 5682-5688.

151. Collawn, J.F., Strangel, M., Kuhn, L.A., Esekogwu, V., Jing, S., Trowbridge, I.S., and Tainer, J.A. (1990) Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell* **63**, 1061-1072.

152. Collawn, J.F., Kuhn, L.A., Liu, L.S., Tainer, J.A., and Trowbridge, I.S. (1991) Transplanted LDL and mannose-6-phosphate receptor internalization signals promote high-efficiency endocytosis of the transferrin receptor. *EMBO J.* **10**, 3247-3253.

153. Gironès, N., Alvarez, E., Seth, A., Lin, I., Latour, D.A., and Davis, R.J. (1991) Mutational analysis of the cytoplasmic tail of the human transferrin receptor: identification of a sub-domain that is required for rapid endocytosis. *J. Biol. Chem.* **266**, 19006-19012.

154. Dickson R.B., Hanover, J.A., Willingham, M.C., and Pastan, I. (1983) Prelysosomal divergence of transferrin and epidermal growth factor during receptor-mediated endocytosis. *Biochemistry* **22**, 5667-5674.

155. Hanover, J.A., Willingham, M.C., and Pastan, I. (1984) Kinetics of transit of transferrin and epidermal growth factor through clathrin-coated membranes. *Cell* **39**, 283-293.

156. Geuze, H.J., Slot, J.W, Strous, G.J.A.M., Lodish, H.F., and Schwartz, A. (1983) Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell* **32**, 277-287.

157. Yamashiro, D.J., Tycko, B., Fluss, S.R., and Maxfield, F.R. (1984) Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell* **37**, 789-800.

158. Karin, M., and Mintz, B. (1981) Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma cells. *J. Biol. Chem.* **256**, 3245-3252.

159. Bleil, J.D., and Bretscher, M.S. (1982) Transferrin receptor and its recycling in HeLa cells. *EMBO J.* **1**, 351-355.

160. Ciechanover, A., Schwartz, A.L., Dautry-Versat, A., and Lodish, H.F. (1983) Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. *J. Biol. Chem.* **258**, 9681-9689.

161. Enns, C.A., Larrick, J.W., Suomalainen, H., Schroder, J., and Sussman, H.H. (1983) Co-migration and internalization of transferrin and its receptor on K562 cells. *J. Cell Biol.* **97**, 579-585.

162. Hamilton, T.A. (1983) Receptor-mediated endocytosis of transferrin in concanavalin A-stimulated rat lymphoblasts. *J. Cell. Physiol.* **114**, 222-228.

163. Hopkins, C.R. (1983) Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell* **35**, 321-330.

164. Klausner, R.D., van Renswoude, J., Ashwell, G., Kempf, C., Schechter, A.N., Dean, A., and Bridges, K.R. (1983) Receptor-mediated endocytosis of transferrin in K562 cells. *J. Biol. Chem.* **258**, 4715-4724.

165. Alam, J., and Smith, A. (1989) Receptor-mediated transport of heme by hemopexin regulates gene expression in mammalian cells. *J. Biol. Chem.* **264**, 17637-17640.

166. Snider, M.D., and Rogers, O.C. (1986) Membrane traffic in animal cells: cellular glycoproteins return to the site of Golgi mannosidase I. *J. Cell Biol.* **103**, 265-275.

167. Regoeczi,E., Chindemi, P.A., Debanne, M.T., and Charlwood., P.A. (1982) Partial resialylation of human asialotransferrin type 3 in the rat. *Proc. Natl. Acad. Sci. USA* **79**, 2226-2230.

168. Stoorvogel, W., Geuze, H.J., Griffith, J.M., and Strous, G.J. (1988) The pathways of endocytosed transferrin and secretory protein are connected in the trans-Golgi reticulum. *J. Cell Biol.* **106**, 1821-1829.

169. Honegger, A.M., Schmidt, A., Ullrich, A., and Schlessinger, J. (1990) Separate endocytic pathways of kinase-defective and -active EGF receptor mutants expressed in same cells. *J. Cell Biol.* **110**, 1541-1548.

170. Weissman, A.M., Klausner, R.D., Rao, K., and Harford, J.B. (1986) Exposure of K562 cells to anti-receptor monoclonal antibody OKT9 results in rapid redistribution and enhanced degradation of the transferrin receptor. *J. Cell Biol* **102**, 951-958.

171. Lesley, J.F., and Schulte, R.J. (1985) Inhibition of cell growth by monoclonal anti-transferrin receptor antibodies. *Mol. Cell. Biol.* **5**, 1814-1821.

172. Crichton, R.R., and Charloteaux-Wauters, M. (1987) Iron transport and storage. *Eur. J. Biochem.* **164**, 485-506.

173. Morgan, E.H. (1983) Effect of pH and iron content of transferrin on its binding to reticulocyte receptors. *Biochim. Biophys. Acta* **762**, 498-502.

174. Dautry-Varsat, A., Ciechanover, A., and Lodish, H.F. (1983) pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA* **80**, 2258-2262.

175. Loh, T.T., Yeung, Y.G., Yeung, D. (1977) Transferrin and iron uptake by rabbit reticulocytes. *Biochim. Biophys. Acta* **471**, 118-124.

176. Glass, J., Nunez, M.T., and Robinson, S.H. (1977) Iron transport from Sepharose-bound transferrin. *Biochem. Biophys. Res. Commun.* **75**, 226-232.

177. Lamb, J.E., Ray, F., Ward, J.H., Kushner, J.P., and Kaplan, J. (1983) Internalization and subcellular localization of transferrin and transferrin receptors in HeLa cells. *J. Biol. Chem.* **258**, 8751-8758.

178. van Renswoude, J., Bridges, K.R., Harford, J.B., and Klausner, R.D. (1982) Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: identification of a nonlysosomal acidic compartment. *Proc. Natl. Acad. Sci. USA* **79**, 6186-6190.

179. Klausner, R.D., Ashwell, G., van Renswoude, J., Harford, J.B., and Bridges, K.R. (1983) Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. USA* **80**, 2263-2266.

