CHARACTERIZATION OF THE GRANULINS AND CORTICOSTATINS, TWO CLASSES OF CYSTINE-RICH PEPTIDES FOUND IN THE HEMATOPOIETIC TISSUES OF A TELEOST FISH, *CYPRINUS CARPIO*, AND THE RAT.

by

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Dédicacé à ma famille. Lise, mon épouse, mes fils, Pascal, Eryck, Alex et Maxime, et mes parents, Léonard et Claire, pour leur amour et leur joie de vivre. Merci.

"Les plus importantes découvertes scientifiques résultent de la patiente observation de petits faits subsidiaires"

André Gide

ABSTRACT

Lower vertebrates such as teleost fish contain numerous biologically active factors which have often only been studied superficially. The corticostatins have recently been discovered in mammalian tissues and shown to have significant effects upon steroidogenesis of adrenal cells *in vitro*. We have examined the possible existence of similar bioactive peptides in tissue extracts of teleost fish and the rat in order to gain insight into important structure/function relationships. Using high performance liquid chromatography fractionation of acidic extracts of heart and hematopoietic tissues of teleost fish and the bone marrow of the rat, we have structurally and biologically characterized two families of cystine-rich polypeptides. These studies demonstrate that the carp and goldfish lack corticostatic peptides but do synthesize a novel class of cystine-rich peptides which we have designated the granulins owing to their apparent localization to the granule fraction of phagocytic leukocytes. Carp granulins-1, -2 and -3 are 57 amino acids in length and possess a unique structural motif composed of 12 cysteines arranged as four pairs flanked by two singletons towards the amino- and carboxyl-termini. Distribution and immunocytochemical studies using a polyclonal antibody to carp granulin-1 demonstrated that it is localized primarily in mononuclear phagocytic cells found in the spleen and head kidney. Significant granulin-1 immunoreactivity was also detected in phagocytic cells in tissues involved in the first line of defense against infection (i.e. gills, intestine and skin). We have also demonstrated the existence of a granulin-like peptide which was co-purified with corticostatin-like peptides from extracts of rat bone marrow. The granulins are part of a larger family of growth modulatory polypeptides which include epithelins, acrogranins, PC cell-derived growth factor (PCDGF), epithelial transforming growth factor (TGFe) and other structurally related peptides. Preliminary studies have shown that carp granulins have growth modulatory effects on epithelial cells *in vitro*. We have in these studies established that the hematopoietic tissues of teleost fish are a rich source of these prototypic forms of this growth factor family.

ABRÉGÉ

Plusieurs facteurs peptidiques que l'on retrouve chez les poissons téléostéens sont plus souvent étudiés superficiellement. Certains facteurs tels les corticostatins ont récemment été caractérisés comme ayant certains effets sur la stéroïdogénèse au niveau de la glande surrénale chez les mammifères. La possibilité de retrouver ces facteurs peptidiques chez les poissons téléostéens (carpe) et le rat et d'étudier les structures primaires importantes pour leurs activités a été entreprise. A cette fin, nous avons utilisé la chromatographie liquide à haute pression pour l'isolation et la caractérisation de deux familles de facteurs peptidiques ayant un haut pourcentage de cystéine. L'extraction acidique des tissus tels le coeur et le système hématopoïétique (la ratte et le rein antérieur) chez le poisson téléostéen (carpe) a révélé que les corticostatins ne sont pas présents dans ces extraits. Par contre, on retrouve un nouveau groupe de polypeptides riche en cystéine étant placé très spécifiquement en une série de 4 paires bornées par deux cystéines simples aux deux extrémités, notamment le N-terminal et le C-terminal. Les trois peptides en question on été surnommés granulin-1, -2 et -3. La ratte de la carpe possède seulement granulin-1, ce qui nous a permi d'établir un anticorps avec une spécificité pour seulement granulin-1. Nous avons déterminé, en utilisant différentes techniques d'immunocytologie, que granulin-1 était détecté dans le cytoplasme du macrophage ou du monocyte de la ratte et du rein antérieur. Aussi, granulin-1 est retrouvé pareillement dans les cellules monocytiques des tissus responsables pour la défense de l'organisme. Dans le cas de la moëlle osseuse du rat, nous avons découvert une structure ressemblant au granulin du poisson. Ce granulin a été copurifié avec les corticostatins dû à son élution chromatographique. Les granulins font partie d'un groupe de polypeptides qui joue certains rôles dans la croissance et le développement des cellules. Cette famille comprend les épithelins, l'acrogranin et d'autres facteurs homologues (PCDGF, TGFe, etc.). Nos études préliminaires confirment que les granulins de la carpe causent certains effets sur la croissance des cellules épithéliales en culture. Alors, nous avons établi que les tissus hématopoïétiques du poisson téléostéen sont une source riche de formes prototypiques pour cette nouvelle famille de facteurs de croissance.

PREFACE

The Guidelines Concerning Thesis Preparation issued by the Faculty of Graduate Studies and Research at McGill University reads as follows:

> "The candidate has the option, subject to the approval of their Department, of including as part of the thesis the text, or duplicated published text, of an original paper or papers. Manuscript-style theses must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than just a collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.
>
> It is acceptable for theses to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instances, connecting texts are mandatory and supplementary explanatory material is always necessary. Photographs or other materials which do not duplicate well must be included in their original form.
>
> While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent and supervisors must attest to the accuracy of the claims at the PhD. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear."

I have chosen to write my thesis according to the above quoted option using four published papers. The thesis is organized in seven chapters. Chapter I is a general introduction and literature review, the references for which are presented at the end of the dissertation. Chapters II to V contain the four manuscripts, each with its own preface, abstract, introduction, materials and methods, results, discussion and references. Chapter VI is a general discussion; its references are found at the end of the thesis. Chapter VII contains the claims to original research.

PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS AND CONTRIBUTIONS MADE BY CO-AUTHORS

CHAPTER II

Belcourt, D., Singh, A., Bateman, A., Lazure, C., Solomon, S. and Bennett, H.P.J. (1992) Purification of cationic cystine-rich peptides from rat bone marrow. Primary structures and biological activity of the rat corticostatin family of peptides. Regulatory Peptides, 40, 87-100.

The candidate was responsible for initiating the research in the study of rat bone marrow as a rich source of members of corticostatin/defensin family of peptides. He was responsible for the complete purification and structural characterization of the rat corticostatins and related peptides. He was also responsible for performing the anti-ACTH biological assay and the lizard skin MSH assay. **Drs. A. Singh** and **A. Bateman** performed and analyzed the cytotoxic assay of the rat corticostatin peptides using established cell lines and procedures. The sequencing using Edman degradation was performed in part by **Dr. C. Lazure**, namely the primary sequencing of rat corticostatin 4 and 5. The preparation and revision of the manuscript was completed by the candidate in consultation with **Dr. H. Bennett**.

CHAPTER III

Bateman, A., Belcourt, D., Bennett, H., Lazure, C. and Solomon, S. (1990) Granulins, a novel class of peptide from leukocytes. Biochemical and Biophysical Research Communications, 173, No. 3, 1161-1168.

During the development of new fields of research, the work between colleagues will often overlap. Due to the importance of both research projects, the rat and human granulins were published as a collaborative study between **Dr. A. Bateman** and the candidate. **Dr. A. Bateman** purified and structurally characterized the human granulins and prepared the greater part of the manuscript. The candidate independently isolated and structurally characterized the rat granulin and supplemented the manuscript with relevent information concerning this work.

Dr. C. Lazure performed microsequencing of the human granulins. Revisions and consultations were elicited from **Drs. H. Bennett** and **S. Solomon.**

CHAPTER IV

Belcourt, D.R., Lazure, C. and Bennet, H.P.J. (1993) Isolation and primary structure of the three major forms of granulin-like peptides from hematopoietic tissues of a teleost fish (*Cyprinus carpio*). Journal of Biological Chemistry, 268 (13), 9230-9237.

The development of this project represents the greater part of the candidate's research efforts. He was responsible for the complete isolation and structural characterization of the carp granulin structures. The microsequencing of peptides was performed in several different laboratories as outlined in chapter 4, however, the major contribution with respect to sequencing came from **Dr. C. Lazure**. The manuscript was prepared and revised for publication by the candidate in consultation with **Dr. H. Bennett**.

CHAPTER V

Belcourt, D.R., Okawara, Y., Fryer, J.N. and Bennett H.P.J. (1995) Immunocytochemical localization of granulin-1 to mononuclear phagocytic cells of *Cyprinus carpio* and *Carassius auratus*. Journal of Leukocyte Biology, 57, 94-100.

The candidate was responsible for performing all cytochemical procedures with the guidance and encouragement from **Dr. Y. Okawara**. **Dr. J. Fryer** assisted with the interpretation of the immunocytochemical data and revision of the manuscript for publication. The candidate was responsible for the manuscript preparation and revision. **Dr. H. Bennett** provided the final revisions to the manuscript prior to submission and publication.

The following co-authored publications and abstracts have also appeared resulting from work performed during the course of my Ph.D.:

Publications:

1. **Belcourt, D.**, Varma, D.R., Toney, K. and Bennett, H.P.J. (1991) Purification of rat pro-atrial natriuretic factor--a simplified scheme using reversed-phase high performance liquid chromatography. Protein Expression and Purification, **1**, 28-32.

2. MacLeod, R.J., Hamilton, J.R., Bateman, A., **Belcourt, D.**, Hu, J., Bennett, H.P.J. and Solomon, S. (1991) Corticostatic peptides cause nifedipine sensitive volume reduction in jejunal villus enterocytes. Proc. Natl. Acad. Sci. (USA), **88**, 552-556.

3. Solomon, S., Hu, J., Zhu, Q., **Belcourt, D.**, Bennett, H.P.J., Bateman, A. and Antakly, T. (1991) Corticostatic peptides. J. Steroid Biochem. Molec. Biol., **40**, 391-398.

Abstracts:

1. **Belcourt, D.**, Bennett, H.P.J.. (1988) Novel cystine-rich peptides from teleost fish (carp) leukocytes. J. Cell Biol., **107**, 629a.

2. **Belcourt, D.**, Bateman, A., Singh, A., Lazure, C., Bennett, H.P.J., and Solomon, S.. Structure and biological activities of rat corticostatin and related peptides. (72nd Annual Meeting of the Endocrine Society, Atlanta, Georgia, pp 276 (abs.), 1990).

3. **Belcourt, D.** and Bennett, H.P.J.. Isolation et caractérisation d'une nouvelle famille de peptides provenant du système hématopoïétique d'un poisson téléostéen (carpe). Congrès de l'ACFAS, 1991.

4. **Belcourt, D.**, Okawara, Y., Fryer, J. and Bennett, H.P.J. Immunocytochemical localization of a granulin-like peptide within hematopoietic tissues of the carp goldfish. CFBS Meeting, 1991.

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ABBREVIATIONS

α-MSH	α-melanotropin
ACTH	corticotropin
Asp-N	Asp-N endoproteinase
BSA	bovine serum albumin
CNBr	cyanogen bromide
EGF	epidermal growth factor
EPI	epithelin
$F(ab')_2$	immunoglobulin G antigen binding domain
Fc	immunoglobulin G constant domain
GRN	granulin
HFBA	heptafluorobutyric acid
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
IS-MS	ion spray mass spectrometry
MED	minimum effective dose
NGF	nerve growth factor
PBS	phosphate buffered saline
PCDGF	PC cell-derived growth factor
PE-Cys	pyridylethyl cysteine
PTH	phenylthiohydantoin
RIA	radioimmunoassay
RP-HPLC	reversed-phase high performance liquid chromatography
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris HCl buffered saline
TFA	trifluoroacetic acid
TGF	transforming growth factor
TGFα	transforming growth factor alpha

TGFß	transforming growth factor beta
TGFe	epithelial cell-derived growth factor
VGF	vaccinia virus growth factor

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CHAPTER I

GENERAL INTRODUCTION

Preface

Endocrine hormones and other humoral substances including tissue growth factors evolved early in vertebrate evolution. So-called lower vertebrates (e.g. teleost fish) have been shown to synthesize polypeptide hormones that are structurally similar to those found in mammals. A granulin-like peptide was originally purified from extracts of heart and spleen tissues of the goldfish. Subsequently, the hematopoietic cells of another teleost fish, the carp, the rat and the human were identified as rich sources of various granulin-like peptides. The growth promoting and modulatory effects of other members of the granulin family including the epithelins and epithelial cell transforming growth factor have recently been reported.

The introduction to my dissertation will focus in part on epidermal growth factor (EGF) as a model of growth modulatory factor. As is the case for EGF, granulins have been discovered in several apparently unrelated biological systems, which will be described in the introduction in order to establish the multifunctional and variant nature of the granulin family of polypeptides.

Epidermal growth factor

2.1 General overview of EGF: Polypeptide growth factors, in general, are soluble proteinacious cellular components which initiate a cascade of events leading to cell growth and/or differentiation. In reviews by Carpenter et al. (1) and Stoschek et al. (2), EGF is described as a polypeptide with a molecular weight of 6032 daltons with growth modulatory activities upon a variety of tissues including the epidermis of the skin. The initial discovery of EGF in the mouse salivary gland by Cohen (3) in the early 1960s occurred during studies of nerve growth factor (NGF). A purified fraction from the salivary gland of male mice caused the eyelids of newborn mice to open prematurely. Based on the capacity of EGF to induce cell proliferation of the basal cells of the skin, the active component (EGF) was characterized. EGF has been shown to be multifunctional and has also been shown to inhibit gastric acid secretion (4). This secondary activity of EGF, in this context known as ß-urogastrone, was apparently not related to its mitogenic actions.

Several different structurally related peptides were also found which demonstrated a vast number of biological activities related to the mitogenic and differentiating potential of EGF. Transforming growth factors (TGFs) were identified in the conditioned medium of sarcoma-transformed cells in the 1970's (5). The transforming growth factor alpha (TGFα) molecule was identified through its ability to bind to the EGF receptor and shown to be homologous to EGF. TGFα caused induction of anchorage independent growth of normal cells in soft agar in the presence of transforming growth factor beta (TGFß), a structurally distinct protein. Also showing significant sequence homology to EGF is a growth factor protein synthesized under the control of the vaccinia virus (VGF), a member of the Pox virus family (6). This peptide is 77 residues in length and is processed from a larger precursor. It is secreted into the medium of vaccinia-infected cells and has been shown to have EGF-like activity. The primary sequences of EGF, TGFα and VGF are shown below:

hEGF NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWLER

hTGF VVSHFNDCPDSHTQF CFH-GTCRFLVQEDKPACVCHSGYVGARCEHADLLA

VGF .....PAIRLCGPEGDGYCLH-GDCIHARDIDGMI CRCSHGYTGI RCQHVVLDVYQRS...

Common motif

.....C-------C---G-C------D---C-C--G--G--C...

A common feature of these structures is a cysteine motif also shown above. This cysteine motif is also found in proteins of the immune complement system (namely Factor X, Factor IX, and Protein C), the plasminogen activator and fibronectin. However, in these contexts, the EGF motifs lack growth factor activity and act as structural elements. These proteins contain the partial sequence CXCXXGXXGXXC, the last part of the cysteine motif found in EGF.

Molecular cloning of EGF and TGFα cDNAs indicated that these molecules are produced as larger precursors which are products of independently regulated and unlinked genes. TGFα is produced in transformed cells whereas EGF is found under normal conditions in most body fluids. One important feature of all EGF-related molecules is the position of the cysteine residues in these molecules. This would suggest important structural requirements involving disulfide bonds. Reduction of EGF abolishes biological activity. The EGF precursor is anchored to the cell surface via a membrane spanning sequence. The extracellular domain is composed of nine sequences homologous to EGF itself. EGF is excised from proEGF by cleavage at arginine and lysine residues by an arginine esterase. Since EGF is the only sequence flanked by basic amino acids, the other eight EGF-like molecules are most likely never cleaved from the remaining precursor although their fate has not been defined.

2.2 EGF receptor: Growth factors like EGF cause cellular growth or differentiation by first interacting with a cell surface receptor. The structural and biological characteristics of the EGF receptor are well known and have been reviewed extensively (2, 7, 8). A cell line derived from a human epidermoid carcinoma (A-431 cells) was found to overexpress the EGF receptor. The EGF receptor was isolated and characterized

using A-431 cells (9). The EGF receptor (170 kDa) is a transmembrane protein comprising an extracellular EGF binding site which is heavily glycosylated and a protein kinase moiety which occupies the intracellular face of the cytoplasmic membrane. The EGF binding site and the intrinsic protein tyrosine kinase activity are linked by a small transmembrane segment.

The first step in receptor activation requires that the ligand (i.e. EGF) bind to the extracellular domain. This binding causes conformational changes in the extracellular domain which allows interaction with other EGF activated receptors (i.e. ligand induced oligomerization) (8, 10). Oligomerized receptors stabilize interaction of the cytoplasmic tyrosine kinase domains and allows transmission of the extracellular signal to the intracellular domain without altering the transmembrane segment. The tyrosine kinase, once activated, is the principal function of the intracellular domain and serves to amplify the EGF signal. The initial kinase activity is generally considered to be the autophosphorylation of the carboxyl-terminal region of the receptor. Autophosphorylation then enhances the capacity for the tyrosine kinase to phosphorylate other substrates within the cytoplasm. In order to have a functional tyrosine kinase activity, a consensus sequence GlyXGlyXXGly$X_{(15\text{-}20)}$Lys must be intact and functional with respect to binding of ATP (8, 10, 11, 12, 13). Failure to have a functional tyrosine kinase activity prevents all signal transduction from occurring, thus attenuating mitogenic and transformation pathways in response to EGF (13, 14). Several downstream events are prevented including calcium mobilization, phosphorylation of ribosomal proteins, DNA synthesis, proto oncogene expression (e.g. c-myc and c-fos) and formation of inositol phosphate (15). Cellular sorting of receptor tyrosine kinase is also dependent on the tyrosine kinase domain. After autophosphorylation, the receptor aggregates are quickly internalized and are targeted for degradation within lysosomes (8, 16). It has been shown that intrinsic kinase activity is responsible for correct sorting, since kinase defective receptors are shuttled back to the surface of the cellular membrane instead of being degraded within the lysosomal fraction (17). Although numerous growth factors have been studied and reviewed in the literature, the biological activities of EGF most closely resembles those of the granulin/epithelin family of growth modulatory proteins including the nature of

target tissues and mode of action.