180. Octave, J.-N., Schneider, Y.-J., Hoffman, P., Trouet, A., and Crichton, R.R. (1982) Transferrin uptake by cultured rat embryo fibroblasts: the influence of lysosomotropic agents, iron chelators, and colchicine on the uptake of iron and transferrin. *Eur. J. Biochem.* **123**, 235-240.

181. Glass, J., and Nunez, M.T. (1986) Amines as inhibitors of iron transport in rabbit reticulocytes. *J. Biol. Chem.* **261**, 8298-8302.

182. Bakkeren, D.L., de Jeu-Jaspars, C.M.H., Kroos, M.J., and van Eijk, H.G. (1987) Release of iron from endosomes in an eary step in the transferrin cycle. *Int. J. Biochem.* **19**, 179-186.

183. Bali, P.K., Zak, O., and Aisen, P. (1991) A new role for the transferrin receptor in the release of iron from transferrin. *Biochemistry* **30**, 324-328.

184. Nuñez, M.T., Gaete, V., Watkins, J.A., and Glass, J. (1990) Mobilization of iron from endocytic vesicles: the effects of acidification and reduction. *J. Biol. Chem.* **265**, 6688-6692.

185. Weaver, J., and Pollack, S. (1990) Two types of receptors for iron on mitochondria. *Biochem. J.* **271**, 463-466.

186. Morgan, E.H. (1989) Calcium chelators induce association with the detergent-insoluble cytoskeleton and functional inactivation of the transferrin receptor in reticulocytes. *Biochim. Biophys. Acta* **981**, 121-129.

187. Nilsen, T. (1991) Effects of calcium on hepatocyte iron uptake from transferrin iron-pyrophosphate and iron-ascorbate. *Biochim. Biophys. Acta (Mol. Cell Res.)* **1095**, 39-45.

188. Seligman, P.A., Kovar, J., Schleicher, R.B., and Gelfand, E.W. (1991) Transferrin-independent iron uptake supports B lymphocyte growth. *Blood* **78**, 1526-1531.

189. Wright, T.L., Brissot, P., Ma, W.-L., Weisiger, R.A. (1986) Characterization of non-transferrin-bound iron clearance by rat liver. *J. Biol. Chem.* **261**, 10909-10914.

190. Sturrock, A., Alexander, J., Lamb, J., Craven, C.M., and Kaplan, J. (1990) Characterization of a transferrin-independent uptake system for iron in HeLa cells. *J. Biol. Chem.* **265**, 3139-3145.

191. Page, M.A., Baker, E., and Morgan, E.H. (1984) Transferrin and iron uptake by rat hepatocytes in culture. *Am. J. Physiol.* **246**, G26-G33.

192. Mattia, E., Rao, K., Shapiro, D.S., Sussman, H.H., and Klausner, R.D. (1984) Biosynthetic regulation of the human transferrin receptor by desferrioxamine in K562 cells. *J. Biol. Chem.* **259**, 2689-2692.

193. Bridges, K.R., and Cudkowicz, A. (1984) Effect of iron chelators on the transferrin receptor in K562 cells. *J. Biol. Chem.* **259**, 12970-12977.

194. Bomford, A., Isaac, J., Roberts, S., Edwards, A., Young, S., and Williams, R. (1986) The effect of desferrioxamine on transferrin receptors, the cell cycle and growth rates of human leukaemic cells. *Biochem. J.* **236**, 243-249.

195. Ward, J.H., Kushner, J.P., and Kaplan, J. (1982) Regulation of HeLa cell transferrin receptors. *J. Biol. Chem.* **257**, 10317-10323.

196. Louache, F., Testa, U., Pelicci, P., Thomopoulos, P., Titeux, M., and Rochant, H.(1984) Regulation of transferrin receptors in human hematopoietic cell lines. *J. Biol. Chem.* **259**, 11576-11582.

197. Bottomley, S.S., Wolfe, L.C., Bridges, K.R. (1985) Iron metabolism in K562 erythroleukemic cells. *J. Biol. Chem.* **260**, 6811-6815.

198. Rudolph, N.S., Ohlsson-Wilhelm, B.M., Leary, J.F., and Rowley, P.T. (1985) Regulation of K562 cell transferrin receptors by exogenous iron. *J. Cell. Physiol.* **122**, 441-450.

199. Rouault, T., Rao, K., Harford, J., Mattia, E., Klausner, R.D. (1985) Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. *J. Biol. Chem.* **260**, 14862-14866.

200. Koeller, D.M., Casey, J.L., Hentze, M.W., Gerhardt, E.M., Chan, L.-N.L., Klausner, R.D., and Harford, J.B. (1989) A cytosolic protein binds to structural elements within the iron regulatory region of the transferrin receptor mRNA. *Proc. Natl. Acad. Sci. USA* **86**, 3574-3578.

201. Müllner, E.W., Neupert, B., and Kühn, L.C. (1989) A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. *Cell* **58**, 373-382.

202. Casey, J.L., Hentze, M.W., Koeller, D.M., Caughman, S.W., Rouault, T.A., Klausner, R.D., and Harford, J.B. (1988) Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. *Science* **240**, 924-928.

203. Müllner, E.W., and Kühn, L.C. (1988) A stem-loop in the 3' untranslated region mediates iron-dependent regulation of the transferrin receptor mRNA stability in the cytoplasm. *Cell* **53**, 815-825.

204. Rouault, T.A., Hentz, M.W., Caughman, S.W., Harford, J.B., and Klausner, R.D. (1988) Binding of a cytosolic protein to the iron-responsive element of human ferritin messenger RNA. *Science* **241**, 1207-1210.

205. Theil, E.C. (1990) Regulation of ferritin and transferrin receptor mRNAs. *J. Biol. Chem.* **265**, 4771-4774.

206. Cox, T.C., Bawden, M.J., Martin, A., May, B.K. (1991) Human erythroid 5-ALA synthase: promoter analysis and identification of an iron-responsive element in mRNA. *EMBO J.* **10**, 1891-1902.

207. Dandekar, T., Stripecke, R., Gray, N.K., Goossen, B., Constable, A., Johansson, H.E., Hentze, M.W. (1991) Identification of a novel iron-responsive element in murine and human erythroid δ-aminolevulinic acid synthase mRNA. *EMBO J.* **10**, 1903-1909.

208. Rouault, T.A., Stout, C.D., Kaptain, S., Harford, J.B., and Klausner, R.D. (1991) Structural relationship between an iron-regulated RNA-binding protein (IRE-BP) and aconitase.*Cell* **64**, 881-883.

209. Hentze, M.W., and Argos, P. (1991) Homology between IRE-BP, a regulatory RNA-binding protein, aconitase, and isopropylmalate isomerase. *Nucleic Acids Res.* **19**, 1739-1740.

210. Kaptain, S, Downey, W.E., Tang, C., Philpott, C., Haile, D., Orloff, D.G., Harford, J.B., Rouault, T.A., and Klausner, R.D. (1991) A regulated RNA binding protein also possesses aconitase activity. *Proc. Natl. Acad. Sci. USA* **88**, 10109-10113.

211. Battistini, A., Coccia, E.M., Marzialli, G., Bulgarini, D., Scalzo, S., Fiorucci, G., Romeo, G., Affabris, E., Testa, U., Rossi, G.B., Peschle, C. (1991) Intracellular heme coordinately modulates globin chain synthesis, transferrin receptor number, and ferritin content in differentiating Friend erythroleukemia cells. *Blood* **78**, 2098-2103.

212. Yeh, C.G., Parmichael, M., and Faulk, W.P. (1982) Loss of transferrin receptors following induced differentiation of HL-60 promyelocytic leukemia cells. *Exp. Cell Res.* **138**, 429-433.

213. Rovera, G., Ferrero, D., Pagliardi, G.L., Vartikar, J., Pessano, S., Bottero, L., Abraham, S., and Lebman, D. (1982) Induction of differentiation of human myeloid leukemias by phorbol diesters. *Ann. N.Y. Acad. Sci.* **397**,211-220.

214. Larrick, J.W., and Cresswell, P. (1979) Modulation of cell surface iron transferrin receptors by cellular density and state of activation. *J. Supramol. Struct.* **11**, 579-586.

215. Neckers, L.M., and Cossman, J. (1983) Transferrin receptor induction in mitogen-stimulated T lymphocytes is required for DNA synthesis and

cell division and is regulated by interleukin-2 (TCGF). *Proc. Natl. Acad. Sci. USA* **80**, 3494-3498.

216. Owen, D., and Kühn, L.C. (1987) Noncoding 3' sequences of the transferrin receptor gene are required for mRNA regulation by iron. *EMBO J.* **6**, 1287-1293.
217. Iacopetta, B.J., Morgan, E.H., and Yeoh, G.C.T. (1983) Receptor-mediated endocytosis of transferrin by developing erythroid cells from fetal rat liver. *J. Histochem. Cytochem.* **31**, 336-344.
218. Wiley, H.S., and Kaplan, J. (1984) Epidermal growth factor rapidly induces a redistribution of transferrin receptor pools in human fibroblasts. *Proc. Natl. Acad. Sci. USA* **81**, 7456-7460.
219. Davis, R.J., Corvera, S., and Czech, M.P. (1986) Insulin stimulates cellular iron uptake and causes a redistribution of intracellular transferrin receptors to the plasma membrane. *J. Biol. Chem.* **261**, 8708-8711.
220. Davis, R.J., Faucher, M., Racaniello, L.K., Carruthers, A., and Czech, M.P. (1987) Insulin-like growth factor I and epidermal growth factor regulate the expression of transferrin receptors at the cell surface by distinct mechanisms. *J. Biol. Chem.* **262**, 13126-13134.
221. Tanner, L.I., and Lienhard, G.E. (1987) Insulin elicits a redistribution of transferrin receptors in 3T3-L1 adipocytes through an increase in the rate constant for receptor externalization. *J. Biol. Chem.* **262**, 8975-8980.
222. Buys, S.S., Keogh, E.A., and Kaplan, J. (1984) Fusion of intracellular membrane pools with cell surfaces of macrophages stimulated by phorbol esters and calcium ionophores. *Cell* **38**, 569-576.
223. McGraw, T.E., Dunn, K.W., and Maxfield, F.R. (1988) Phorbol ester treatment increases the exocytic rate of the transferrin receptor recycling pathway independent of serine-24 phosphorylation. *J. Cell Biol.* **106**, 1061-1066.
224. Zerial, M., Suomalainen, M., Zanetti-Schneider, M., Schneider, C., and Garoff, H. (1987) Phosphorylation of the human transferrin receptor by protein kinase C is not required for endocytosis and recycling in mouse 3T3 cells. *EMBO J.* **6**, 2661-2667.

225. Davis, R.J., and Meisner, H. (1987) Regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts. *J. Biol. Chem.* **262**, 16041-16047.

226. May, W.S., Jacobs, S., and Cuatrecasas, P. (1984) Association of phorbol ester-induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL60 cells. *Proc. Natl. Acad. Sci. USA* **81**, 2016-2020.

227. Klausner, R.D., Harford, J., van Renswoude, J. (1984) Rapid internalization of the transferrin receptor in K562 cells is triggered by ligand binding or treatment with a phorbol ester. *Proc. Natl. Acad. Sci. USA* **81**, 3005-3009.

228. Iacopetta, B., Carpentier, J.L., Pozzan, T., Lew, D.P., Gorden, P., and Orci, L. (1986) Role of intracellular calcium and protein kinase C in the endocytosis of transferrin and insulin by HL60 cells. *J. Cell Biol.* **103**, 851-856.

229. Alcantara, O., Phillips, J.L., and Boldt, D.H. (1986) Phorbol diesters and transferrin modulate lymphoblastoid cell transferrin receptor expression by two different mechanisms *J. Cell. Physiol.* **129**, 329-335.

230. Sorokin, L.M., Morgan, E.H., and Yeoh, G.C.T. (1988) Differences in transferrin receptor function between normal developing and transformed myogenic cells as revealed by differential effects of phorbol ester on receptor distribution and rates of iron uptake. *J. Biol. Chem.* **263**, 14128-14133.