The mammalian granulins

Granulins were initially isolated from cells found in the circulatory system. This novel class of cystine-rich polypeptides were isolated from human leukocytes and rat bone marrow (18). Chapter III describes the initial isolation and characterization of these peptides. In brief, they are cystine-rich (≈ 20%) polypeptides, 57 amino acids in length and characterized by a novel cysteine motif:

$$CX_{5-6}CX_5CCX_8CCX_6CCX_5CCX_5CX_{5-6}C$$

In order to elucidate the biological significance of these peptides, Bhandari et al., in three separate publications, reported the cloning (19), genomic localization of the precursor encoding the human preprogranulin (20), and the cDNA sequence of rat preprogranulin in addition to its tissue distribution and localization as assessed by Northern blot and *in situ* hybridization analysis (21). The following section will provide a review of what is known of human granulins.

3.1 Isolation and sequence of the granulin precursor: Bhandari et al. (19) reported the cDNA encoding the human granulin precursor. Using oligonucleotide primers derived from the amino-terminal and mid-region of human granulin-A followed by PCR amplification and screening human genomic DNA, two products were isolated. One product was used to screen a human bone marrow cDNA library to obtain a full length cDNA sequence encoding the human granulin precursor. The predicted messenger RNA (mRNA) of 2062 bp including 5′ and 3′ noncoding regions contained a 5′ AUG initiation site and a 3′ poly (A) tail with the poly (A) signal (AAUAAA).

The deduced amino acid sequence of the human preprogranulin consisted of 593 residues containing a probable signal peptide of 17 residues. It contained the sequences of granulins (GRN) A, B, C, D previously identified by protein sequencing together with 3 other predicted structures related to the granulins. These structures are presented in Figure 1 along with intervening sequences linking the different granulin structures within the preprogranulin sequence.

MWTLVSWVALTAGLVAGTRCPDGQFCPVACCLDPGGASYSCCRPLLD[1]KWPTT
LSRHLGGPCQVDAHCSAGHSCIFTVSGTSSCCPFPEAVACGDGHHCCPRGFHC
SADGRSCF[2]QRSGNNSVGAIQCPDSQFECPDFSTCCVMVDGSWGCCPMPQAS
CCEDRVHCCPHGAFCDLVHTRCI[3]TPTGTHPLAKLPAQRTNRAVALSSSVMCPD
ARSRCPDGSTCCELPSGKYGCCPMPNATCCSDHLHCCPQDTVCDLIQSKCL[4]SK
ENATTDLLTKLPAHTVGDVKCDMEVSCPDGYTCCRLQSGAWGCCPFTQAVCCE
DHIHCCPAGFTCDTQKGTCE[5]QGPHQVPWMEKAPAHLSLPDPQALKRDVPCDN
VSSCPSSDTCCQLTSGEWGCCPIPEAVCCSDHQHCCPQRYTCVAEGQCQ[6]RGS
EIVAGLEQMPARRGSLSHPRDIGCDQHTSCPVGGTCCPSQGGSWACCDLPHAV
CCEDRQHCCPAGYTCNVKARSCE[7]KEVVSAQPATFLARSPHVGVKDVECGEGH
FCHDNQTCCRDNRQGWACCPYAQGVCCADRRHCCPAGFRCARRGTKCL[8]RRE
APRWDAPLRDPALRQLL

Figure 1 Human preprogranulin primary sequence. Amino acid sequences underlined are the 7½ granulins deduced from the full length cDNA of human preprogranulin. The superscripts correspond to the following granulins: 1 = Paragranulin; 2 = Granulin G; 3 = Granulin F; 4 = Granulin B; 5 = Granulin A; 6 = Granulin C; 7 = Granulin D; and 8 = Granulin E.

Bhandari et al. (19) noted the potential N-glycosylation sites and the Pro-Ala dipeptide site located between some domains that are possibly involved in the proteolytic processing of progranulin. Cleavage of the progranulin to individual granulins has yet to be studied and the biochemical basis for this process remains unclear. They also reported the presence of only one gene in human genomic DNA as assessed by Southern blot analysis. This gene seems to be conserved in those mammalian species studied to date. Northern analysis of a 2.3 kb transcript for the human granulin precursor was shown to be expressed in myelogenous leukemic cell lines, 3T3 fibroblasts and epithelial cell lines, the latter giving the highest signal. The granulin precursor was also shown to be expressed in rabbit kidney and to a lesser extent in the spleen. Low levels were detected in liver, lung, heart, thymus and brain. Contrary to the initial assumption that granulins were only

found in leukocytes and related cells, it would seem the gene is widely expressed in many tissues, especially those of epithelial character.

Further studies by Bhandari et al. (21) on the tissue distribution and cellular localization of the rat granulin precursor in-vivo suggested that the expression of preprogranulin was restricted mainly to hematopoietic cells and relatively few epithelial cell types. They reported the isolation and characterization of the cDNA encoding the rat preprogranulin and described its homology to the human preprogranulin. They also studied tissue distribution and gene expression using quantitative analysis of the 2.3 kb transcript. Tissues with varying amounts of epithelial cells were studied showing that the level of expression of the granulin gene was proportional to the quantity of epithelium present. However, *in situ* hybridization revealed that granulin gene expression was restricted to lymphocytes in the spleen, hepatocytes in the liver and epithelial cells in the kidney.

Bhandari et al. (20) also reported that the human granulin gene is located on chromosome 17 and is comprised of 12 exons. It can be seen from the structural organization, that each granulin domain is encoded by two exons of different sizes within the genomic sequence.

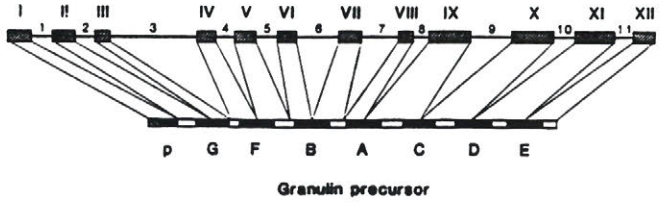

Figure 2 Structural organization of the human granulin gene. Exons are indicated by hatched boxes and introns by lines. Exons I, II, IV, VI and VIII are α-exons, exons III, V, VII and XII are ß-exons and exons IX, X and XI are ßα-exons. The organization of the granulin precursor was determined from its cDNA (19). Closed boxes represent the granulin domains, open boxes represent the intervening spacer regions and the hatched box represents the signal sequence.

Although no biological activity has been reported for the granulin polypeptides,

it has become apparent that the granulins are homologous to the epithelins, a family of growth modulatory peptides isolated from rat kidney. The epithelins will now be discussed to show the data accumulated thus far on their structural and biological importance.

Epithelin family of peptides

Cellular proliferation is a complex process under the control of negative and positive regulatory elements. The development of epithelial cells and the functions of those cells are under the control of numerous polypeptide growth factors. Recently, the epithelins, a novel growth factor family of peptides, have been isolated and structurally characterized following the observation of their biological effects upon certain epithelial cell types (22). The cDNA for the epithelin precursor has been cloned (23) and a cell surface receptor molecule has been partially characterized (24). The following sections will briefly describe this family of peptides.

4.1 Epithelins-1 and -2: Structural and biochemical characterization: Epithelin 1 and 2 were determined to be cystine-rich polypeptides with intact disulfide bridges critical for their biological activity (22). Initial characterization of the epithelins in crude tissue extracts indicated that the activity was resistant to denaturing conditions such as 1M acetic acid, 1M ammonium hydroxide, 6M Urea and heat (56°C, 30 min.). However, reduction and alkylation and treatment with glycosidases, lipases, trypsin, and endoproteinases Lys-C and Glu-C (V8) decreased bioactivity.

The two peptides were initially isolated from 430 g of frozen rat kidneys. Crude extracts of the kidney tissue were obtained using an acid-ethanol extraction medium containing enzyme inhibitors to prevent degradation of active factors. A series of centrifugations, chloroform extraction, dialysis and lyophilization procedures resulted in the production of 4.55 g of crude extract. This extract was subjected to a six step purification protocol based on gel permeation and reversed-phase high performance liquid chromatography and resulted in the isolation of epithelin-1 and -2. The active fractions were purified to apparent homogeneity by following their activities using an *in vitro* A431

cell bioassay (described below). The apparent molecular weights of epithelin-1 and -2 were ≈ 4.5 kDa, but when subjected to pyridylethylation, the apparent molecular weight rose to ≈ 13 kDa. This data suggested that the high cystine content (presumably in the form of disulfide bridges) resulted in a highly compact structure most likely necessary for biological activity. Both gel permeation chromatography and SDS-PAGE were used in the assessment of molecular size.

The S-pyridylethylated proteins were subjected to gas-phase microsequencing and the amino-terminal sequences for epithelin-1 and -2 were determined. These partial sequences (shown below) clearly indicated that epithelins-1 and -2 were novel peptides with a unique arrangement of cysteine residues.

Epithelin 1	VKC-DLEVSCPDGYTCCRLNTG...
Epithelin 2	VVCPDAKTQCPDDSTCCELPTG...

4.2 Biological characterization of epithelins: Three different bioassays were employed to follow and characterize the biological properties of the epithelins (22). The A431 cell bioassay consisted of measuring the inhibition of proliferation of a tumor cell-line by tissue extracts and HPLC fractions. A431 cells are derived from a human epidermoid carcinoma of the vulva. The growth inhibitory activity measured by GIA units corresponds to the amount of factor required to inhibit ^{125}I-labelled deoxyuridine incorporation into A431 cells by 50%. In this assay, epithelin-1 (ED_{50} ≈ 21 nM) showed a 36 fold greater potency in decreasing DNA synthesis as compared to epithelin-2 (ED_{50} ≈ 750 nM). Epithelins also showed varying effects on other human tumour and non-tumour cell-lines and cultures of several non-human cell lines.

The BALB/MK cell bioassay consists of measuring cell number in the absence or presence of different factors. Proliferation of these murine keratinocyte-derived cells is dependent on certain growth factors such as EGF. When these cells were exposed to the epithelins, they underwent proliferation in the presence of epithelin-1 but not epithelin-2. When cells were stimulated with epithelin-1, co-incubation with epithelin-2 decreased this response by 50% at a concentration of ≈ 7 nM. Anchorage independent growth of rat kidney cells (NRK-SA6) grown in the presence of TGFß is induced by EGF-like

molecules. Epithelin-1 had a similar mitogenic effect on these cells which could also be inhibited by epithelin-2 by 50% at a concentration of 85 nM. The authors concluded that the epithelins had growth inhibitory effects on A431 cells, epithelin-1 being more active than epithelin-2. Also in the proliferation of murine keratinocyte cells, epithelin-1 and -2 had opposing effects namely epithelin-2 antagonized the mitogenic effects of epithelin-1. The authors also suggested that these effects are not mediated by the EGF receptor since epithelin-1 or -2 could not displace ^{125}I EGF from its receptor in binding studies. They concluded that epithelin-1 and -2 most likely act by interaction with their own cell surface receptor.

4.3 Epithelin precursor cloning and characterization: The initial data on the characterization of the epithelins suggested important roles for these peptides in cellular proliferation of certain cell types *in vitro*. In order to understand further their role in normal epithelial cell growth, Plowman et al. (23) proceeded to the cloning of the cDNAs encoding preproepithelin from three mammalian species. The rat, mouse and human cDNAs encoding the complete preproepithelins were obtained by screening λgt 10 libraries using PCR technologies and protocols. They reported the complete composite structure for rat and human epithelin cDNAs as being 2153 bp and 2152 bp long respectively, and having 5′ and 3′ untranslated regions, consistent with a 2.3 kb transcript observed by Northern analysis. The predicted structures for rat human and mouse preproepithelin are 589, 593 and 589 residues long respectively, giving predicted molecular masses of approximately 61.6 kDa. The epithelin precursors contained 7½ copies of a novel cysteine motif, identical to that characteristic of the granulin structures (i.e. $CX_{5-6}CX_6CCX_8CCX_6CCX_5CCX_5CX_{5-6}C$). Compared to the rat, there is 86% (mouse), 75% (human) predicted amino acid sequence homology. The predicted sequence of human preproepithelin was identical to that of human preprogranulin previously reported (19). The processing of the granulin/epithelin precursor to generate bioactive fragments has not been studied but most likely requires post translational cleavage of the protein precursor.

Northern analysis indicated that epithelins are widely expressed. A 2.3 kb

transcript was shown predominantly in adult kidney, placenta and colon but was also present in heart, duodenum and cerebral cortex. It was detected in numerous normal cell lines of epithelial and mesenchymal origin and in various tumour cell lines.

Recombinant forms of the complete preproepithelin or epithelin-1 and -2 were generated using expression vectors employing the cytomegalovirus immediate-early promotor. The recombinant form of progranulin was not processed to smaller forms suggesting that COS cells lack the correct processing enzymes. The precursor was devoid of biological activity. However, expressed epithelin-1 and -2 showed the predicted biological activities, namely recombinant epithelin-1 and -2 inhibited A431 cell proliferation, whereas, epithelin-2 inhibited the proliferative response of normal cell lines to epithelin-1.

4.4 Biochemical analysis of epithelin receptor: The biochemical analysis of a membrane bound binding protein for the epithelins has been reported. Culouscou et al. (24) demonstrated the existence of a specific cell surface receptor crucial to the understanding of how epithelin-1 and -2 act as growth modulators for epithelial cells. The authors characterized the epithelin receptor in human breast carcinoma cells (MDA-MB-468) using binding and cross-linking experiments. Two binding sites were identified, a high affinity site with a dissociation constant of $\approx 2 \times 10^{-10}$ M with 290 receptors/cell and a low affinity site with a dissociation constant of $\approx 10^{-8}$ M with 32,000 receptors/cell. They investigated whether epithelins interact with this specific receptor expressed on the surface of target cells. It was shown that epithelins-1, -2 and -3 bind a single receptor population. Specifically ^{125}I-epithelin-1 binds directly to the human breast carcinoma cell line (MDA-MB-468). Epithelins-1, -2 and -3 showed an equal competitive displacement of ^{125}I-epithelin-1 from its binding site in a dose dependent manner. This suggested that all three epithelins bind to the same receptor sites. Other cytokines and growth factor tested in the competition assay could not displace ^{125}I-epithelin-1. An experiment using disuccinimidyl suberate, a bifunctional cross-linking reagent, to chemically link ^{125}I-epithelin-1 to its binding site revealed a single 140-145 kDa protein on SDS-PAGE. The formation of this complex was prevented in the presence of excess epithelin-1, -2 and -3

in co-incubation experiments. Since there is a single binding species of receptor and since epithelins-1 and -2 displace ^{125}I-epithelin-1 equally, it is possible that they cause different conformational changes within the receptor. This could explain the differences in the biological activities of epithelin-1 and -2 described previously, ie. different potencies as growth inhibitors and the antagonistic effect of epithelin-2 upon the growth stimulatory effect of epithelin-1.

PC cell-derived growth factor (PCDGF)

The precursor for the granulin/epithelin growth factor has recently been purified and cloned from a highly tumorigenic cell line (PC cells) (25). PC cells are derived from a strain of C3Hm teratoma-derived adipogenic cell line 1246 which requires insulin for proliferation and differentiation. Using an *in vitro/in vivo* shuttle technique employing insulin free medium, it was possible to isolate a highly tumorigenic cell line designated as the PC cell line. These cells produce and secrete autocrine growth stimulators and differentiation inhibitors. From the conditioned medium of PC cells grown to confluency (i.e. high density plating), a factor was isolated and designated PC cell derived growth factor (PCDGF) to describe its biological activity, namely a mitogenic effect upon Balb/c 3T3 fibroblast and its autocrine growth effects upon low density plated PC cells. The factor was isolated by a series of low pH, protein precipitation and centrifugation steps, followed by molecular sieve chromatography. The active material $\approx$ 90 kDa was fractionated using a phenyl-Sepharose affinity chromatography column that under initial conditions retained the active component, and was then eluted with 50% ethyleneglycol. Assessment of homogeneity by SDS-PAGE revealed a single band of 88 kDa apparent molecular weight. After treatment with N-glycosidase F, the apparent molecular weight of PCDGF on SDS-PAGE decreased by 20 kDa indicating a core protein structure of 68 kDa.

Preliminary structure determination of the trypsinized and alkylated CNBr treated PCDGF, yielded sequence information which shared homologies with the granulin/ epithelin precursors. PCDGF, a mouse derived factor was found to be identical to the mouse epithelin precursor given the limited amount of primary structure information on

the fragmented PCDGF.

Characterization of the biological activity of purified PCDGF was assessed by examining its cell proliferation stimulating activity on Balb/c 3T3 fibroblast. In this assay, PCDGF has significant mitogenic effects in stimulating DNA synthesis (ED_{50} = 2.5 ng/well). It had no effect on Balb/mk cells previously shown to be stimulated by epithelin 1 and 2 (22). PCDGF also acts as an autocrine stimulator for the producer cell namely the PC cells in increasing DNA synthesis by 50% at 2.5 ng/ml.