231. Kohno, H., Taketani, S., and Tokunaga, R. (1986) Phorbol ester-induced regulation of transferrin receptors in human leukemia K562 cells. *Cell Struct. Funct.* **11**, 181-190.

232. Larrick, J.W., Enns, C., Raubitschek, A., and Weintraub, H. (1985) Receptor-mediated endocytosis of human transferrin and its cell surface receptor. *J. Cell. Physiol.* **124**, 283-287.

233. Gironès, N., and Davis, R.J. (1989) Comparison of the kinetics of cycling of the transferrin receptor in the presence and absence of bound diferric transferrin. *Biochem. J.* **264**, 35-46.

234. Sainte-Marie, J., Vidal, M., Bette-Bobillo, P., Philippot, J.R., and Bienvenue, A. (1991) The influence of transferrin binding to L_2C guinea pig leukemic lymphocytes on the endocytosis cycle kinetics of its receptor. *Eur. J. Biochem.* **201**, 295-302.

235. Hradilek, A., and Neuwirt, J. (1987) Iron uptake from transferrin and transferrin endocytic cycle in Friend erythroleukemic cells. *J. Cell. Physiol.* **133**, 192-196.

236. Ponka, P., and Neuwirt, J. (1969) Regulation of iron entry into reticulocytes. I. Feedback inhibitory effect of heme on iron entry into reticulocytes and on heme synthesis. *Blood* **33**, 690-707.

237. Iacopetta, B., and Morgan, E. (1984) Heme inhibits transferrin endocytosis in immature erythroid cells. *Biochim. Biophys. Acta* **805**, 211-216.

238. Ponka, P., Schulman, H.M., and Martinez-Medellin, J. (1988) Haem inhibits iron uptake subsequent to endocytosis of transferrin in reticulocytes. *Biochem. J.* **251**, 105-109.

239. Cox, T.M., O'Donnell, M.W., Aisen, P., and London, I.M. (1985) Hemin inhibits internalization of transferrin by reticulocytes and promotes phosphorylation of the membrane transferrin receptor. *Proc. Natl. Acad. Sci. USA* **82**, 5170-5174.

240. Ponka, P., Wilczynska, A., Schulman, H.M. (1982) Iron utilization in rabbit reticulocytes: a study using succinylacetone as an inhibitor of heme synthesis. *Biochim. Biophys. Acta* **720**, 96-105.

241. Adams, M.L., Ostapiuk, I., and Grasso, J.A. (1989) The effects of inhibition of heme synthesis on the intracellular localization of iron in rat reticulocytes. *Biochim. Biophys. Acta* **1012**, 243-253.

242. Hradilek, A., Borova, J., Fuchs, O., and Neuwirt, J. (1981) The effect of heme on intracellular iron pools during differentiation of Friend erythroleukemia cells. *Biochim. Biophys. Acta* **678**, 373-380.

243. Hamilton, T.A., Wada, H.G., and Sussman, H.H. (1979) Identification of transferrin receptors on the surface of human cultured cells. *Proc. Natl. Acad. Sci. USA* **76**, 6406-6410.

244. Fernandez-Pol, J.A., and Klos, D.J. (1980) Isolation and characterization of normal rat kidney cell membrane proteins with affinity for transferrin. *Biochemistry* **19**, 3904-3912.

245. Leibman, A., and Aisen, P. (1977) Transferrin receptor of the rabbit reticulocyte. *Biochemistry* **16**, 1268-1272.

246. van Bockxmeer, F.M., and Morgan, E.H. (1977) Identification of transferrin receptors in reticulocytes. *Biochim. Biophys. Acta* **468**, 437-450.

247. Ecarot-Charrier, B., Grey, V.L., Wilczynska, A., and Schulman, H.M. (1980) Reticulocyte membrane transferrin receptors. *Can. J. Biochem.* **58**, 418-426.

248. Seligman, P.A., Schleicher, R.B., Allen, R.H. (1979) Isolation and characterization of the transferrin receptor from human placenta. *J. Biol. Chem.* **254**, 9943-9946.

249. Enns, C.A., and Sussman, H.H. (1981) Characterization of the transferrin-binding protein in a human trophoblast. *Placenta* **3**, 23-32.

250. Trowbridge, I.S., and Omary, M.B. (1981) Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci. USA* **78**, 3039-3043.

251. van Driel, I.R., Stearne, P.A., Grego, B., Simpson, R.J., and Goding, J.W. (1984) The receptor for transferrin on murine myeloma cells: one-step purification based on its physiology and partial amino acid sequence. *J. Immunol.* **133**, 3220-3224.

252. Turkewitz, A.P., Amatruda, J.F., Borhani, D., Harrison, S.C., and Schwartz, A.L. (1988) A high yield purification of the human transferrin receptor and properties of its major extracellular fragment. *J. Biol. Chem.* **263**, 8318-8325.

253. Omary, M.B., and Trowbridge, I.S. (1981) Biosynthesis of the human transferrin receptor in cultured cells. *J. Biol. Chem.* **256**, 12888-12892.

254. Schneider, C., Sutherland, R., Newman, R., and Greaves, M. (1982) Structural features of the cell surface receptor for transferrin that is recognized by the monoclonal antibody OKT9. *J. Biol. Chem.* **257**, 8516-8522.

255. van Agthoven, A., Goridis, C., Naquet, P., Pierres, A., and Pierres, M. (1984) Structural characteristics of the mouse transferrin receptor. *Eur. J. Biochem.* **140**, 433-440.

256. Jing, S., and Trowbridge, I.S. (1987) Identification of the intermolecular disulfide bonds of the human transferrin receptor and its lipid-attachment site. *EMBO J.* **6**, 327-331.

257. McClelland, A., Kuhn, L.C., and Ruddle, F.H. (1984) The human transferrin receptor gene: genomic organization, and the complete primary structure of the receptor deduced from a cDNA sequence. *Cell* **39**, 267-274.

258. Schneider, C., Owen, M.J., Banville, D., and Williams, J.G. (1984) Primary structure of human transferrin receptor deduced from the mRNA sequence. *Nature* **311**, 675-678.