The authors concluded that PCDGF is a cystine-rich, glycosylated protein produced and secreted by PC cells, and that this protein is homologous to members of the previously characterized granulin/epithelin family.

Epithelial cell-derived growth factor (TGFe)

6.1 Isolation and characterization: A novel growth factor activity responsible for the induction of anchorage-independent growth of SW-13 cells was initially identified and partially characterized by Halper and Moses (26). They showed that SW-13 cells, human adenocarcinoma of the adrenal cortex, were induced to grow in soft agar by epithelial tissue-derived growth factor-like peptides. This section will describe the biological studies that led to the characterization of the apparently novel factor termed Epithelial cell Transforming Growth Factor (TGFe).

Initially, it was observed that certain human epithelial malignant cell lines produced and secreted polypeptides into the surrounding growth medium which demonstrated growth factor-like characteristics. SW-13 cells respond to these factors by growing in soft agar. Conditioned medium from SW-13 cells could also cause the producer cell (SW-13) to acquire anchorage-independent growth resulting in an autostimulatory phenomenon. Halper and Moses (26) showed that one major peak of activity with an apparent molecular weight of 20-22 kDa was clearly responsible for this SW-13 proliferative activity and that it was distinct and separable from other epithelial tissue-derived growth factors. Many of these growth factors share common features namely that they are heat and acid stable polypeptides containing disulfide bonds as indicated by their sensitivity to reduction. These factors can stimulate DNA synthesis and

cause morphological changes in cells in monolayer culture as well as stimulate anchorage dependent cells (SW-13) to grow in soft agar.

Further characterization of TGFe activity by Halper and Moses (27) included the purification and biological characterization of a TGFe-like activity isolated from bovine kidney. Acid-ethanol extracts of bovine kidney were fractionated using molecular sieve chromatography and reversed-phase HPLC. They purified a polypeptide with an apparent molecular weight of 23-25 kDa according to SDS-PAGE. This TGFe-like molecule derived from normal cells stimulated SW-13 cells to grow in soft agar. It was shown that TGFe activity was different from other known growth factors such as TGFß, TGFα, EGF, acidic and basic FGF and bombesin by comparing their biological and physical characteristics. Although SW-13 cells are the most sensitive to TGFe, some squamous cell carcinomas like A431 and D562 cells were an order of magnitude less sensitive.

Through further characterization of the factors that control SW-13 cell growth, Halper and Carter (28) showed that heparin, an anticoagulant, inhibits the growth of SW-13 cells in soft agar and monolayer cultures. It also inhibited the TGFe and bFGF stimulated growth of SW-13 cells. TGFe was shown to partly rescue the effect of heparin on these cells. They proposed that growth factor like molecules and heparin-like molecules in the extracellular matrix control the growth of SW-13 cells in a complex fashion.

6.2 Mitogenic effect of TGFe on normal fibroblastic and epithelial cell lines: As described by Brown and Halper (29), TGFe acts as mitogen for both fibroblastic and epithelial cell lines. They reported that TGFe stimulated growth of three fibroblast and four epithelial cell lines grown in monolayer culture. The mitogenic effect of TGFe monitored by ^{3}H-thymidine incorporation and cell number was dose dependent and comparable to other mitogens, ie. EGF. The fibroblastic cell lines require a continuous exposure to TGFe, suggesting that it is a progression factor and not a competence factor which can cause maximal DNA synthesis with transient exposures. EGF, a progression factor behaves in a similar manner to TGFe. Like other mitogens, (e.g. EGF), DNA synthesis does not occur immediately in response to TGFe but begins after 10-14 hours

with a maximum response occuring at 19-22 hours after mitogen exposure. Since TGFe stimulates both anchorage-dependent and independent growth for normal fibroblastic and epithelial cell lines, it has the capacity to play critical roles as a paracrine mitogen and an autocrine growth factor.

6.3 Purification to homogeneity and physicochemical characterization of TGFe: Although many biological studies on TGFe were reported, there exists very little information concerning the primary sequence of TGFe. As previously stated, it was observed to be a cystine containing protein of 22-25 kDa which was heat and acid stable but sensitive to reducing agents. Major difficulties have been encountered in the purification of this protein due to the low concentration found in bovine tissues.

Initial purification by Halper (27) permitted only initial biochemical and biological characterization. However, it was Parnell et al. who purified TGFe to homogeneity from bovine kidney acid ethanol extracts (30). Using a series of batch ion exchange chromatography, molecular sieve chromatography and heparin-Sepharose affinity chromatography procedures followed by RP-HPLC, TGFe was purified to 90% purity or greater but yielding only 10-15 ng. The molecular weight assigned by SDS-PAGE was 22-25 kDa as previously reported (27). However, the low intensity staining of the active band suggested that it was a glycoprotein.

The first primary sequence data was published by Parnell (31) who used a similar protocol to the previous purification of TGFe (30), except for the deletion of the heparin-Sepharose chromatography procedure and the addition of a final High Performance Electrophoresis Chromatography (HPEC) step. The purified TGFe was characterized in terms of amino acid composition, amino-terminal sequence analysis and determination of the presence of glycosylation. The amino-terminal sequence was reported as DVK(X)DMEVS(X)PD(X)YT... which is identical to that of granulin A. The amino acid analysis showed high levels of glutamate, aspartate, glycine and proline residues, with an estimated total of 218 amino acids. The total number of amino acids is consistent with the apparent molecular weight of 22-25 kDa, only if glycosylation is present. Enzymatic deglycosylation of TGFe using N-glycanase, neuramidase and O-glycanase decreased the

molecular weight from 25 kDa to 20 kDa giving evidence that TGFe is a glycoprotein. Only treatment with the enzyme N-glycanase resulted in removal of all glycosylated portions of TGFe suggesting the presence of mostly N-linked oligosaccharides.

Other investigators have also reported the isolation and characterization of TGFe from bovine kidney as a 22-25 kDa protein with proliferative activity for SW-13 cells (32). However, their purification of TGFe from human SW-13 cells differed significantly from that outlined above. They reported a band in the 59 kDa range as compared to Halper et al. (27) who reported a 22-25 kDa band when TGFe was isolated from human SW-13 cells. Both proteins, however, showed similar activity with respect to proliferation of SW-13 cells, causing anchorage independent cell growth and stimulation of DNA synthesis. Dunnington (33) also identified a form of TGFe with a molecular weight in the 14 kDa range in extracts of bovine kidney. A difference in glycosylation or differences in the actual size of the protein due to different processing could reconcile these various observations.

Although the authors did not know of the existence of the granulin/epithelin family of growth modulatory peptides, it seems likely that the amino-terminal sequence for TGFe is homologous to that found for the human granulin/epithelin-1 family. Also, the 20 kDa estimated size for the deglycosylated TGFe would predict that TGFe begins with the granulin/ epithelin-1 amino-terminal sequence located midway within the precursor and finishing at the end of the known precursor for the granulin/epithelin family of growth modulatory proteins.

Acrogranin

Spermatogenesis is a complex process of differentiation which requires progressive spermatogenic cell remodeling. The acrosome is formed during this process and this entails changes in nuclear structure and gene regulation, which is beyond the scope of this introduction. In brief, the acrosome is a sperm specific organelle forming the anterior layer of the sperm head. During the complex interaction of the egg and the sperm during fertilization, the sperm acrosome undergoes a secretory response which releases several hydrolytic enzymes needed for penetration of the sperm into the egg.

7.1 Acrogranin, an acrosomal cysteine-rich glycoprotein: During studies on spermatogenesis using an acrosomal marker protein (34), a 67 kDa glycoprotein was identified and partially characterized from extracts of guinea pig sperm cells. Initial immunocytochemical data suggested that acrogranin first appears in proacrosomal granules of spermatocytes (pachytene spermatocytes) and is present throughout development from spermatocytes to round spermatids (mature sperm cells).

Further studies by Baba et al. (34) showed, using immunoblotting techniques, detectable levels of acrogranin in the testis and epididymis only, suggesting it to be a testis-specific glycoprotein. Immunofluorescent localization studies showed that acrogranin appeared to be concentrated in the acrosome of the developing spermatid and pachytene spermatocytes.

Baba et al. (34) isolated several clones encoding acrogranin from guinea pig and mouse testicular cDNA libraries. Selected clones were sequenced and the deduced amino acid sequences for guinea and mouse acrogranin were reported along with putative signal sequences and 3′-untranslated regions. The mouse and guinea pig forms of acrogranin were shown to be cysteine-rich polypeptide molecules of 61,613 and 62,953 daltons respectively. Overall the amino acid sequences are very similar (63% identity) with both having high cysteine content. Noticeable was the presence a motif comprised mainly of cysteine residues which was observed to be repeated seven and a half times within the acrogranin prepro-polypeptide. Comparison of these acrogranin molecules to known protein structures and cDNA sequences in the GenBank database, demonstrated that they were homologues of human granulins and rat epithelins described previously. All protein structures were shown to be well conserved especially within the repeated cysteine motif with an exception found in the guinea pig structure (i.e. deletion in the fifth repeat and two insertions in the second and fourth repeats).

Like the observed wide expression of human granulins and rat epithelins, the acrogranin mRNA was shown to be expressed in many tissues studied in the mouse and guinea pig. Prepubertal mice lacking pachytene spermatocytes were studied and it was reported that acrogranin was expressed in early germ cell of the testis. Interestingly, the germ cells or the spermatocytes apparently cannot process the acrogranin polypeptide to

smaller forms since the complete precursor molecule is found throughout spermatogenesis.

7.2 Exon/Intron organization of the acrogranin/granulin/epithelin gene: In order to understand what controls expression of the granulin/epithelin/acrogranin gene, Baba et al. (35) reported the exon/intron organization of the mouse gene along with the putative promoter region. They showed results describing a mouse genomic fragment having 13 exons separated by 12 introns with a 5′-flanking region of 1 Kbp. Consensus sequences for regulatory elements were also present in the 5′-flanking region along with selective degradation of mRNA signals (A-T rich sequence). A CCAT sequence was shown to be a functional promotor sequence. This data was consistent with the work of Bhandari et al. (20).

These investigators have not shown any biological characterization of the acrogranin precursor with regards to spermatogenesis and the role of the acrosome. They, however, speculate possible roles for the precursor due to its presence throughout spermatogenesis pointing to new biological functions for this class of polypeptide in their precursor form.

eNAP-1, an antimicrobial polypeptide isolated from Equine neutrophils

Defensins are antimicrobial peptides and function as part of the non oxidative mechanisms of defense against pathogenic and non-pathogenic microorganism. Antimicrobial polypeptides are described in detail in Chapter II of my thesis and will not be dealt with in depth here. Equine (horse) neutrophils have recently been studied and shown not to contain these defensin-like peptides (36). Antimicrobial activity in extracts of these neutrophils have been studied and interestingly one active component has been partially biologically and structurally characterized. Amino acid and partial amino acid sequence analysis of this polypeptide designated eNAP-1 have been reported. The sequence shown was obtained by Edman microsequencing of the reduced and pyridylethylated peptide:

eNAP-1 DVQCGEGHFCHDXQTCCRASQGGXACCPYSQGVCCADQRHCCPVGF

eNAP-1 $CX_5\ CX_5CCX_8CCX_6CCX_5CCX_4$...

GRN/EPI $CX_{5\text{-}6}CX_5CCX_8CCX_6CCX_5CCX_5CX_{5\text{-}6}C$

It was observed that this polypeptide is an equine representative of the granulin/epithelin family of peptides having the conserved cysteine motif at least within its amino-terminal sequence.

The limited data on the antimicrobial activity of eNAP-1 seems to indicate some antibacterial specificity. It appears that gram positive bacteria namely *S. zooepidemicus* are more susceptible than gram negative bacteria (ie: *E. coli*, *P. aeruginosa* and *K. pneumoniae*). Growth modulatory acitivities for eNAP-1 have not yet been reported. It, however, seems likely that it would have similar effects to the granulin/epithelins due to its striking homology to this family of peptide.

Insect granulin/epithelin from *Locusta migratorias* (PMP-D1)

Three novel peptides were isolated from the pars intercerebralis of the brain of the insect *Locusta migratoria* (37). Structural analysis revealed peptides of 35, 36 and 54 residues in length. Peptides one and two have 6 cystines cross-linked in intramolecular disulfide bonds, whereas the third peptide of 54 residues has twelve cystines all in disulfide bonds as confirmed by its molecular mass of 5776 daltons. This peptide designated PMP-D1 has the following primary sequence:

PMP-D1 SCTEKTCPGTETCCTTPQGEEGCCPYKEGVCCLDGIHCCPSGTVCDEDHRRCIQ

PMP-D1 $CX_4\ CX_5CCX_8CCX_6CCX_5CCX_5CX_6\ C$

GRN/EPI $CX_{5\text{-}6}CX_5CCX_8CCX_6CCX_5CCX_5CX_{5\text{-}6}C$

From the primary sequence of PMP-D1 it was clear that it is homologous to the granulin/ epithelin family of growth modulatory polypeptides. No biological studies were performed but it can be inferred from its sequence that the cysteine motif from PMP-D1 is conserved and that this peptide should have biological importance. The cysteine motif is well conserved, again demonstrating the ancient phylogenetic origins of the granulin/ epithelin family.

Cysteine proteinases of rice seeds (oryzains)

The germinating rice seed contains three distinctive cysteine proteinases with similarities to a series of well characterized animal proteinases (38). The deduced amino acid

sequences for oryzains α, ß and λ are distinct but share common structural features. Apart from the catalytic triad Cys^{25}-His^{159}-Asn^{175} oryzains α and ß have 70% similarity and show 5% similarity to papain and actimidin. Oryzain λ is similar to aleurain (85%) and cathepsin (60%). A striking feature of oryzains α and ß is the presence of a cysteine sequence motif which is similar to that found in the granulin/epithelin family of proteins.

Oryzain α	.....$CCX_6CCX_5CCX_6CX_6$ C...
Oryzain ß	.....$CCX_6CCX_5CCX_6CX_5$ C...
GRN/EPI	$CX_{5-6}CX_5CCX_8CCX_6CCX_5CCX_5CX_{5-6}C$

This motif is found in the carboxyl-terminal region of orizain α and ß and coincides with the carboxyl-terminal half of the conserved granulin/epithelin motif. Although the significance of this similarity is unknown, it does reflect on the ancient phylogenetic origins of this structural motif. The presence of this motif does not necessarily imply a homology between these genes. Many studies are needed to show any functional relationship between the cysteine-rich domain of the oryzain and the granulin/epithelin family.

My introduction reviewed the current status of granulin-like peptides, in order to emphasize the various contexts in which they have been characterized. It also discussed the growth modulatory activities which have been attributed to this class of protein. The following chapters will show my contributions to the study of defensin/corticostatin peptide family and to the granulin/epithelin class of peptides. Emphasis should be placed upon the work described in Chapters IV and V which illustrate structural, biochemical and immunocytochemical characterization of teleost granulins.

CHAPTER II

PURIFICATION OF CATIONIC CYSTINE-RICH PEPTIDES FROM RAT BONE MARROW. PRIMARY STRUCTURES AND BIOLOGICAL ACTIVITY OF THE RAT CORTICOSTATIN FAMILY OF PEPTIDES

Preface

Structural elements within peptides which confer biological activity are of primary importance in protein chemistry. This chapter will show, in part, the important amino acid sequences which permit the corticostatins to bind selectively to the corticotropin (ACTH) receptor and antagonize its *in vitro* effects. Also, showing the existence of corticostatins in other species, such as the rat, validates previous studies completed on corticostatins isolated from rabbit tissues in the Endocrine laboratory. This study was undertaken in parallel with the investigation of the nature of cystine-rich peptides found in the hematopoietic tissues of a teleost fish, the carp, which is the subject of Chapter IV of this thesis. It transpired that while no defensin-like peptides were found in the carp, this species proved to be a rich source of granulins. The corticostatin/defensin family are the major cystine-rich peptides found in hematopoietic cells of the rat and their characterization is the subject of this chapter. Another cystine-rich peptide which proved to be rat granulin was also isolated during this study and is the subject in part of chapter III.

Abstract

Seven cationic, cystine-rich peptides of 29 to 32 amino acid residues have been purified from extracts of rat bone marrow (R-1, R-1a, R-1b, R-2, R-3, R-4 and R-5). Structural analysis clearly indicated that all seven peptides belong to the corticostatin/defensin family of leukocyte-derived peptides known to participate in oxygen-independent killing of phagocytosed bacteria. For R-1 to R-5, six cysteine residues were found at characteristic and highly conserved positions. R-1a and R-1b were partially characterized and appear to be structural variants of R-1. Aside from the conserved cysteines, there is a remarkable degree of structural diversity evident within the sequences of those members of the corticostatin/defensin family characterized so far. The structures of the peptides that we have purified can be compared directly with the sequences obtained for rat defensins isolated from extracts of peritoneal neutrophils (Lehrer, Ganz and Selsted, Cell, 64, 229-230, 1991). Some discrepancies are apparent which can be explained in terms of proteolytic cleavage of several of these peptides at both amino- and carboxyl-termini. The corticostatins owe their bioactivity to their ability to compete with corticotropin for occupancy of the corticotropin receptor (Zhu, Hu, Mulay, Esch, Shimasaki and Solomon, Proc. Natl. Acad. Sci., 85, 592-596, 1988). The potency of these peptides can be expressed in terms of their capacity to inhibit the steroidogenic response of isolated rat adrenocortical cells half-maximally stimulated by corticotropin (i.e. at the ED_{50} concentration for corticotropin in this assay, namely 33 pM). In this assay, the rat peptides R-1, R-2 and R-3 were shown to be inactive. In contrast, the more cationic peptides R-4 and R-5 were found to inhibit steroidogenesis. R-4 was somewhat less active than rabbit corticostatin (IC_{50} 25 nM) showing an IC_{50} value of 50 nM. R-5 appeared to be significantly less potent than R-4. The lower yield of R-5 precluded an accurate estimate of the corticostatic potency of this peptide. R-4 differs in structure from R-5 in having an arginine to serine substitution at position 7. It can be concluded that an arginine at this position accounts, at least in part, for the corticostatic activity of R-4. When tested for their cytotoxic activity using Chinese hamster ovary cells in culture, R-4 was shown to be active (IC_{50} 2 μM) while R-1 and R-3 were inactive. None of the rat peptides was found to antagonize the biological activity of α-melanotropin in an anolis skin bioassay.