259. Stearne, P.A., Pieterz, G.A., and Goding, J.W. (1985) cDNA cloning of the murine transferrin receptor: sequence of trans-membrane and adjacent regions. *J. Immunol.* **134**, 3474-3479.

260. Gerhardt, E.M., Chan, L.-N.L., Jing, S., Qi, M., and Trowbridge, I.S. (1991) The cDNA sequence and primary structure of the chicken transferrin receptor. *Gene* **102**, 249-254.

261. Adam, M., Rodriquez, A., Turbide, C., Larrick, J., Meighen, E., and Johnstone, R.M. (1984) In vitro acylation of the transferrin receptor. *J. Biol. Chem.* **259**, 15460-15463.

262. Jing, S., and Trowbridge, I.S. (1990) Nonacylated human transferrin receptors are rapidly internalized and mediate iron uptake. *J. Biol. Chem.* **265**, 11555-11559.

263. Alvarez, E., Gironès, N., and Davis, R.J. (1989) Intermolecular disulfide bonds are not required for the expression of the dimeric state and functional activity of the transferrin receptor. *EMBO J.* **8**, 2231-2240.

264. Enns, C.A., Clinton, E.M., Reckhow, C.L., Root, B.J., Do, S.-I., and Cook, C. (1991) Aquisition of the functional properties of the TfR during its biosynthesis. *J. Biol. Chem.* **266**, 13272-13277.

265. Do, S.-I., Enns, C., and Cummings, R.D. (1990) Human transferrin receptor contains O-linked oligosaccharides. *J. Biol. Chem* **265**, 114-125.

266. Reckhow, C.L., and Enns, C.A. (1988) Characterization of the transferrin receptor in tunicamycin-treated A431 cells. *J. Biol. Chem.* **263**, 7297-7301.

267. Hunt, R.C., Riegler, R., and Davis, A.A. (1989) Changes in glycosylation alter the affinity of the human transferrin receptor for its ligand. *J. Biol. Chem.* **264**, 9643-9648.

268. Ralton, J.E., Jackson, H.J., Zanoni, M., and Gleeson, P.A. (1989) Effect of glycosylation inhibitors on the structure and function of the murine transferrin receptor. *Eur. J. Biochem.* **186**, 637-647.

269. Williams, A.M., and Enns, C.A. (1991) A mutated transferrin receptor lacking asparagine-linked glycosylation sites shows reduced

functionality and an association with binding immunoglobulin protein. *J. Biol. Chem.* **266**, 17648-17654.

270. Hoe, M.H., and Hunt, R.C. (1992) Loss of one asparagine-linked oligosaccharide from human transferrin receptors results in specific cleavage and association with the endoplasmic reticulum. *J. Biol. Chem.* **267**, 4916-4923.
271. Johnstone, R.M., Adam, M., Turbide, C., and Larrick, J. (1984) Phosphorylation of the transferrin receptor in isolated sheep reticulocyte plasma membranes. *Can. J. Biochem. Cell Biol.* **62**, 927-934.
272. May, W.S., and Tyler, G. (1987) Phosphorylation of the surface transferrin receptor stimulates receptor internalization in HL60 leukemic cells. *J. Biol. Chem.* **262**, 16710-16718.
273. Hunt, R.C., Ruffin, R., and Yang, Y.-S. (1984) Alterations in the transferrin receptor of human erythroleukemic cells after induction of hemoglobin synthesis. *J. Biol. Chem.* **259**, 9944-9952.
274. Rothenberger, S., Iacopetta, B.J., and Kuhn, L.C. (1987) Endocytosis of the transferrin receptor requires the cytoplasmic domain but not its phosphorylation site. *Cell* **49**, 423-431.
275. Laskey, J.D., Ponka, P., and Schulman, H.M. (1986) Control of heme synthesis during Friend cell differentiation: role of iron and transferrin. *J. Cell. Physiol.* **129**, 185-192.
276. Ponka, P., and Schulman, H.M. (1985) Acquisition of iron from transferrin regulates reticulocyte heme synthesis. *J. Biol. Chem.* **260**, 14717-14721.
277. Ponka, P., and Schulman, H.M. (1985) Regulation of heme synthesis in erythroid cells: hemin inhibits transferrin iron utilization but not protoporphyrin synthesis. *Blood* **65**, 850-857.
278. Riddle, R.D., Yamamoto, [illegible]., Engel, J.D. (1989) Expression of δ-aminolevulinate synthase in avian cells: separate genes encode erythroid-specific and nonspecific isozymes. *Proc. Natl. Acad. Sci.USA* **86**, 792-796.
279. Elferink, C.J., Sassa, S., and May, B.K. (1988) Regulation of 5-aminolevulinate synthase in mouse erythroleukemic cells is different from that in liver. *J. Biol. Chem.* **263**, 13012-13016.

280. Hoffman, L.M., and Ross, J. (1980) The role of heme in the maturation of erythroblasts: the effect of inhibition of pyridoxine metabolism. *Blood* **55**, 762-771.

281. Mager, D., and Bernstein, A. (1979) The role of heme in the regulation of the late program of Friend cell erythroid differentiation. *J. Cell. Physiol.* **100**, 467-480.

282. Lo, S.C., Aft, R., and Mueller, G.C. (1981) Role of nonhemoglobin heme accumulation in the terminal differentiation of Friend erythroleukemia cells. *Cancer Res.* **41**, 864-870.

283. Chen, J.-J., and London, I.M. (1981) Hemin enhances the differentiation of mouse 3T3 cells to adipocytes. *Cell* **26**, 117-122.

284. Ishii, D.N., and Maniatis, G.M. Hemin promotes rapid neurite outgrowth in cultured mouse neuroblastoma cells. (1978) *Nature* **274**, 373-374.

285. Bruns, G.P., and London, I.M. (1965) The effect of hemin on the synthesis of globin. *Biochem. Biophys. Res. Commun.* **18**, 236-242.

286. Adamson, S.D., Herbert, E., and Godchaux, W. (1968) Factors affecting the rate of protein synthesis in lysate systems from reticulocytes. *Arch. Biochem. Biophys.* **125**, 671-683.