Introduction

Members of the corticostatin/defensin family of peptides have recently been purified from human, rabbit and guinea pig neutrophils and macrophages. It is now appreciated that these basic 29 to 34 amino acid peptides are the low molecular weight lysosomal cationic proteins originally described by Zeya and Spitznagel (1,2). The cationic proteins participate in the non-oxygen dependent killing of phagocytosed bacteria. During this process bacteria are engulfed by phagocytes and the resulting phagosome fuses with lysosomes. The cationic peptides or defensins bind to the bacterial cell wall and disrupt its integrity resulting in cell lysis and death. Defensins act primarily at the initial neutral pH of the phagosome. The pH of the phagosome subsequently falls permitting the activation of lysosomal enzymes (e.g. lactoferrin and lysozyme). Concomitant with this action is the activation of oxygen-dependent killing mechanisms (3). Defensins have been found to have wide bactericidal activities against both gram-positive and gram-negative bacteria (4). These peptides have also been found to have anti-fungal and anti-viral activity (5,6) and more recently, cytotoxic activity (7,8). In addition, members of this peptide family have been shown to have mast cell degranulating activity (9), are chemotactic for monocytes (10) and cytostatic activity towards promyelocytic tumour cells (11).

To date, four human defensins (HP-1 to HP-4), six rabbit defensins (NP-1, NP-2, NP-3a, NP-3b, NP-4 and NP-5) , two guinea pig defensins and four rat defensins have been purified and characterized (8-17). In addition, the mRNA encoding members of the human, rabbit and guinea pig defensin/corticostatin family have been cloned and sequenced (18-21). The most striking feature of these structures is the absolute conservation of the position of the six cysteine residues within all these peptides. Some members of the family are closely related and constitute structural subsets. For instance, human HP-1, 2 and 3 differ by only one amino acid residue (10). Similarly rabbit NP-1 and 2 differ only at residue 13 (11). Aside from these similarities, there is remarkable sequence diversity in those amino acids flanking the conserved cysteines. The cationic nature of these peptides is also highly variable. They range from a net positive charge of +8 for NP-1 to +2 for HP-3 (17).

An intriguing development in this field has been the purification and characterization of corticostatic peptides from extracts of fetal and adult rabbit lung (22). Four peptides (CS-I to CS-IV) have been purified which have the ability to antagonize the steroidogenic action of corticotropin by competing for receptor occupancy (22,23). The characterization of CS-I as a competitive inhibitor of the steroidogenic activity of ACTH has recently been confirmed by others (24). CS-I is the most potent corticostatin having an IC_{50} of 2.5 X 10^{-8}M with respect to inhibition of corticosterone production by isolated rat adrenal cells in response to 33 pM corticotropin (i.e. the ED_{50} concentration for this hormone) (22). Not all members of this cystine-rich polypeptide family are corticostatic. Aside from the rabbit peptides CS-I to CS-IV, the only members of the family to have corticostatic activity are the human peptide HP-4 (IC_{50} = 7 X 10^{-7}M) (8) and two guinea pig corticostatins (IC_{50} 2.5 X 10^{-7}M) (15). A third unrelated corticostatic peptide from the guinea pig has also been characterized (IC_{50} 2 X 10^{-6}M) (15).

An abundant mRNA has been shown to be expressed in mouse intestinal crypt epithelium which encodes a member of the corticostatin/defensin family (25). These observations prompted an investigation of the properties of the corticostatin/defensin family with respect to volume regulation of villus cells prepared from guinea pig intestinal epithelium. This study showed that the corticostatins, but not other members of this peptide family, were active as L-type Ca^{2+} channel agonists in this bioassay system (26).

In order to gain a better understanding of what structural elements within these peptides are responsible for their biological activities, the homologous rat peptides were purified and their anti-ACTH and cytotoxic properties were defined. Bone marrow was chosen as the potential source of these peptides since this lymphoid tissue is known to be rich in mature resident macrophages and polymorphonuclear leukocytes. Also, rabbit femur marrow has been shown to be a source of mRNA encoding two members of the corticostatin/defensin family (20). Since the ACTH and α-melanotropin (α-MSH) receptors share binding characteristics, we also investigated the ability of corticostatic peptides to antagonize the α-MSH receptor in an Anolis skin bioassay for melanotropic activity (27).

Materials and Methods

4.1 Animals: Male Sprague-Dawley rats (150g-200g) were obtained from Charles River (St. Constant, Quebec, Canada). Anolis carolinensis were obtained from a local pet store. They were kept in a terrarium at 21°C and fed Tenebrio larvae.

4.2 Tissue Preparation and Extraction: Bone marrow was obtained from the femurs of 250 male Sprague-Dawley rats. Femurs were cut at one end and the marrow was aspirated using a 10ml syringe containing extraction medium (1M hydrochloric acid, 5% formic acid (v/v), 1% trifluoracetic acid (TFA) (v/v) and 1% sodium chloride (w/v)) (28). The mixture was homogenized and centrifuged at 2000 X g for 10 min and the crude supernatant was concentrated and desalted using C_{18} Sep-Pak cartridges (Waters Associates, Milford, MA, USA) as described previously (28).

4.3 Purification of rat peptides (R-1, R-1a, R-1b, R-2, R-3, R-4, R-5): High performance liquid chromatography (HPLC) was performed on a Waters Associates HPLC system (Milford, MA, USA) and all HPLC solvents and reagents were prepared as described previously (28). The concentrated eluate from the extraction procedure was fractionated by reversed-phase HPLC using a Waters C_{18} μBondapak column and a linear gradient of 8% to 48% acetonitrile containing 0.1% TFA throughout, over one hour at a flow rate of 1.5 ml per minute. All fractions were screened using amino acid analysis and the regions with the highest cystine content were purified by a shallow gradient of 20-40% acetonitrile containing 0.13% heptafluorobutyric acid (HFBA) throughout, over one hour at a flow rate of 1.5 ml per minute. Further purification was performed on the same reversed-phase HPLC column using a linear gradient of 16% to 28% acetonitrile containing 0.1% TFA throughout over one hour at a flow rate of 1.5 ml per minute. Individual cystine rich peaks were further purified using two Waters I-125 gel permeation columns connected in series (29). The columns were loaded using a reversed-phase trace enrichment technique and eluted isocratically using 40% acetonitrile containing 0.1% TFA at a flow rate of 1 ml per minute as described previously (30).

4.4 Pyridylethylation of cysteine residues and tryptic fragmentation: Pyridylethylation of peptides was achieved according to a previously published procedure (31). Peptides were solubilized in a 1M Tris HCl, 8M guanidinium chloride buffer containing 4mM EDTA at pH 8.5. The solution was saturated with nitrogen and the disulfide bonds were reduced using 10mM ß-mercaptoethanol for 1 hour at 37°C. This was followed by the addition of 4µl of 4-vinyl pyridine, under nitrogen and the reaction was allowed to proceed for 1 hour. The pyridylethylated peptides were purified by reversed-phase HPLC using a linear gradient over one hour of 8% to 48% acetonitrile containing 0.1% TFA throughout at a flow rate of 1.5 ml per minute. Successful derivatization was assessed by amino acid analysis and by monitoring absorbance of HPLC eluates at 254 nm. Peptide R-1a was digested using trypsin (TPCK-treated, Sigma), at an enzyme to substrate ratio of 1:50 (w/w), at 37°C in 100µl 50mM ammonium bicarbonate buffer (pH 8.3) for 4 hours. Fragments were purified by reversed-phase HPLC as described previously (28).

4.5 Amino acid analysis and peptide sequencing: Peptides were subjected to vapour hydrolysis in 6N HCl at 105°C for 18 hrs using a Waters Pico-Tag work station. The hydrolysates were analyzed using a Beckman System 6300 Amino Acid Analyzer (Beckman Instruments, Palo Alto, CA., USA).

The pyridylethylated derivative of each peptide was subjected to gas phase microsequencing using an Applied Biosystems gas-phase sequenators (Applied Biosystems, Foster City, CA, USA) either located at the McGill University protein-sequencing core facility located at the Shriner's Hospital for Crippled Children or at the Montreal Clinical Research Institute.

4.6 Ion spray mass spectrometry (IS-MS): Ion spray mass spectra of purified peptides and tryptic fragments for R-1a were obtained using an API III mass spectrometer with ion spray interface (SCIEX, Thornhill, Ontario, Canada) located at the Montreal Biotechnology Research Institute of the National Research Council of Canada. We gratefully acknowledge the help of Yasuo Konishi and Bernard Gibbs for performing the

mass determinations.

4.7 Biological assays:

a) Rat adrenal cell bioassay: The rat adrenal cell preparation is a modification of the method of Sayers et al (32,33). Briefly, the adrenals were removed from decapitated male Sprague-Dawley rats (150g-200g) and were decapsulated. The adrenals were minced and placed for 1 hour in a Hams F-12 buffer (Flow Laboratories) containing collagenase (2 mg/ml) and DNAse (0.25 mg/ml) to disperse the tissue. This was followed by washing of the cells in the same buffer and density centrifugation over a 2% BSA solution (w/v). The cell concentration was adjusted to 3.5-4 X 10^5 cells/ml and 0.5 ml aliquots were allowed to pre-incubate for 1 hour at 37°C in 95% O_2/ 5% CO_2 atmosphere. This was followed by addition to the cell suspension of ACTH alone or different peptides plus 33 pM ACTH. After a 2 hour incubation period the steroids were extracted in 2 ml of methylene chloride and the levels of corticosterone were determined using a radioimmunoassay kit purchased from BioRad (Montreal, Quebec, Canada). Peptides were tested for their corticostatic potency relative to highly purified rabbit CS-I kindly provided by Dr. Q. Zhu of the Endocrine Laboratory.

b) Anolis skin bioassay: The assay was performed as described by Tilders et al (27). Briefly, the skin of uniform green colour was removed from decapitated lizards (Anolis carolinensis) and cut into square pieces of approximately 5 mm. The pieces of skin were equilibrated in medium (8.3g NaCl, 0.33g KCl, 0.16g $CaCl_2$-$2H_2O$, 0.21g $MgCl_2$-$6H_2O$, 0.1g $NaHCO_3$ and 10 mg bovine serum albumin per litre of double distilled water). Serial dilutions of the test peptides were placed in different wells of multiwell culture plates and the fragments of skin were introduced. A positive result was indicated by a green to brown colour change and the results were assessed by two different investigators.

c) Cell culture and cytotoxic assay: Chinese Hamster Ovary cells (CHO-K1) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown under the conditions recommended by ATCC. Thymidine incorporation and cell number measurements were performed as previously described (8).

Results and Discussion

5.1 Isolation and characterization of rat bone marrow peptides: The rat femur marrow extract was subjected to reversed-phase HPLC using TFA as counter-ion. This initial purification is shown in Figure 1A. The column eluate was monitored for UV absorbance at 210 nm while individual column fractions were assessed in terms of their cystine content and their ability to inhibit ACTH-stimulated steroidogenesis in the rat adrenal cell bioassay. A large peak of UV absorbing material eluting between 22 and 28 minutes was found to possess both high cystine content and corticostatic activity (results not shown). These fractions were subjected to further reversed-phase HPLC using HFBA as counter-ion (Figure 1B). The cystine-rich material was now resolved into two components. The major proportion eluted between fractions 37 and 46 (designated peak I) while a smaller proportion eluted between fractions 49 and 55 (designated peak II) (Figure 1B). Fractions from the peak I were pooled separately and repurified by reversed-phase HPLC using TFA as counter-ion (not shown). Material contained within peak I now resolved into five components (R-1, R-1a, R-1b, R-2, R-3)[1], whereas material contained within peak II resolved into two components (R-4, R-5) which were individually purified as described in the Materials and Methods. Purified peptides were analyzed for their amino acid content and in most cases, their molecular weights were determined by ion-spray mass spectrometry. Table 1 shows the amino acid compositions of all seven rat peptides. The chromatographic behaviour of the seven cystine-rich peptides in the HFBA solvent system could be rationalized in terms of their arginine content. While R-1, R-1a, R-1b, R-2 and R-3 have 4, 5, 4, 6 and 6 arginines per mole of peptide respectively, R-4 and R-5 have 10 and 9 respectively. Previous studies from this laboratory have demonstrated that reversed-phase HPLC solvents containing HFBA can be used to enhance the resolution of peptides based upon their overall basic charge (28). Thus, it was apparent that R-4 and R-5 were resolved from R-1, R-1a, R-1b, R-2 and R-3 using the HFBA solvent system because of their greater arginine content (Fig. 1B). All seven peptides have high cystine content indicating that they were members of the corticostatin/defensin family of peptides. While some differences in amino acid compositions were evident (Table 1), it was apparent that R-1 and R-1a were closely

related, R-1a having extra glutamate and arginine residues relative to R-1. R-1b and R-1 have similar amino acid compositions but distinctly different chromatographic elution positions and observed masses (Table 1). The compositions of R-2 and R-3 could be grouped together, R-2 having extra valine and threonine residues relative to R-3. Similarly, R-5 differed from R-4 in apparently having a serine for arginine substitution.

The purified peptides were subjected to pyridylethylation and characterized by gas-phase sequencing. Complete primary structures were obtained for R-1, R-2, R-3, R-4 and R-5 (Figure 2). The sequence data obtained confirm that the rat peptides are members of the corticostatin/defensin family. Comparison of these new sequences with those of other members of the family indicate that they all have identical cysteine backbone structures (Figure 2). The structures of R-2, R-3, R-4 and R-5 were confirmed by ion spray mass spectrometric analysis of their molecular weights. Table 1 shows their expected and determined molecular weights. Unfortunately, there was no R-1 remaining following biological characterization of this peptide to permit the determination of its mass. Amino acid analysis of R-1a and R-1b indicate that they are closely related in structure to R-1. R-1b was partially sequenced and the first 19 residues proved to be identical to R-1 (data not shown). There was insufficient material remaining to provide a complete sequence. While structurally related to R-1, R-1b was found to be 103 mass units greater in molecular weight (Table 1). Since their amino acid compositions are very similar, it is not clear what accounts for their different masses. R-1a yielded no sequence information indicating that it was amino-terminally blocked. Relative to R-1, R-1a has extra arginine and glutamate residues. The observed mass of 3443 is consistent with a core sequence identical to R-1 with a pyroglutamate residue at the amino-terminus and an arginine residue at the carboxyl-terminus. To test this hypothesis, R-1a was subjected to pyridylethylation, digested with trypsin, and the resultant fragments separated by reversed-phase HPLC (data not shown). Peptides corresponding to the amino- and carboxyl-termini were tentatively identified by amino acid and mass spectrometric analysis of the tryptic fragments (data not shown). The putative carboxyl-terminal fragment contained arginine, leucine and pyridylethyl-cysteine. The observed mass of 704.5 is consistent with the sequence LCCR. The putative amino-terminal fragment contained glutamate, alanine,

pyridylethyl-cysteine, tyrosine and arginine. The observed mass of 968.0 is consistent with the sequence pEACYCR but in a doubly oxidized state (i.e. 32 mass units higher than expected). Both masses take into account the pyridylethylation of the cystine residues (i.e. an extra 105.14 mass units per cystine). We were unable to pursue the characterization of R-1a further because all the peptide had been consumed. R-2 and R-3 differ only in the presence of a valine-threonine amino-terminal extension evident for R-2. R-1 differs from R-2 and R-3 at the amino-terminal region. In agreement with their amino acid compositions, R-4 and R-5 differ only by an arginine to serine subsitution at residue 7. There is a striking similarity between the rat peptide R-1 and the human peptide HP-1 (Figure 2). Seventeen of the thirty residues are identical in the two peptides representing 56.6% similarity. This is the highest degree of similarity that has been observed between members of the corticostatin/defensin family of peptides from different species. It is possible that the seven rat peptides represent only three gene products. The observed structural heterogeneity may be accounted for in terms of proteolytic processing of R-1a to form R-1 and of R-2 to form R-3. Also, allelic variation could account for R-4 and R-5 and for R-1 and R-1b.