287. Zucker, W.V., and Schulman, H.M. (1968) Stimulation of globin-chain initiation by hemin in the reticulocyte cell-free system. *Proc. Natl. Acad. Sci. USA* **59**, 582-589.

288. Beuzard, Y., Rodvien, R., and London, I.M. (1973) Effect of hemin on the synthesis of hemoglobin and other proteins in mammalian cells. *Proc. Natl. Acad. Sci. USA* **70**, 1022-1026.

289. Lodish, H.F., and Desalu, O. (1973) Regulation of synthesis of non-globin proteins in cell-free extracts of rabbit reticulocytes. *J. Biol. Chem.* **248**, 3520-3527.

290. Gross, M., and Rabinowitz, M. (1972) Control of globin synthesis in cell-free preparations of reticulocytes by formation of a translational repressor that is inactivated by hemin. *Proc. Natl. Acad. Sci. USA* **69**, 1565-1568.

291. Mizuno, S., Fisher, J.M., Rabinovitz, M. (1972) Hemin control of globin synthesis: action of an inhibitor formed in the absence of hemin on the reticulocyte cell-free system and its reversal by a ribosomal factor. *Biochim. Biophys. Acta* **272**, 638-650.

292. Farrell, P.J., Balkow, K., Hunt, T., Jackson, R.J., and Trachsel, H. (1977) Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. *Cell* **11**, 187-200.

293. Matts, R.L., Levin, D.H., and London, I.M. (1986) Fate of reversing factor during restoration of protein synthesis by hemin or GTP in heme-deficient reticulocyte lysates. *Proc. Natl. Acad. Sci. USA* **83**, 1217-1221.

294. Fagard, R., and London, I.M. (1981) Relationship between phosphorylation and activity of heme-regulated eukaryotic initiation factor 2α kinase. *Proc. Natl. Acad. Sci. USA* **78**, 866-870.

295. Chen, J.-J., Yang, J.M., Petryshyn, R., Kosower, N., and London, I.M. (1989) Disulfide bond formation in the regulation of eIF-2α kinase by heme. *J. Biol. Chem.* **264**, 9559-9564.

296. Etlinger, J.D., and Goldberg, A.L. (1980) Control of protein degradation in reticulocytes and reticulocyte extracts by hemin. *J. Biol. Chem.* **255**, 4563-4568.

297. Haas, A.L., and Rose, I.A. (1981) Hemin inhibits ATP-dependent ubiquitin-dependent proteolysis: Role of hemin in regulating ubiquitin conjugate degradation. *Proc. Natl. Acad. Sci. USA* **78**, 6845-6848.

298. Aft, R.L., and Mueller, G.C. (1984) Heme-mediated oxidative degradation of proteins. *J. Biol. Chem.* **259**, 301-305.

299. Stadtman, E.R. (1991) Ascorbic acid and oxidative inactivation of proteins. *Am. J. Clin. Nutr.* **54**, 1125S-1128S.

300. Lui, S.-C., Zhai, S., Lawler, J., and Palek, J. (1985) Hemin-mediated dissociation of erythrocyte membrane skeletal proteins. *J. Biol. Chem.* **260**, 12234-12239.

301. Chou, A.C., and Fitch, C.D. (1981) Mechanism of hemolysis induced by ferriprotoporphyrin IX. *J. Clin. Invest.* **68**, 672-677.

302. Shviro,Y., and Shaklai, N. (1987) Glutathione as a scavenger of free hemin: a mechanism of preventing red cell membrane damage. *Biochem. Pharmacol.* **36**,3801-3807.

303. Koch, P.A., Gardner, F.H., Gartrell, J.E., and Carter, J.R. (1975) Biogenesis of erythrocyte membrane proteins: in vitro studies with rabbit reticulocytes. *Biochim. Biophys. Acta* **389**, 177-187.

304. Lodish, H.F., and Small, B. (1975) Membrane proteins synthesized by rabbit reticulocytes. *J. Cell Biol.* **65**, 51-64.

305. Chang, H., Langer, P.J., and Lodish, H.F. (1976) Asynchronous synthesis of erythrocyte membrane proteins. *Proc. Natl. Acad. Sci. USA* **73**, 3206-3210.

306. Krasnow, S.H., Pielichowski, H.J., Caro, J., Burka, E.R., and Ballas, S.K. (1981) Membrane proteins synthesized by human reticulocytes and thei precursors. *Br. J. Haematol.* **47**, 171-183.

307. Reichstein, E., and Blostein, R. (1975).Arrangement of human erythrocyte membrane proteins. *J. Biol. Chem.* **250**, 6256-6263.

308. Mishell, B.B., and Shiigi, S.M. (1980) Lysis of red blood cells with Tris-buffered ammonium chloride. In: *Selected Methods in Cellular Immunology* (B.B. Mishell and S.M. Shiigi, eds.) W.H. Freeman, San Francisco, p. 23.

309. Mans, R.J., and Novelli, G.D. (1961) Filter paper disk method for measuring incorporation of radioactive amino acids into protein. *Arch. Biochem. Biophys.* **94**, 48-53.

310. Baker, E., Shaw, D.C., and Morgan, E.H. (1968) Isolation and characterization of rabbit serum and milk transferrins: evidence for difference in sialic acid content only. *Biochemistry* **7**, 1371-1378.

311. Hunter, W.M., and Greenwood, F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* **194**, 459-496.

312. Adam, M., Wu., C., Turbide, C., Larrick, J., and Johnstone, R.M. (1986) Evidence for a pool of non-recycling transferrin receptors in peripheral sheep reticulocytes. *J. Cell. Physiol.* **127**, 8-16.

313. Beaufay, H., Amar-Costesec, A., Feytmans, E., Thinès-Sempoux, D., Wibo, M., Robbi, M., and Berthet, J. (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* **61**, 188-200.

314. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**, 119-130.

315. Edge, A.S.B., Faltynek, C.R., Hof, L., Reichert, L.E., Jr., and Weber, P. (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal. Biochem.* **118**, 131-137.

316. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

317. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**, 2606-2617.

318. Cabantchik, and Rothstein. (1974) Membrane proteins related to anion permeability of human red blood cells. *J. Memb. Biol.* **15**, 207-226.