5.2 Biological activities of the rat corticostatin/defensin family of peptides: In screening the HPLC column fractions for biological activity, we observed that the corticostatic and cytotoxic activities co-eluted with those fractions found to be rich in cystine (not shown). The purified rat peptides were therefore tested individually in the two assays to assess their spectrum of activities. Each peptide was tested in the rat adrenal cell bioassay for its ability to inhibit the steroidogenic activity of human ACTH in the rat adrenal assay. The activities were compared to that of the rabbit corticostatic peptide CS-I. Not all the rat peptides were corticostatic. Table 2 shows that while a concentration of 1 μM CS-I completely inhibited the steroidogenic response of dispersed adrenal cells elicited by 33 pM ACTH, the rat peptides R-1, R-2 and R-3 showed no corticostatic activity up to 1 μM concentration. R-4 and R-5 were found to be corticostatic at 1 μM. R-4 completely suppressed the steroidogenic activity of ACTH at this concentration while R-5 reduced corticosterone output by 50%. Figure 3 illustrates the ability of R-4 to inhibit

ACTH stimulated steroidogenesis in a dose-dependent manner. The inhibition curve for R-4 was found to be parallel to that of CS-I. The observed EC_{50} for CS-I (25 nM) was comparable to that found previously (22). The R-4 peptides was found to be slightly less potent with an EC_{50} value of 50 nM being observed. Our results suggest that R-5 has a considerably lower corticostatic potency than R-4 (Table 2) but because of the lower yields of this peptide, a full dose response curve was not obtained. In comparing the structures of R-4 and R-5 (Fig. 2), we can conclude that the arginine residue at position 7 is important for corticostatic activity.

Figure 4 illustrates the ability of R-4 and not R-1 or R-3 to inhibit ^{3}H-thymidine uptake in CHO K1 nontransformed cells. This activity of R-4 is similar to HP-1 in that it has the same ED_{50}=2μM and a similar dose response curve. This effect was also confirmed by a representative drop in cell number at the higher concentration of peptide HP-1 and R-4.

Aside from the strict conservation of the cysteine back-bone, there is a remarkable lack of amino acid sequence conservation within this family of peptides. However, these peptides can be divided into two groups based upon their bioactivities. Those having no corticostatic activity (e.g. HP-1, HP-2 and HP-3) do have antibacterial and cytotoxic activity. Those having very little antibacterial and cytotoxic activity (e.g. the rabbit corticostatins) have relatively potent corticostatic properties (Fig. 3). The corticostatic activity of the peptides can also be related to the extent and positioning of basic amino acids like arginine since all corticostatic peptides with high potencies are very basic molecules (i.e. CS-I and R-4). The results from the present study suggest that cationic charge alone is not responsible for the corticostatic activity and that it is critical for corticostatic peptides to have basic amino acids within positions 6 to 8 in the primary sequence. In view of the fact that rabbit CS-I acts as a competitive inhibitor of the action of ACTH (34), it is possible that this C-terminal extension and other N-terminal residues interferes with the ability of ACTH to bind efficiently with its receptor. The 4-10 heptapeptide core sequence of ACTH is capable of promoting full adrenal receptor activation. However, this sequence acts only as a weak agonist and it is the basic Lys_{15} Lys_{16} Arg_{17} Arg_{18} region that is responsible for enhanced receptor binding (35). It is

likely that the corticostatins compete with this sequence of ACTH for receptor binding.

The cytotoxic effect, although ill understood, seems to parallel the antimicrobial nature of the defensins. We have shown that R-4 is both cytotoxic and corticostatic, and for this reason, it must have within its structure the basic requirements to kill chinese hamster ovary cells and to specifically inhibit ACTH binding without affecting adrenal cell viability. R-4 (RatNP-1) has also been shown to be highly antimicrobial (16). R-4 is the only member of the corticostatin/defensin family to have potent cytotoxic, corticostatic, antibacterial and "L" type Ca^{2+} channel agonist activity (16,26). This peptide constitutes an excellent model for examining the various biological properties of the corticostatin/defensin family through structure/activity studies.

There is a close structural relationship between ACTH and its biosynthetic derivative α-melanotropin (α-MSH or α-N-acetyl $ACTH_{1-13}$ amide). It follows that ACTH and α-MSH receptors may share some binding and structural characteristics. They are both G protein linked receptors that act through the activation of adenylate cyclase. They both have an absolute requirement for extracellular Ca^{2+} to facilitate ligand binding (35,36). We have undertaken a preliminary study to determine what effect the corticostatins and the other related peptides have upon activation of the melanophore receptor. Using an anolis lizard skin in vitro bioassay (27), we have tested these peptides for both their agonist and antagonist properties. The melanotropic activity of peptides can be expressed as the mean molar concentration required to induce a green to brown change in skin colour. The potencies shown below are mean minimum effective doses (MED) ± standard deviation. The bioassay shows exquisite sensitivity to the action of α-MSH showing an MED of $9.5 \times 10^{-11}M \pm 4 \times 10^{-12}M$ (n=5). As expected, the MED for ACTH is approximately 100 times higher at $1.3 \times 10^{-8}M \pm 4.3 \times 10^{-9}M$ (n=4). A variety of peptides including vasotocin, bombesin, angiotensin II and ß-endorphin were without activity up to 10µg/ml. Somewhat unexpectedly, the rat peptides and both corticostatin (i.e. CS-I) and HP-1 proved to be weak agonists in the α-MSH bioassay. The following MED values were observed: CS-I $4.8 \times 10^{-6}M \pm 3.5 \times 10^{-7}M$ (n=8), HP-1 $7.5 \times 10^{-6}M \pm 7 \times 10^{-7}M$ (n=3). Neither peptides demonstrated any antagonistic properties with respect to α-MSH action up to concentrations approaching their MED values. The possibility that

members of the corticostatin/defensin family have α-MSH-like activity should be viewed with caution. The potencies displayed by these naturally occurring peptides is so low that a minute contamination with an α-MSH-like peptide could account for the activity observed. However, we can, with confidence, state that none of these peptides appear to be melanotropin receptor antagonists. This is consistent with the data showing no antagonistic effect of CS-I on α-MSH stimulated corticosterone production in the rat adrenal assay (36).

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Footnote

1 We have named the rat peptides according to their elution positions on reversed-phase HPLC (see Fig. 1). The designations R-1 to R-5 have been used in an abstract (35) and a paper in which the peptides were used as biological standards (26). Recently, similar peptides (RatNP-1 to RatNP-4) have been purified from rat peritoneal neutrophils and in several instances assigned different numbers (16,17). To be consistent with our own publications, we use our own numbering system in this paper. To compare our nomenclature with the other publications the following peptides can be considered to be identical (our designation in bold print): **R-3** and RatNP-3, **R-4** and RatNP-1, **R-5** and RatNP-2. **R-1**, **R-1a**, **R-1b** and **R-2** were not identified in the previous studies (16,17). Similarly, RatNP-4 which was identified previously was not found in the present study.

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Table 1 Amino acid composition (molar ratios) and mass for the seven members of the rat corticostatin/defensin family

	Asx	Thr	Ser	Glu	Gly	Ala	Cys	Val	Ile	Leu	Tyr	Phe	Arg	Yield (nmoles)	Mass[a] (determined)	Mass[b] (predicted)
R1	1.1 (1)[c]	0.9 (1)	0.8 (1)	1.1 (1)	4.8 (5)	3.1 (3)	4.8 (6)	1.1 (1)	1.4 (2)	2.9 (3)	1.8 (2)	0.0 (0)	3.9 (4)	ND[d]	ND	3176.76
R-1a	1.0 (1)	1.0 (1)	1.1 (1)	2.2 (2)	4.6 (5)	2.9 (3)	5.2 (6)	0.9 (1)	1.5 (2)	3.2 (3)	1.6 (2)	0.0 (0)	5.5 (5)	36	3443.07 ± 0.10	3444.06
R-1b	1.1	1.0	1.2	1.2	4.5	2.9	4.5	1.0	1.1	3.1	1.6	0.0	4.2	25	3279.58 ± 0.39	3275.90
R-2	1.2 (1)	2.0 (2)	4.5 (4)	1.2 (1)	3.1 (3)	1.3 (1)	6.0 (6)	0.8 (1)	0.7 (1)	3.5 (3)	0.9 (1)	1.2 (1)	6.2 (6)	112	3465.51 ± 0.43	3466.08
R-3	1.0 (1)	1.5 (1)	4.6 (4)	1.4 (1)	3.4 (3)	1.4 (1)	4.5 (6)	0.0 (0)	0.3 (1)	3.5 (3)	0.5 (1)	1.0 (1)	5.8 (6)	273	3265.49 ± 0.43	3265.84
R-4	0.0 (0)	1.5 (2)	0.8 (1)	1.0 (1)	4.4 (4)	1.1 (1)	4.0 (6)	0.6 (1)	0.4 (1)	2.1 (2)	2.2 (2)	0.9 (1)	9.0 (9)	49	3829.03 ± 0.29	3829.53
R-5	0.0 (0)	1.0 (2)	1.7 (2)	1.3 (1)	4.2 (4)	1.0 (1)	5.8 (6)	0.7 (1)	0.5 (1)	2.2 (2)	2.5 (2)	0.9 (1)	8.0 (8)	18	3760.10 ± 0.24	3760.42

a. Observed masses are average values obtained from multiply charged ions ± standard deviation.
b. Expected masses were calculated using average masses for each amino acid and primary sequences shown in Figure 2.
c. The expected amino acid composition values for each peptide is shown in parentheses.
d. Not determined. All of R-1 was used to determine its primary structure and its biological activities.

Table 2 Corticostatic activities for members of the rat corticostatin/defensin family and the rabbit CSI. Values represent corticosterone (B) production as a % of that observed following stimulation with 33 pM ACTH (n=3).

Treatment	% B production	S.E.
ACTH alone	100	0.51
ACTH + R-1 (10^{-6}M)	96.3	0.72
ACTH + R-2 (10^{-6}M)	96.2	1.18
ACTH + R-3 (10^{-6}M)	97.0	0.93
ACTH + R-5 (10^{-6}M)	49.0	1.53
ACTH + R-4 (10^{-6}M)	2.0	0.20
ACTH + CSI (10^{-6}M)	2.2	0.53
Basal (i.e., no peptide)	1.5	0.35

Figure 1 Reversed-phase HPLC of an ODS-silica extract of the marrow obtained from 250 rat femurs. Panel A: The initial extract was loaded onto a C_{18} μBondapak column which was eluted over 1 hour at 1.5 ml/min with a linear gradient from 8 to 48% acetonitrile containing 0.1% TFA throughout. Material eluting between 22 and 28 minutes was found to have both corticostatic and cytotoxic activity and to be rich in cystine and was purified further. Panel B: Fractions 22 to 28 inclusive were reloaded onto the same HPLC column which was eluted over 1 hour at 1.5 ml/min with a linear gradient from 20 to 40% acetonitrile containing 0.13% HFBA throughout. Material designated I (R-1, R-1a, R-1b, R-2, R-3) and II (R-4, R-5) were subjected to further purification as outlined in the Materials and Methods section.

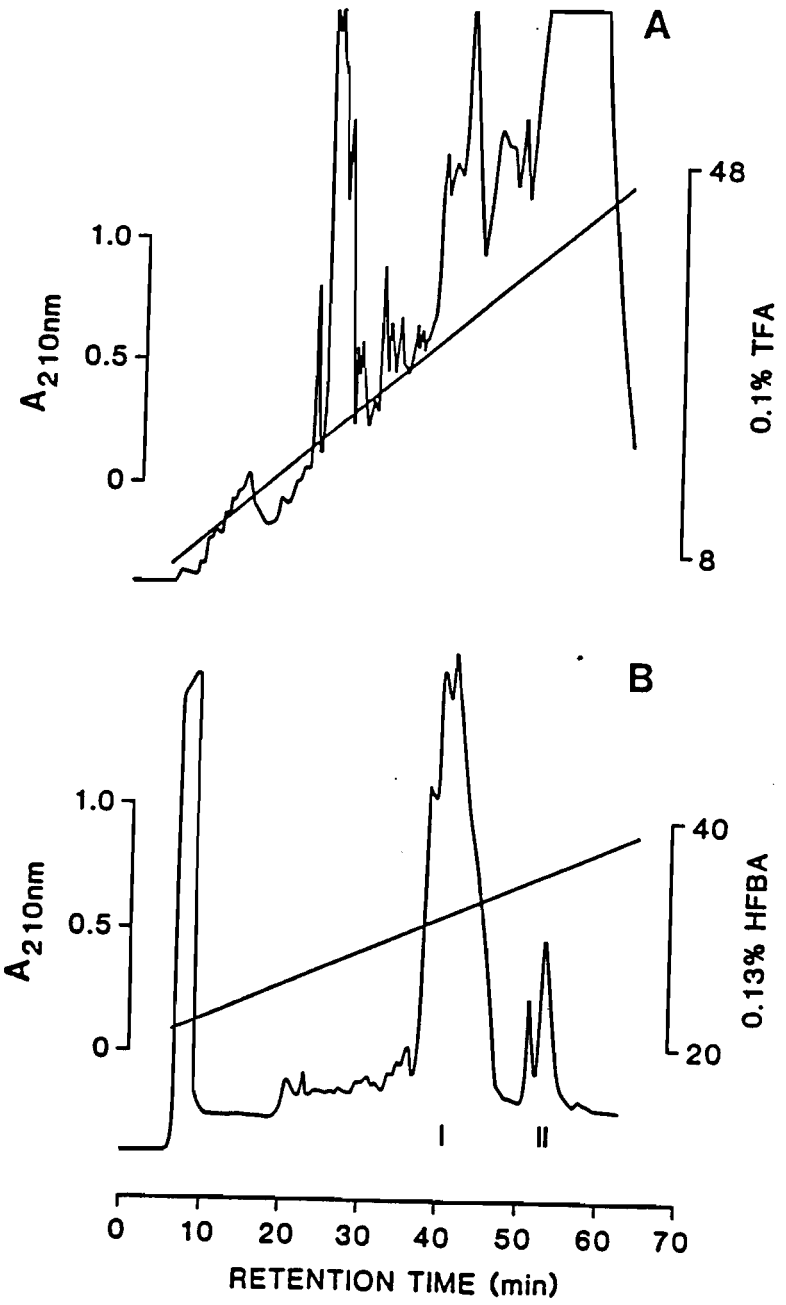

A
1.0
0.5
0
A_{210nm}
48
8
0.1% TFA
B
1.0
0.5
0
A_{210nm}
40
20
0.13% HFBA
I
II
0
10
20
30
40
50
60
70
RETENTION TIME (min)

Figure 2 The primary sequences of the five rat members of the corticostatin/defensin family. The sequences of human HP-1 and the rabbit CS-I are also shown for comparison. Standard one letter code used: A=alanine, C=cysteine, D=aspartate, E=glutamate, F=phenylalanine, G=glycine, H=histidine, I=isoleucine, K=lysine, L=leucine, M=methionine, N=asparagine, P=proline, Q=glutamine, R=arginine, S=serine, T=threonine, V=valine, W=tryptophan, Y=tyrosine.

Rat	R-1	ACYCRIGACVSGERLTGACGLNGRIYRLCC
	R-2	VTCSCRTSSCRFGERLSGACRLNGRIYRLCC
	R-3	CSCRTSSCRFGERLSGACRLNGRIYRLCC
	R-4	VTCYCRRTRCGFRERLSGACGYRGRIYRLCCR
	R-5	VTCYCRSTRCGFRERLSGACGYRGRIYRLCCR
Human	HP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
Rabbit	CS-1	GICACRRRFCPNSERFSGYCRVNGARYVRCCSRR

Figure 3 Corticostatic effect of R-4 as compared to the rabbit corticostatin (CS-I). Dispersed adrenal cells were incubated with 33 pM ACTH alone or together with increasing concentrations of R-4 or CS-I. Corticosterone production was measured and expressed as a percent of the maximal output. The results shown are average values for three experiments ± standard deviation (n=3). Dose response curve for CS-I represented by open circles and R-4 by closed circles.(CS-I IC_{50} =25nM, R-4 IC_{50} =50nM.) IC_{50} values for these peptides are significantly different at $P < 0.05$.

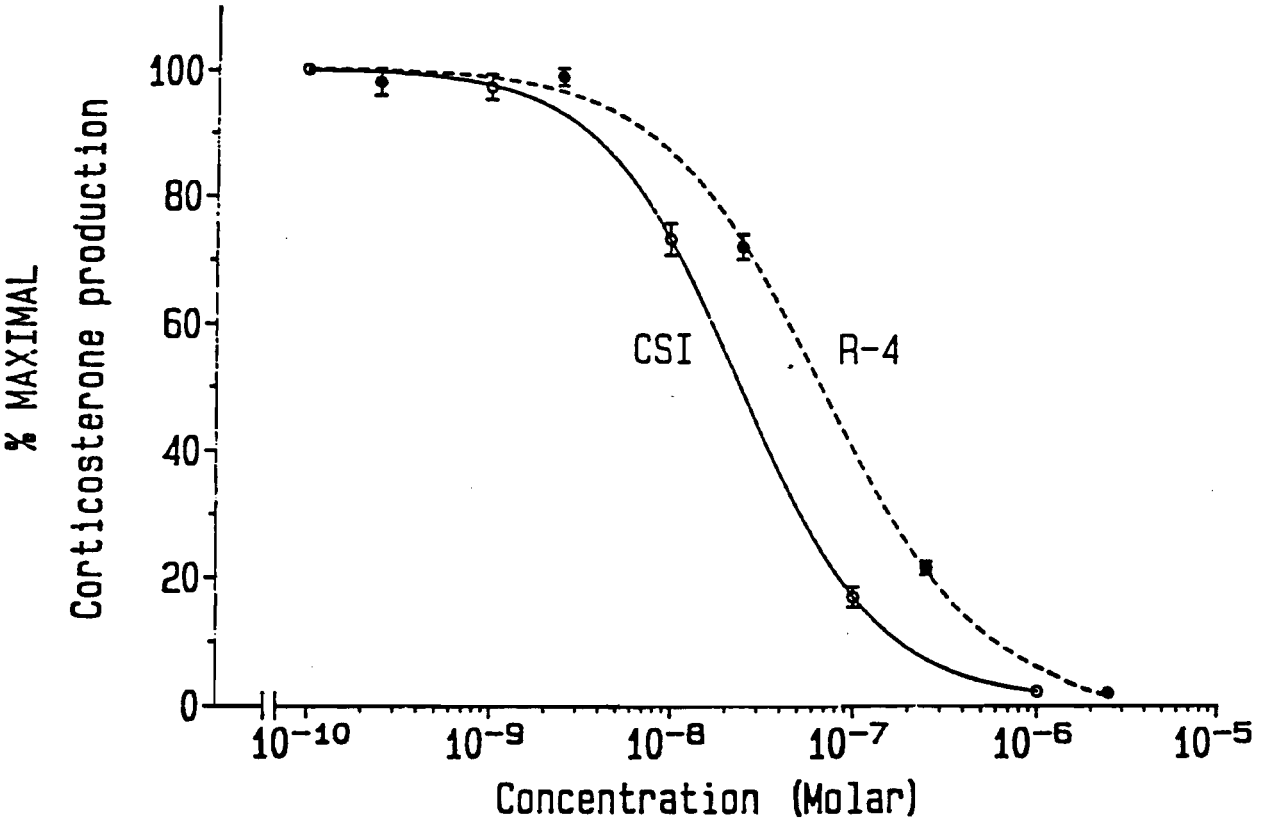
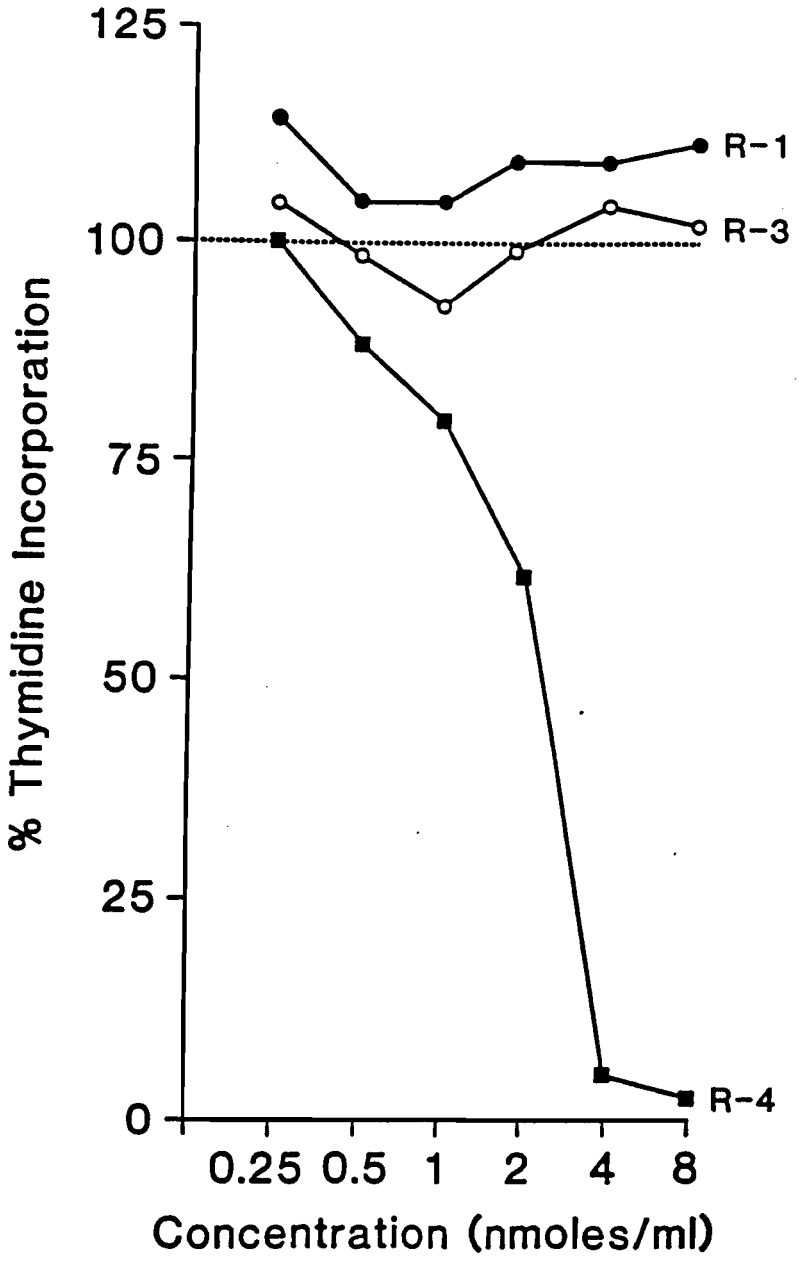

% MAXIMAL
Corticosterone production
100
80
60
40
20
0
CSI
R-4
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Concentration (Molar)

Figure 4 Cytotoxic assay of rat R-4 in Chinese hamster ovary cells (K1, nontransformed). Measured is the percent thymidine incorporation in cells after a 24 hour incubation with the test peptides. Estimates are averages of triplicate values from a single representative experiment.


125
100
75
50
25
0
% Thymidine Incorporation
R-1
R-3
R-4
0.25 0.5 1 2 4 8
Concentration (nmoles/ml)

CHAPTER III

GRANULINS, A NOVEL CLASS OF PEPTIDE FROM LEUKOCYTES

Preface

This chapter represents collaborative work between Andrew Bateman and the candidate and establishes the existence of granulin-like peptides in mammals. Following the initial finding of a prototypic form of granulin in teleost fish (Belcourt, D. and Bennett, H.P.J. (1988) J. Cell Biol. 107, 629a), it was of interest to verify the existence of this peptide in other species. For my part, rat bone marrow was chosen for the isolation knowing it would be a good representative source of hematopoietic tissue. This tissue also yielded large amounts of members of the corticostatin/defensin family. The characterization of these peptides was the subject of Chapter II. Simultaneously, A. Bateman, working on human leukocytes prepared from human blood, was successful in isolating novel structures related to teleost granulins. This combination of rat and human granulin purification and structure determination was published together to show the existence of homologous peptides in two mammalian species.

Abstract

We report the isolation and characterization of a novel class of leukocyte peptides with possible cytokine-like activities which we call granulins. They are cystine-rich with molecular weights of approximately 6Kda, except for granulin D, which appears to be a dimer. We present the sequence of one member of this family, a 56 residue peptide, granulin A, and amino-terminal sequences for three other granulins from human peripheral leukocytes. A fifth related peptide was isolated and partially sequenced from rat bone marrow, suggesting that at least some of the granulin in peripheral leukocytes is preformed in the marrow. Rat granulin, and human granulin A, are closely related, showing that the granulin structures are highly conserved between species.

Introduction

It has recently become clear that leukocytes are peptidergic cells. Neutrophil granules contain large amounts of basic, cystine-rich peptides of 29 to 34 amino acids, that have been variously called defensins (1), corticostatins (2), myeloid-related sequences (3), and cryptidins (4). Some of these peptides are antimicrobial agents at micromolar concentrations (5), and it was initially thought that their only biological activity was in non-oxidative, non-enzymatic, destruction of phagocytosed microorganisms. More recently, however, we have shown that corticostatins have potential regulatory functions, including the ability to inhibit the action of the hormone adrenocorticotropin on glucocorticoid secretion (2,6,7) and to stimulate nifedipine-sensitive L-type Ca^{2+} channels in villus enterocytes (8). It has also been reported that a human defensin is a monocyte chemotactic agent (9). Other granulocyte-associated peptides have also been shown to have regulatory activities. For example, hemoregulatory peptide 1 is a granulocyte-associated thiol containing pentapeptide, with potent inhibitory actions on myelopoiesis (10). Several groups have reported the existence of immunomodulatory or cytokine-like activities associated with neutrophil extracts or supernatants (11,12,13,14). These activities include mast cell degranulation, chemotaxis, and the inhibition of myelopoetic-colony formation. Despite these reports, and the evidence for regulatory actions associated with known granulocyte peptides, few systematic attempts to characterize the regulatory molecules of the granulocyte seem to have been made. Granulocyte-enriched extracts contain several cystine-rich components at levels approximately three orders of magnitude lower than the defensin/corticostatins. From their compositional analysis and chromatographic behaviour these peptides appeared unrelated to any known hormone, including the defensin/corticostatins. In view of the potential role of granulocyte-derived peptides both as immunoregulatory molecules, and in host resistance, it was clearly important to characterize these peptides. Here we report the isolation and characterization a family of novel leukocyte-associated peptides that are cystine-rich, of approximately 6 Kda, and that may be cytokines.

Materials and Methods

4.1 Tissue Sources: Blood was taken from healthy volunteers, prepared and fractionated using Ficoll-Hypaque (Pharmacia, Upsalla, Sweden) as previously described (6). Differential counts were obtained from the Hematology Department, Royal Victoria Hospital. For structural studies the first wash peritoneal exudate from patients with peritonitis was used as a source of leukocytes. Typically, this comprises from 75 to 95% neutrophils. Crude granule preparations were made by lysing the cells in Hank's buffered saline solution using a Cole Palmer Ultrasonic Homogeniser 4710 Series. Preparations were inspected visually under a microscope to ensure complete cell lysis. The cellular debris was pelleted by centrifugation at 500 x g for 10 mins, and the supernatant inspected to ensure the complete removal of broken cells. The supernatant was then pelleted by centrifugation for 20 minutes at 5000 x g, and the crude granule pellet washed twice in HBSS. The two human granule peptides, HP-1 and HP-4, and the granule enzyme lysozyme, were used as granule markers, and the cytoplasmic peptide thymosin-ß-4 was used as a marker for cytoplasmic contamination.

4.2 Extraction and Purification: Whole cell preparations or crude granule fractions were extracted by sonication using an acidic high-salt extraction medium as described elsewhere (15). The extract was then centrifuged at 2000 x g for 15 mins, and the pellets re-extracted. Pooled supernatants were then adsorbed on SepPak C_{18} cartridges (Waters Associates, Milford, Mass) and eluted in 5 to 10 ml 80% acetonitrile in 0.1% TFA and the eluate lyophilized. The SepPak eluate was fractionated by reversed-phase high preformance liquid chromatography (RP-HPLC) using a Waters C_{18} µBondapak column (7.8mm x 30cm) eluted over a three hour period using a gradient of 0 to 80% acetonitrile in 0.1% TFA throughout at an elution rate of 1.5ml min^{-1}. Aliquots of the eluted fractons were then screened by amino acid analysis.The fractions of interest were further purified by size-exclusion HPLC using two I-125 ProteinPak columns (Waters) connected in series, eluted isocratically in 40% acetonitrile in 0.1% TFA at 1 ml min^{-1} (16). Partially purified peptides were then purified to homogeneity using a second C-18 µBondapak HPLC column (3.9mm x 30cm), with a gradient of 10 to 40% acetonitrile in 0.1% TFA

throughout at 1.5 ml min^{-1} for 90 mins. The rat peptide was purified from the aspirated femural bone marrow of 50 Sprague-Dawley rats (Charles Rivers, St Constant, Quebec), and extracted directly as outlined above. SepPak eluates were fractionated on a C_{18} μBondapak column using a gradient of 4 to 48% acetonitrile in 0.1% TFA throughout over 1 hour at 1.5 ml min^{-1}. Fractions were screened by amino acid analysis, and granulin-like material further purified using the same column with a gradient of 20 to 40% acetonitrile in 0.13% HFBA throughout over one hour (15). Final purification was by size-exclusion HPLC as described above. The rat granulin was purified from the same tissue extracts which we used for the purification of the rat corticostatins/defensins see chapter II.

4.3 Amino Acid Analysis and Microsequencing: For amino acid analysis aliquots of the peptide were lyophilized in borosilicate glass tubes and hydrolyzed in an evacuated reacti-vial for 16 hours at 105°C with 6N HCl. Amino acid analysis was performed using a model 6300A Analyser (Beckman Instruments, Palo Alto, CA). For microsequence analysis purified peptides were reduced with 10 mM dithiothreitol, or 2-mercaptoethanol, in 8M guanidine-HCl, 1 mM EDTA, 0.25M Tris, pH 8.5 for 1.5 hours at 37°C and then pyridylethylated with 2 μl 4-vinylpyridine (Aldrich Chemicals) under the same conditions.

The S-pyridylethylated peptides were then purified using a gradient of 5% to 60% acetonitrile containing 0.1% TFA throughout over 60 minutes with an initial 40 minute isocratic stage at 5% acetonitrile to elute polymeric vinylpyridine side products. The derivatized peptides were then submitted directly to sequence analysis or further processed by enzymatic digestion. The amino acid sequence determinations were carried out with an Applied Biosystem gas-phase sequenator (model 470A) as described in (17) but using a sequence program adapted from Speicher (18). The resulting phenylthiohydantoin (PTH)-amino acids were analysed by RP-HPLC on the on line PTH-analyser (Applied Biosystem model 120A) and/or a stand alone Varian HPLC unit as described previously (17). The PTH-yields for each standard were normalized according to a PTH-NorLeucine internal standard while the initial and repetitive yields were obtained by linear regression from the yields of selected stable PTH-derivatives. Sequence analysis of rat granulin and

its fragments was undertaken at the McGill Peptide and Protein Sequencing facility located in the laboratory of Dr Michel van der Rest at the Shriners Hospital for Crippled Children in Montreal.

4.4 Enzymatic Digestion: S-pyridylethylated peptides were digested using trypsin (TPCK-treated, Sigma), chymotrypsin (Sigma), and *S. aureus* V8 protease (Sigma), at enzyme to substrate ratios of approximately 1 to 50 by weight. Digestions were performed at 37°C in 100µl 50mM ammonium bicarbonate buffer, pH 8.3 for 3 hours, and terminated by the addition of 1ml of 0.1% TFA. The proteolytic fragments were then fractionated by RP-HPLC on a C_{-18} µBondapak column using a gradient of 0 to 40% acetonitrile in 0.1% TFA throughout over 60 mins at 1.5ml min^{-1}. Fractions were collected, aliquots removed for amino acid analysis, and then stored frozen at -80°C.

Results

The RP-HPLC profile of a typical extract of human inflammatory cells is shown in Figure 1. In addition to HP-1 and HP-4, several low abundance components are present. On the basis of amino acid composition analysis of these components we identified three low abundance components that had unusually high levels of cystine. These are labelled A, B, and C/D, and were present in both whole cell extracts (Fig 1B) and crude granule preparations (Fig 1A). Each of these extracts was then further purified using size-exclusion HPLC, revealing that the component C/D contained two peptides, one of which, D, eluted as a larger molecule than the other three. Each peptide was further purified on RP-HPLC. Their amino acid compositions are given in Table 1. The purified peptides were S-pyridylethylated, and amino terminal sequence analyses were performed, revealing that the four peptides were distinct but related molecules with no homology to any known protein. Because these peptides were associated with the granule fraction, we call them granulins A, B, C, and D. Granulin D was run on reducing and non-reducing SDS-PAGE, and ran as a smaller molecule after reduction, indicating that it is probably a dimer. Only one peptide was recovered after S-pyridylethylation of granulin D suggesting that it is a homodimer. However, until a full sequence is determined we cannot

exclude the possibility that it is a heterodimer of closely related subunits. The rat defensin/corticostatins elute in the same region of the chromatogram as the granulins (19). Rat granulin (marked with a bar in Fig 3A) was purified to apparent homogeneity by a further RP-HPLC step using HFBA as the counterion (Fig 3B) and subjected to structural analysis essentially as described for the human peptides.

Granulin A is the most abundant of the human granulins, and was chosen for more detailed analysis. S-pyridylethylated peptide was digested with trypsin, chymotrypsin or *S. aureus* V8 protease. The fragments were isolated by HPLC, one fifth aliquots analysed by amino acid analysis, and appropriate fragments were then submitted to gas phase Edman microsequencing. Final recovery of the digestion products was between 150 and 300 picomoles. The overlap of the granulin A fragments is described in the legend to figure 4 together with similar data for the rat granulin. The two sequences are highly conserved, as would be expected for regulatory molecules. Inflammatory exudates and bone marrow preparations are mixtures of cells, the exudates containing typically 70 to 95% granulocytes. When leukocytes from the blood of healthy donors was fractionated by density gradient centrifugation, granulins could be detected in the granulocyte pellet, but not in the interface where the mononuclear cells partition (data not shown).

Discussion

In this report we have described the isolation and characterization of a novel family of leukocyte associated cystine-rich peptides, which we call granulins. We have sequenced one human granulin, A, and identified three other human granulins, B,C, and D. We have partially sequenced a fifth granulin isolated from rat bone marrow . The most striking feature of their primary sequence is the high content of oxidised cystine, over 20%, suggesting that the secondary structure of the granulins are an essential determinant of their biological activity. This is supported by evidence of rigid evolutionary constraint on their structures; the rat partial sequence is almost identical with human granulin A .

When the sequences were entered into the National Biomedical Research Foundation PIR data bank no homologies were found with other proteins, indicating that granulins are a novel polypeptide family. After the research reported here was completed,

however, two amino terminal sequences were published, epithelin 1 and 2, isolated from the rat kidney, which are homologous with the granulins. The kidney peptides are putative cytokines that have growth inhibitory and stimulatory properties on some epithelial cells *in vitro* (20). Rat granulin and the reported amino terminal sequence of epithelin 1 differ at only one residue; epithelin lacks the amino terminal glutamyl residue of rat granulin A. At present it is not certain if both peptides are the product of the same, or different genes. It is also too early to determine whether the epithelins are intrinsic to renal cells, or if they are derived from blood borne cells trapped in the kidney. It is clear, however, that the two peptides from the kidney are members of a larger family, the granulins, and that a major source of the granulins, and probably also the epithelins, is from circulating leukocytes.