319. Cleveland, D.W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**, 1102-1106.

320. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

321. Romero, P.A., Saunier, B., and Herscovics, A. (1985) Comparison between 1-deoxynojirimycin and N-methyl-1-deoxynojirimycin as inhibitors of oligosaccharide processing in intestinal epithelial cells. *Biochem. J.* **226**, 733-740.

322. Oi, V.T., and Herzenberg, L.A. (1980) Immunoglobulin-producing hybrid cell lines. In: *Selected Methods in Cellular Immunology* (B.B. Mishell and S.M. Shiigi, eds). W.H. Freedman, San Francisco,pp. 357-359.

323. Morris, S.J., Sarkar, D.P., White, J.M., and Blumenthal, R. (1989) Kinetics of pH-dependent fusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells: measurement by dequenching of fluorescence. *J. Biol. Chem.* **264**, 3972-3978.

324. Magnani, M., Rossi, L., Bianchi, M., Serafini, G., and Stocchi, V. (1988) Role of hexokinase in the regulation of erythrocyte hexose monophosphate pathway under oxidative stress. *Biochem. Biophys. Res. Commun.* **155**, 423-428.

325. Stanley, P.E., and Williams, S.G. (1969) Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem.* **29**, 381-392.

326. Nielands, J.B. (1955) Lactate dehydrogenase of heart muscle. *Method. Enzymol.* **1**, 449-454.

327. Hamaguchi, H., and Cleve, H. (1972) Solubilization of human erythrocyte membrane glycoproteins and separation of the MN glycoprotein from a

glycoprotein with I, S, and A activity. *Biochim. Biophys. Acta* **278**, 271-280.

328. Markwell, M.A.K., Hass, S.M., Tolbert, N.E., and Bieber, L.L. (1981) Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol.* **72**, 296-303.
329. Davis, L.G., Dibner, M.D., Battey, J.F. (1986) *Basic Methods in Molecular Biology*. Elvesier, New York, p. 129.
330. Ward, J.H., Jordon, I., Kushner, J.P., and Kaplan, J. (1984) Heme regulation of HeLa cell transferrin receptor number. *J. Biol. Chem.* **259**, 13235-13240.
331. Johnstone, R.M., Adam, M., and Pan, B.T. (1984) The fate of the transferrin receptor during maturation of sheep reticulocytes in vitro. *Can. J. Biochem. Cell Biol.* **62**, 1246-1254.
332. Kirschner-Zilber, I., Rabizadeh, E., and Shaklai, N. (1982) The interaction of hemin and bilirubin with the human red cell membrane. *Biochim. Biophys. Acta* **690**, 20-30.
333. Neuwirt, J., Ponka, P., and Borova, J. (1972) Evidence for the presence of free and protein-bound nonhemoglobin heme in rabbit reticulocytes. *Biochim. Biophys. Acta* **264**, 235-244.
334. Cox, T.M., O'Donnell, M.W., Aisen, P., and London, I.M. (1985) Biosynthesis of the transferrin receptor in rabbit reticulocytes. *J. Clin. Invest.* **76**, 2144-2150.
335. Schulman, H.M. (1968) Hemoglobin synthesis during rabbit reticulocyte maturation *in vitro*. *Biochim. Biophys. Acta* **155**, 253-261.
336. Trimble, R.B., and Maley, F. (1984) Optimizing hydrolysis of N-linked high-mannose oligosaccharides by endo-β-N-acetylglucosaminidase H. *Anal. Biochem.* **141**, 515-522.
337. Plummer, T.H., Elder, J.H., Alexander, S., Phelan, A.W., and Tarentino, A.L. (1984) Demonstration of peptide:N-glycosidase F activity in endo-β-N-acetylglucosaminidase F preparations. *J. Biol. Chem.* **259**, 10700-10704.
338. Merkle, R.K., and Cummings, R.D. (1987) Lectin affinity chromatography of glycopeptides. *Methods in Enzymology* **138**, 232-259.

339. Diaz, R., Mayorga, L.S., Weidman, P.J., Rothman, J.E., and Stahl, P.D. (1989) Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature* **339**, 398-400.

340. Misumi, Y., Miki, K., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* **261**, 11398-11403.

341. Ulmer, J.B., and Palade, G.E. (1989) Targetting and processing of glycophorins in murine erythroleukemia cells: use of brefeldin A as a perturbant of intracellular traffic. *Proc. Natl. Acad. Sci. USA* **86**, 6992-6996.

342. Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801-813.

343. Duden, R., Griffiths, G., Frank, R., Argos, P., and Kreis, T.E. (1991) β-COP, a 110 kD protein associated with non-clathrin coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell* **64**, 649-665.

344. Orci, L, Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J.G., Lippincott-Schwartz, J., Klausner, R.D., and Rothman, J.E. (1991) Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell* **64**, 1183-1195.

345. Morrow, B., and Rubin, C.S. (1987) Biogenesis of glycophorin A in K562 human erythroleukemia cells. *J. Biol. Chem.* **262**, 13812-13820.

346. Howard, R.J., Smith, P.M., and Mitchell, G.F. (1979) Identification of differences between the surface proteins and glycoproteins of normal mouse (Balb/c) and human erythrocytes*J. Memb. Biol.* **49**, 171- 178.

347. Wreschner, D., Mishan-Dahan, H., Raab, M., and Herzberg, M. (1977) Interaction between membrane properties and protein synthesis: in vitro synthesis of membrane proteins during erythropoiesis. *Exp. Cell Res.* **108**, 321-330.

348. Harris, E.D., and Johnson, C.A. (1969) Incorporation of glucosamine-^{14}C into membrane proteins of reticulocytes. *Biochemistry* **8**, 512-518.

349. Hendil, K.B. (1980) Intracellular degradation of hemoglobin transferred into fibroblasts by fusion with red blood cells. *J. Cell. Physiol.* **105**, 449-460.

350. Doxsey, S.J., Sambrook, J., Helenius, A., and White, J. (1985) An efficient method for introducing macromolecules into living cells. *J. Cell Biol.* **101**, 19-27.