Rat granulin was isolated from bone marrow, indicating that granulins are of myeloid origin. Whether granulins are also synthesized in circulating leukocytes remains to be determined. Granulins were extracted from granulocyte rich preparations, and are recoverable from the granulocyte pellet after Ficoll-Hypaque density gradient centrifugation (data not shown). This suggests that their cellular origin may be the neutrophil, however we cannot exclude the possibilty that other granulocytes such as eosinophils, or contaminating monocytes, contribute to the granulin content of these extracts. Extracts of human platelets contained no detectable granulins (data not shown). It is possible that each granulin belongs to a distinct cell type, or that sub-classes of the same cell-type contain different granulins. These are issues best answered using immunolocalization procedures. The granulins co-purify in a crude granule extract. Granulocytes have several different granule subclasses, including a true secretory compartment (21) that can be activated independently of phagocytosis. The availability of suitable immunoassay techniques will allow us to unambiguously locate the granulins to a cell type, and a subcellular compartment.

In conclusion, the granulins are a novel family of cystine-rich immunoinflammatory peptides. Their presence in circulating leukocytes and inflammatory exudates, and their structural similarity with the rat epithelins (20), suggests possible roles in inflammation, wound repair, and tissue remodeling.

Acknowledgements

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Table 1. The amino acid compositions of purified granulins. The recovery of cystine in this system is variable, and is between 65 to 80%. Values have not been corrected for background contamination or oxidation. Tryptophan was not determined. Predicted values for granulin (GRN) A from the gas-phase sequence determinations are given in brackets.

Amino Acid	GRN A	GRN B	GRN C	GRN D	Rat GRN
ASX	5.7 (5)	6.5	5.2	5.4	4.0
THR	4.9 (5)	2.5	2.6	2.9	5.1
SER	2.3 (2)	5.1	6.2	4.6	1.2
GLU	7.4 (6)	3.4	8.1	7.3	5.6
PRO	3.2 (3)	5.6	5.0	4.5	2.4
GLY	5.7 (5)	3.7	4.8	6.2	5.5
ALA	2.8 (3)	2.0	1.9	4.5	2.7
CYS	7.0 (12)	7.2	7.2	9.1	8.2
VAL	3.0 (3)	1.9	2.9	2.2	2.2
MET	0.7 (1)	1.5	0	0	0
ILE	0.95 (1)	1.0	0.85	0.8	0.7
LEU	1.1 (1)	3.7	1.0	2.6	3.2
TYR	0.9 (1)	1.0	1.1	1.0	1.1
PHE	2.0 (2)	0	0	0	1.7
HIS	2.5 (2)	1.7	2.8	3.6	2.3
LYS	2.0 (2)	2.3	0	1.8	1.9
ARG	1.3 (1)	2.3	1.0	3.0	1.7

Figure 1 Purification of human granulins A, B, C and D. Figure 1A shows the HPLC chromatogram of a crude granule extract from inflammatory exudate cells, and B shows the chromatogram of a whole cell extract. The position of the granulins are marked by arrows. Note the absence in A of thymosinß-4, a cytoplasmic marker peptide. The granule peptide markers HP-1 and HP-4 were identified as previously described (6), lysozyme was identified by amino terminal sequence analysis (unpublished). Thymosinß-4 and its oxidation product were identified by Fast Atom Bombardment mass spectrometry (unpublished).

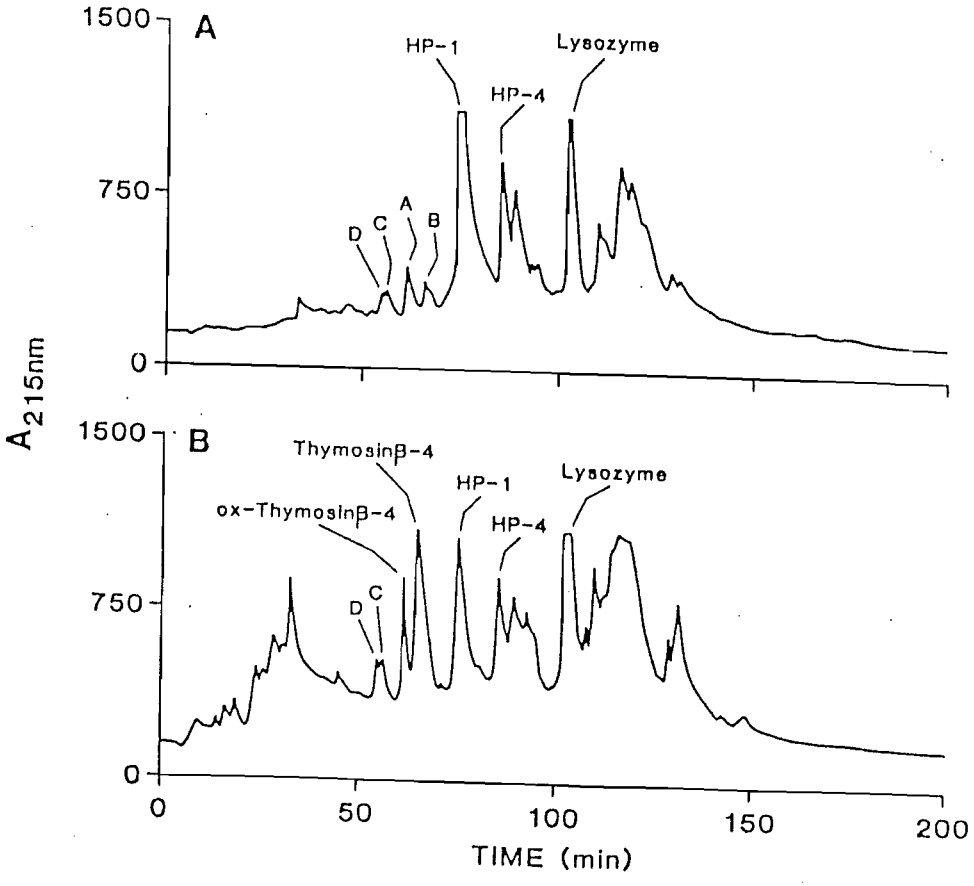
1500
A
HP-1
Lysozyme
HP-4
750
D
C
A
B
0
A_{215nm}
1500
B
Thymosinβ-4
HP-1
Lysozyme
ox-Thymosinβ-4
HP-4
750
D
C
0
0
50
100
150
200
TIME (min)

Figure 1.

Figure 2 Size-exclusion purification of granulin A, (panel A), granulin B, (panel B), and granulins C and D, (panel C). Size markers were substance P, CLIP, and $ACTH_{1-39}$. Apparent m.wts for native granulin A, 2700; granulin B,3200; granulin C, 1700; and granulin D, 3900.

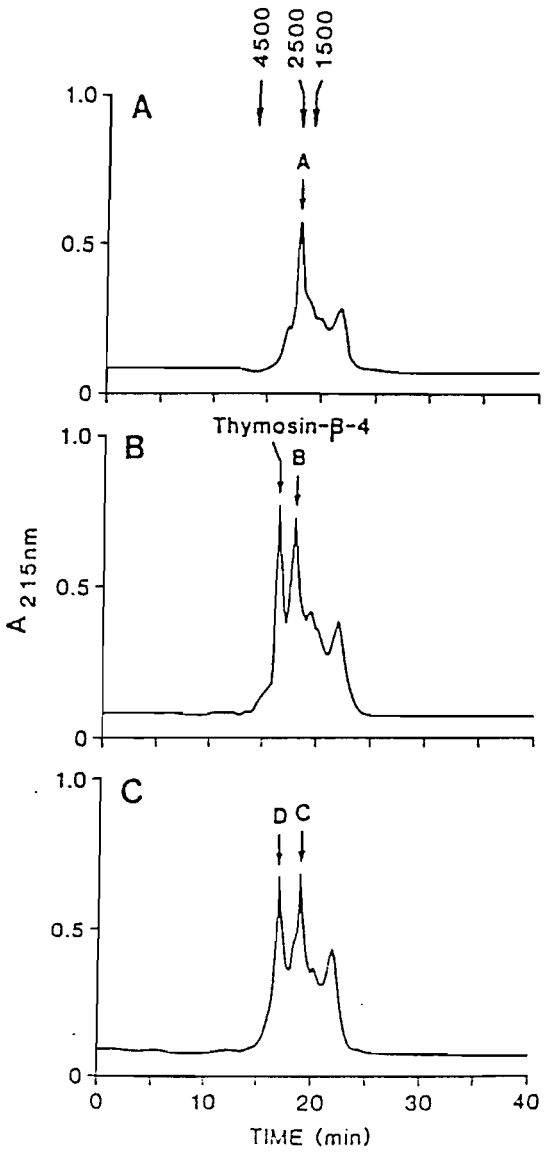
4500
2500
1500
A
1.0
0.5
0
A
Thymosin-β-4
B
1.0
0.5
0
B
A 215nm
C
1.0
0.5
0
D C
0
10
20
30
40
TIME (min)

Figure 2.

Figure 3 Purification of rat granulin from bone marrow. Panel A shows the first HPLC chromatogram in acetonitrile/TFA, and B shows the second step of purification in acetonitrile/HFBA. The bar in A corresponds to the region where the rat granulin elutes, and its elution position in B is marked with an arrow.

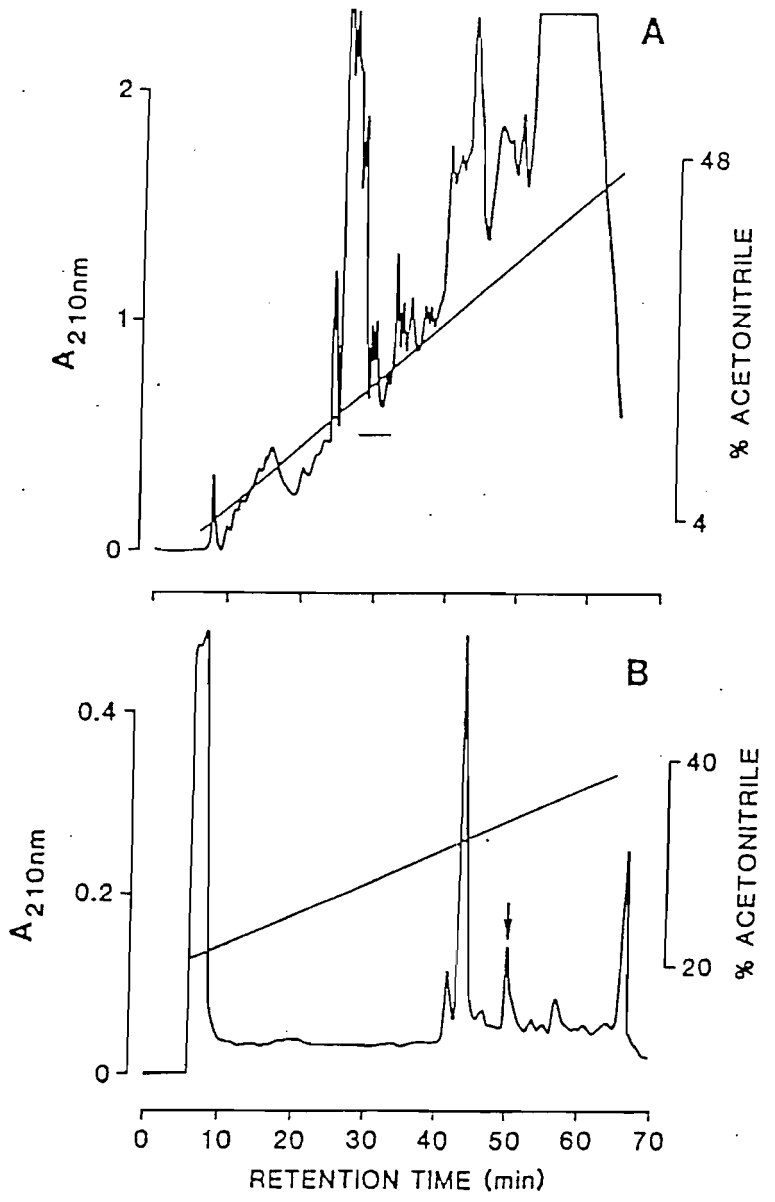
A
B
2
1
0
0.4
0.2
0
A_{210nm}
48
4
40
20
% ACETONITRILE
0 10 20 30 40 50 60 70
RETENTION TIME (min)

Figure 3.

Figure 4 Structural analysis of five members of the granulin family. The proposed structure for granulin A was determined by overlapping two amino terminal sequences, (1-11), and (1-23); tryptic fragments (4-18), (19-52), and V8 protease fragment (37-56), and chymotryptic fragment (47-56). The proposed structure of rat granulin is based partly upon the direct sequencing of the peptide itself (i.e. the sequence 1 through 21) and its proteolytic fragments (i.e. fragments corresponding to 4 to 18, 19 to 31, 32 to 46, and 32 to 51 sequences). Alignment of the fragments is based upon the clear homology of rat granulin with human granulin A. The sequence of residues 26 to 31 (in parenthesis) could not be determined and the sequence shown is based upon the amino acid composition of fragment 19 to 31. This fragment is a major tryptic cleavage product. No evidence of arginine was found, however it is possible that the fragment contains a lysine residue which co-elutes with pyridylethyl-cysteine in the amino acid analysis system. B signifies either aspartic acid or asparagine, and X signifies an unassigned residue.

Granulin A: DVK**C**-DMEVS**C**PDGYT**CC**RLQ SGAWG **CC**PFTQ AV**CC**EDHIH**CC**PAGFT**C**DTQKGT**C**E

Rat Granulin: EVK**C**-DL EVS**C**PDGYT**CC**RLNTGAWG(**CC**PFSB)AV**CC**EDHIH**CC**PAGFT**C**XTQ.....

Granulin B: VM**C**P DARSR**C**PDGHT**CC**ELPSGK YG **CC**P.....

Granulin C: VP**C**-DXV SS**C**PSSDT**CC**QLTSGEHG **CC**PIPEAV**C**....

Granulin D: I G**C**DQXDTSS**CC**PDG....

CHAPTER IV

ISOLATION AND PRIMARY STRUCTURE OF THE THREE MAJOR FORMS OF GRANULIN-LIKE PEPTIDES FROM HEMATOPOIETIC TISSUES OF A TELEOST FISH (*CYPRINUS CARPIO*)

Preface

The preceding chapter discussed the isolation and partial structural characterization of rat and human granulins, a novel class of cystine-rich polypeptides. This chapter presents the full structural characterization of three granulins purified from tissues of a teleost fish, the carp. These peptides are found in the fish hematopoietic tissues (i.e. spleen and head kidney) and are synthesized in abundance in these tissues. These prototypic forms of granulin constitute excellent models for structural and biological studies.

Abstract

Granulins are a new family of growth modulatory peptides recently purified from hematopoietic tissues of various species. Rat and human granulin have been structurally characterized (Bateman, A., Belcourt, D., Bennett, H.P.J., Lazure C. and Solomon S. (1990) Biochem. Biophys. Res. Commun. 173, 1161-1168) and the cDNA encoding human preprogranulin has been cloned and sequenced (Bhandari, V., Palfree R.G.E. and Bateman A. (1992) Proc. Nat. Acad. Sci. U.S.A. 89, 1715-1719). The homologous rat structures called epithelins have been isolated from kidney extracts and shown to have growth modulatory activities upon epithelial cells in culture (Shoyab, M., McDonald, V.L., Byles, C., Todaro, G.J. and Plowman, G.D. (1990) Proc. Nat. Acad. Sci. 87, 7912-7916). Granulins are 57 residue polypeptides having 12 cysteines arranged in a novel structural array of four pairs flanked by two singletons towards the amino- and carboxyl-termini. A prototypic form of these growth modulatory peptides was isolated from the hematopoietic organs of two teleost fishes, the goldfish (Carassius auratus) and the carp (Cyprinus carpio) using reversed-phase high performance liquid chromatography techniques (Belcourt, D. and Bennett, H.P.J. (1988) J. Cell Biol. 107, 629a). We now report the primary sequences of three structurally related forms of granulin isolated from extracts of the spleen and head kidney of the carp. Ion spray mass spectrometric analysis revealed the molecular weights of granulin-1, -2 and -3 to be 6276.8, 6523.1 and 6302.9 daltons, respectively. These mass determinations corroborated the observed primary sequences and demonstrated that each peptide was monomeric in nature with all cystines linked via intramolecular disulfide bridges. A rabbit polyclonal antibody was raised against carp granulin-1 to develop a radioimmunoassay for this peptide, which showed no significant cross reactivity with granulin-2 and -3. The distribution of carp granulin-1 was studied by screening purified tissue extracts for immunoreactive peptides. A single form of immunoreactive granulin-1 was identified in all carp tissues studied including spleen, head kidney, heart, skin, gills and gut. These studies have established that members of the granulin/epithelin family are found in a lower vertebrate and may serve important growth modulatory functions throughout the vertebrate kingdom.