351. DeLoach, J.R. (1987) Dialysis method for entrapment of proteins into resealed red blood cells. *Methods in Enzymology* **149**, 235-242.

352. Blight, G.D., and Morgan, E.H. (1987) Receptor-mediated endocytosis of transferrin and ferritin by guinea pig reticulocytes: uptake by a common endocytic pathway. *Eur.J.Cell Biol.* **43**, 260-265.

353. Chitambar, C.R., and Zivkovic, Z. (1989) Release of soluble transferrin receptor from the surface of human leukemic HL60 cells. *Blood* **74**, 602-608.

354. Baynes, R.D., Shih, Y.J., Hudson, B.G., and Cook, J.D. (1991) Characterization of transferrin receptor released by K562 erythroleukemia cells. *Proc. Soc. Exp. Biol. Med.* **197**, 416-423.

355. Chitambar, C.R., Loebel, A., and Noble, N.A. (1991) Shedding of transferrin receptor from rat reticulocytes during maturation in vitro: soluble transferrin receptor is derived from receptor shed in vesicles. *Blood* **78**, 2444-2450.

356. Rutledge, E.A., Mikoryak, C.A., and Draper, R.K. (1991) Turnover of the transferrin receptor is not influenced by removing most of the extracellular domain. *J. Biol. Chem.* **266**, 21125-21130.

357. Kruh, J., and Borsook, H. (1956) Hemoglobin synthesis in rabbit reticulocytes in vitro. *J. Biol. Chem.* **220**, 905-915.

358. Ponka, P., Schulman, H.M., and Wilczynska, A. (1982) Ferric pyridoxal isonicotinoyl hydrazone can provide iron for heme synthesis in reticulocytes. *Biochim. Biophys. Acta* **718**, 151-156.

359. Testa, U., Petrini, M., Quaranta, M.T., Pelosi-Testa, E., Mastroberardino, G., Camagna, A., Boccoli, G., Sargiacomo, M., Isacchi, G., Cozzi, A., Arosio, P., and Peschle, C. (1989) Iron up-modulates the expression of transferrin receptors during monocyte-macrophage maturation. *J. Biol. Chem.* **264**, 13181-13187.

360. Chan, L.-N.L., and Gerhardt, E.M. (1992) Transferrin receptor gene is hyperexpressed and transcriptionally regulated in differentiating erythroid cells. *J. Biol. Chem.* **267**, 8254-8259.

361. Heynen, M.J., and Verwilghen, R.L. (1982) A quantitative ultrastructural study of normal rat erythroblasts and reticulocytes. *Cell Tissue Res* **224**, 397-408.

362. Lucas, J.J., and Nevar, C. (1978) Loss of mannosyl phosphoryl polyisoprenol synthesis upon conversion of reticulocytes to erythrocytes. *Biochim. Biophys. Acta* **528**, 475-482.

363. Parodi, A.J., and Martin-Barrientos (1977) Glycosylation of endogenous proteins through dolichol derivatives in reticulocyte plasma membranes. *Biochim. Biophys. Acta.* **500**, 80-88.

364. Hunt, R.C., and Marshall, L.M. (1981) Membrane protein redistribution during differentiation of cultured human erythroleukemic cells. *Mol. Cell. Biol.* **1**, 1150-1162.

365. Bonanou-Tzedaki, S.A., Sohi, M., and Arnstein, H.R.V. (1981) Regulation of erythroid cell differentiation by haemin. *Cell Differ.* **10**, 267-279.

366. Taketani, S., Kohno, H., Naitoh, Y., and Tokunaga, R. (1987) Isolation of the hemopexin receptor from human placenta. *J. Biol. Chem.* **262**, 8668-8671.

367. Wyse, J.W., and Butterfield., D.A. (1989) Interaction of hemin with erythrocyte membranes: alterations in the physical state of the major sialoglycoprotein. *Biochim. Biophys. Acta.* **979**, 121-126.

368. Bennett, V., and Stenbuck, P.J. (1980) Association between ankyrin and the cytoplasmic domain of band 3 isolated from the human erythrocyte membrane. *J. Biol. Chem.* **255**, 6424-6432.

369. Hargreaves, W.R., Giedd, K.N., Verkleij, A., and Branton, D. (1980) Reassociation of ankyrin with band 3 in erythrocyte membranes and lipid vesicles. *J. Biol. Chem.* **255**, 11965-11972.

370. Pasternack, G.R., Anderson, R.A., Leto, T.L., and Marchesi, V.T. (1985) Interactions between protein 4.1 and band 3: an alternative binding site for an element of the membrane skeleton. *J. Biol. Chem.* **260**, 3676-3683.

371. Anderson, R.A., and Lovrien, R.E. (1984) Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. *Nature* **307**, 655-658.

372. Bunn, H.F., and Jandl, J.H. (1968) Exchange of heme among hemoglobins and between hemoglobin and albumin. *J. Biol. Chem.* **243**, 465-475.

373. Davies, K.J.A., and Goldberg, A.L. (1987) Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol. Chem.* **262**, 8220-8226.

374. Strapazon, E., and Steck, T.L. (1977) Interaction of the aldolase and the membrane of human erythrocytes. *Biochemistry* **16**, 2966-2971.

375. Thorburn, D.R., and Beutler, E. (1989) Decay of hexokinase during reticulocyte maturation: is oxidative damage a signal for distruction? *Biochem. Biophys. Res. Commun.* **162**, 612-618.

376. Alvarez, E., Gironès, N., and Davis, R.J. (1990) A point mutation in the cytoplasmic domain of the transferrin receptor inhibits endocytosis. *Biochem. J.* **267**, 31-35.

377. Paulson, J.C., and Colley, K.J. (1989) Glycosyltransferases: structure, localization, and control of cell type-specific glycosylation. *J. Biol. Chem.* **264**, 17615-17618.

378. Fischer, S., Nunez, M.T., Hifer, M.A., Delaunay, J., Piau, J.P., Tortolero, M., and Schapira, G. (1979) Studies of the plasma membrane during maturation of the mammalian erythrocyte. *Biochimie* **61**, 7-15.