Introduction

While investigating whether teleost fishes synthesize homologs of the defensin family of antibacterial peptides found in hematopoietic tissues of mammals, we identified a novel cystine-rich polypeptide. This polypeptide was found in abundance in the spleen of both goldfish and carp. Partial sequence analysis revealed a novel array of multiple pairs of cysteines (1). We speculated that the cells that synthesize this cystine-rich peptide might be located within the melanomacrophage centres found in the hematopoietic tissues of teleost fish. These structures play an important role in the immune response of fish and function as clearing units for invading organisms, antigen presentation and possibly act as germinal centres for different classes of lymphocytes (2-4). Since this new class of peptide was found in the hematopoietic tissues of carp, we investigated the nature of low molecular weight peptides found in the equivalent tissue in rats. Whole rat bone marrow was extracted and screened for the presence of peptides rich in cystine (5,6). A rat homolog of the carp polypeptide was identified (5). A parallel study of the nature of cystine-rich polypeptides found in the granule fraction of human peritoneal neutrophils, yielded further members of this peptide family (5). These peptides were named granulins to indicate the organelle from which they were isolated upon subcellular fractionation of human neutrophils. Sequence analysis revealed that the structures of the rat and human granulins were homologous to that found in carp especially with respect to the position of cysteine residues. Coinciding with the characterization of the rat and human granulins, epithelin 1 and 2 were purified from extracts of rat kidney and shown to have pleiotropic growth modulatory effects upon cultures of epithelial cells in vitro. Both epithelin 1 and 2 were found to inhibit growth of cultured A431 tumour cells while epithelin 1 stimulated the growth of murine keratinocytes in primary culture (7). The amino-terminal sequence of epithelin 1 was identical to that of rat granulin, but lacked the amino-terminal glutamic acid. This homology suggested possible biological functions for the granulin family of peptides as modulators of cell growth. Recently the human granulin cDNA has been cloned and sequenced and found to contain seven structurally related granulin domains encoded within a common precursor (8). The rat and mouse epithelin preprosequences have also been cloned and shown to contain tandem repeats of seven granulin-like

domains (9).

This paper presents the work that has been completed on the isolation of the major forms of carp granulin and their structural characterization. The amino acid sequences of carp granulin-1, -2 and -3 are presented together with their amino acid compositions and determined molecular masses. A polyclonal antibody to carp granulin-1 has been raised in rabbits and used to study its tissue distribution.

Materials and Methods

4.1 Tissue extraction and peptide purification: Carp (Cyprinus carpio), approximately 5 to 7 pounds, were purchased at a local fish market (Waldman's, Montreal, Quebec, Canada) and goldfish (Carassius auratus) tissues were generously supplied by Dr. J. Fryer of the Department of Anatomy, University of Ottawa. The spleen and head kidney were dissected from the fish, minced and dispersed in phosphate buffered saline (PBS, pH 7.2) by repeated aspiration into a 10 ml plastic syringe fitted with an 18 gauge needle. Large aggregates were allowed to settle under gravity and discarded. The cell and matrix suspension was collected and centrifuged at 3000 rpm for 15 minutes. Dispersal of cells and matrix was repeated three times with fresh PBS. At each step the deeply pigmented supernatant was discarded. Microscopic examination revealed that the final pellet contained macrophage-like cells attached to a fibrous matrix. Trypan blue exclusion showed 95% cell viability.

The harvested cells and associated matrix were extracted in 35 ml of acidic extraction medium consisting of 1M HCl containing 5% formic acid (v/v) 1% NaCl (w/v) and 1% TFA (v/v) using a Polytron tissue homogenizer (Brinkmann, Mississauga, Ontario, Canada) and centrifuged at 3000 rpm for 15 minutes (10). The pellets were re-extracted with an equal volume of the acidic medium and the combined supernatants were subjected to reversed-phase enrichment using octadecylsilyl-silica C_{18} SepPak cartridges (Waters Associates, Milford, MA) as described previously (10).

The purification of carp granulins was achieved by reversed-phase high performance liquid chromatography (RP-HPLC) using a Waters Associates liquid chromatography system consisting of two Model 510 high performance pumps and an

automated gradient controller. Column eluates were monitored simultaneously for UV absorbance at 210 nm using a Waters Model 450 variable wavelength detector and at 254 or 280 nm using a Model 330 Beckman fixed wavelength detector (Beckman Instruments, Palo Alto, CA). All chromatography was performed using Waters C_{18} μBondapak RP-HPLC columns which were eluted with linear gradients of increasing acetonitrile (as indicated in the legends to Fig. 1 and Fig. 2) over one hour at a standard flow rate of 1.5 ml per minute. All RP-HPLC solvents and reagents were prepared as described previously (10). Peptides were purified using three RP-HPLC steps employing 0.1% trifluoroacetic acid (TFA), 0.13% heptafluorobutyric acid (HFBA) and finally 0.1% TFA throughout as counterion according to a previously published protocol (10). All fractions were screened using amino acid analysis and the regions with the highest cystine content were purified to apparent homogeneity. Fractions rich in cystine were subjected to final purification by gel permeation HPLC using two Waters I-125 columns connected in series (11). The gel permeation HPLC columns were loaded using a reversed-phase trace enrichment technique (12) and eluted isocratically using 40% acetonitrile containing 0.1% TFA as described previously (11).

4.2 Amino acid analysis: Peptides were subjected to vapour hydrolysis using 6N HCl containing 1% phenol, at 105°C (in vacuo) for 18 hours using a Waters Pico-Tag work station. Tryptophan estimation was done using a modified liquid hydrolysis method essentially as described (13). Briefly, samples of 5 μg of peptide were hydrolyzed in 10 μl of 3 N mercaptoethanesulfonic acid (Pierce Co., Rockford, Il) for 18 hours at 105°C (in vacuo), followed by addition of 3 μl of 10 N NaOH to adjust the pH to 4.5. All hydrosylates were analyzed using a Beckman System 6300 automated amino acid analyzer.

4.3 Pyridylethylation of cysteine residues: Pyridylethylation of peptides was achieved according to a previously published procedure (14). Peptides were solubilized in a 1 M Tris HCl, 8 M guanidinium chloride buffer containing 4 mM EDTA at pH 8.5. The solution was saturated with nitrogen and the disulfide bonds were reduced with

10 mM 2-mercaptoethanol for 1 hour at 37°C. This was followed by the addition of 4 µl of 4-vinylpyridine, under nitrogen atmosphere and the reaction was allowed to proceed for 1 hour at 37°C. Pyridylethylated cysteine (PE-Cys) derivatives were purified by RP-HPLC using a linear gradient of 8 to 48% acetonitrile over one hour, containing 0.1% TFA throughout. Successful derivatization was assessed by monitoring absorbance at 254 nm due to the alkylated cysteines and confirmed by amino acid analysis of column fractions. All subsequent reactions and sequencing were undertaken using the pyridylethylated derivatives of the carp granulin peptides.

4.4 Cyanogen bromide (CNBr) cleavage: The PE-Cys derivative of carp granulin-1 was dried and resolubilized in 80% formic acid. A crystal of CNBr was added to the tube which was flushed with nitrogen and cleavage allowed to proceed overnight at room temperature (14). Fragments were purified by RP-HPLC using a gradient of 8 to 48% acetonitrile over one hour, containing 0.1% TFA throughout.

4.5 Endoproteinase Asp-N cleavage: Asp-N enzyme sequencing grade (Sigma Chemical Co., St. Louis, MO.) was used as described by the manufacturer. The enzyme was dissolved in double distilled water to yield a 50 mM Tris HCl buffer solution, pH 7.5 containing 2 µg of enzyme/100 µl. This was added to 20 µg of the PE-Cys derivatives of granulin-1, -2 and -3. The digestions were allowed to proceed overnight at 37°C. Fragments generated by cleavage at aspartic acid residues were purified by RP-HPLC using a gradient of 0 to 48% acetonitrile over one hour, containing 0.1% TFA throughout.

4.6 Microsequencing of carp granulin-1, -2 and -3: Sequencing of the PE-Cys derivative of granulin-1 and of its CNBr and Asp-N cleavage fragments, were undertaken using automated Edman degradation using an Applied Biosystems 470A gas-phase sequenator (Applied Biosystems, Foster City, CA), located at the Shriner's Hospital for Crippled Children, Montreal, Quebec. The structure of granulin-1 was confirmed by solid phase microsequencing using a Sequelon Aryl Amine membrane (MilliGen/ Biosearch, Millipore Corp.). This membrane consists of a polyvinylidene difluoride

(PVDF) matrix which has been derivatized with aryl amine groups. The peptide was dissolved using 20 µl of a 0.1% TFA, 30% acetonitrile solution and applied to an aryl amine disc placed on a Mylar sheet and heating block at 55°C. After drying, the reaction was activated using 5 µl of a 0.1 M (2-N-morpholino) ethansulfonic acid, 15% acetonitrile solution containing 10 mg/ml of (1-ethyl-3[3-dimethylaminopropyl]) carbodiimide, pH 5.0 at room temperature for 20 minutes. The coupled PE-Cys derivative of granulin-1 was then sequenced on a 6600 Milligen ProSequencer System located at Milligen/Bioresearch, Milford, MA using methods previously described (15). Sequencing of carp granulin-2 was performed using an Applied Biosystems 470A gas phase sequenator located at the Clinical Research Institute of Montreal using methods described previously (16). Carp granulin-2 was sequenced essentially as described for granulin-1 with the exception that the CNBr cleavage was performed directly on a Sequelon Aryl Amine membrane and the amino- and carboxyl-terminal fragments were sequenced simultaneously. The two C-terminal fragments obtained from Asp-N digestion of granulin-2 coeluted upon HPLC purification and were sequenced simultaneously. The PE-Cys derivative of carp granulin-3 was covalently attached to a Sequelon Aryl Amine membrane as described above and subjected to solid phase sequencing using an Applied Biosystems 471A gas phase sequenator located at the Shriner's Hospital, Montreal, Quebec. The structure for carp granulin-3 was completed by sequencing two carboxyl-terminal Asp-N cleavage fragments as described previously (16).

4.7 Ion-spray mass spectrometry (IS-MS): Ion spray mass spectra of purified granulin-1, -2 and -3 were obtained using an API III triple stage mass spectrometer with ion spray interface (SCIEX, Thornhill, Ontario, Canada) located at the Montreal Biotechnology Research Institute of the National Research Council of Canada. Briefly, lyophilized peptide samples are redissolved in 10% acetic acid and infused through a stainless steel capillary (100 µm internal diameter) at a flow rate of 1 µl per minute. A stream of air (pneumatic nebulization) is introduced to assist in the formation of submicron droplets (17). These droplets are evaporated at the interface by nitrogen gas, producing a series of multiply charged ions which are detected by the analyzer. Simple

algorithms correlate the charges produced by peptides and proteins to their molecular weights. The mass to charge ratio (m/z) of each of these ions produces a molecular weight estimate. These estimates were averaged to give an observed molecular weight. Each sample was scanned approximately 25 times over 188 sec, each scan being added to the next.

4.8 Development of a radioimmunoassay for carp granulin-1: Granulin-1 was isolated from the spleens of 12 carp essentially as described above. This yielded a total of approximately 3 mg of peptide which was conjugated to 3 mg Keyhole Limpet Hemocyanin (KLH, Sigma) using 0.25% (vol/vol) glutaraldehyde (Pierce) at room temperature for 2 hours. The reaction was stopped by the addition of 0.2 M lysine (Sigma). The conjugated peptide was stored frozen until used for immunization. Polyclonal antibodies were raised in rabbits according to a protocol approved by the McGill University Animal Care Committee. Two New Zealand White rabbits (Reimen Fur Ranch, St. Agatha, Ontario, Canada) were injected every 4 weeks and blood was tested 2 weeks after each injection. The primary injection consisted of 500 µg of KLH-conjugated granulin-1 emulsified in Ribi adjuvant System (R-730) with 0.5 mg Cell Wall Skeleton, according to the manufacturers instructions (Ribi Immunochem Research, Hamilton, Mo). A total of 1 ml per rabbit was injected, 300 µl intraperitoneally and 350 µl intramuscularly in each of the two hind legs. Booster injections were given using 250 µg of conjugated granulin-1 and Ribi adjuvant (R-700) without Cell Wall Skeleton at 4, 8 and 12 weeks. Bleedings were done by inserting an 18 gauge needle in the mid vein of the ear and 20 ml samples were withdrawn in glass tubes, allowed to coagulate overnight at 4°C and centrifuged to remove the clot. Serum was frozen at -20°C.

Carp granulin-1 was radiolabeled according to previously described methods (18). Dried peptide (5 µg) was solubilized with 4 µl of $Na^{125}I$ (Amersham, 5mCi/10 µl) and 40 µl of 0.1 M phosphate buffer, pH 7.4. The reaction was activated by the addition of 10 µl of chloramine-T (1.7 mg/ml) for 10 seconds and was stopped using 20 µl sodium metabisulfite (0.8 mg/ml). The radio-iodinated peptide was purified using a pre-column chromatography method as described previously (19). Antisera plus ^{125}I granulin-1 and

unlabelled carp granulin-1 standards or samples were mixed in appropriate dilutions and allowed to incubate for 2 hours at room temperature in a 50 mM PBS (pH 7.2) containing 0.1% bovine serum albumin. The free and bound ^{125}I carp granulin-1 were separated using 50 mM phosphate buffer containing 0.5% charcoal and 0.05% Dextran and centrifuged at 3000 rpm for 15 minutes. Bound ^{125}I granulin-1 contained in the supernatants was measured by an LKB 1277 Gamma Master gamma counter (Fisher Scientific, Montreal, Quebec) and quantified by an LKB RiaCalc program. Distribution studies were performed using analytical RP-HPLC analysis of carp and goldfish tissue extracts as described above. HPLC fractions were tested in duplicate in the RIA for granulin-1.

Results

5.1 Purification and structural characterization of carp granulin-1, -2 and -3.

Carp spleen, like its mammalian equivalent, is heavily pigmented and composed of dense connective tissue. Upon dispersal of this tissue in PBS, we could not obtain free cells but the resulting mixture consisted of a collagenous matrix together with strongly adherent macrophage-like cells which were 95% viable. During this dispersal procedure large amounts of soluble dark red pigment were discarded. Pilot studies had indicated that this pigment interfered with HPLC procedures. The cell plus matrix mixture was extracted and subjected to a reversed-phase enrichment procedure employing C_{18} SepPak cartridges (10). An initial RP-HPLC purification step was employed using 0.1% TFA as counterion (Fig. 1A). Upon amino acid analysis of individual fractions, a major peak (marked by an arrow, Fig. 1A) containing the highest molar ratio of cystine was observed and subjected to further chromatography. Our purification protocol capitalized on the overall charge of the peptides. By changing the counterion from 0.1% TFA to 0.13% HFBA, basic molecules could be resolved from less basic, neutral and acidic peptides (10). Figure 1B shows the second step of purification using this counterion. Carp granulin-1, the peptide of interest, was retained longer on RP-HPLC in this system thus resolving it from less basic peptides. Figure 1C shows the third step of the purification of granulin-1 (designated GRN1) in which 0.1% TFA was again used as counterion. A final

purification step consisting of gel permeation HPLC yielded a peptide that, upon further chromatography by RP-HPLC maintained a constant amino acid composition (data not shown). The amino acid composition of granulin-1, the major form of granulin isolated from the spleen, is shown in Table 1 and features a cystine content of approximately 20%, a lack of lysine residues and the presence of five histidine residues.

While the spleen extracts yielded only one major form of granulin, three peptides were isolated from carp head kidney, namely granulin-1 and two other forms granulin-2 and -3 (designated GRN-2 and GRN-3 in Fig 2). Figure 2 shows the purification of the major head kidney forms of granulin using RP-HPLC. All forms of granulin coeluted in the first step of purification (indicated by the arrow in Fig. 2A). Granulin-2 and -3 behaved differently in the HFBA system which is a reflection of these peptides having greater overall basic charges relative to granulin-1. The amino acid compositions for the head kidney peptides are shown in Table 1 where they can be compared to that of granulin-1 derived from spleen. All forms of carp granulin lack lysine and have a high cystine content of approximately 20%, however granulin-2 and -3 have a greater arginine content. Using a shallow gradient of acetonitrile, granulin-2 was resolved from granulin-3 by RP-HPLC containing 0.1% TFA as counterion (Fig. 2C). Table 1 shows the relative yields for each of these peptides. While granulin-1 is the major form isolated from the spleen, it is apparent that granulin-3 predominates in the head kidney. The determined masses for these peptides (see Table 1) confirmed that granulin-1 is the same peptide in both tissues and that granulin-2 and -3 are distinct but related peptides only found in the head kidney. All peptides were found to have masses in the 6 kDa range indicating their monomeric nature with all cystines linked via intramolecular disulfide bridges.

5.2 Primary sequencing of carp granulin-1, -2 and -3: All three forms of granulin were subjected to pyridylethylation prior to sequencing or fragmentation. This consistently produced one peak upon RP-HPLC which absorbed strongly at 254 nm (data not shown). Amino acid analysis of this material clearly indicated that all cystines were reduced and alkylated as indicated by the presence of pyridylethyl-cysteine (PE-Cys) and the complete absence of cystine residues (data not shown). Reduction and

pyridylethylation was necessary in order to obtain fragments from either CNBr or Asp-N cleavage. The CNBr cleavage at methionine gave rise in each case to two fragments which are consistent with the presence of one methionine residue in each peptide. The Asp-N cleavage of granulin-1 generated three fragments, one derived from the amino-terminus and two others from the carboxyl-terminus. The amino-terminal fragment coeluted with the carboxyl-terminal fragment upon RP-HPLC (data not shown) and were sequenced simultaneously. The sequencing data for granulin-1 is presented in Table 2 as the yield of the phenylthiohydantoin (PTH) amino acid observed at each cycle and summarized in Figure 5A. Confirmation of the granulin-1 sequence using a solid-phase sequencing approach, is also shown in Table 2 and Figure 5A as N-term[a]. Structural analysis of the carp granulin-1 using an Aryl Amine membrane and the solid phase sequencing technology employed by the Milligen 6300 ProSequencer yielded the most sequencing information with very little carry over evident during consecutive cycles. The extended repetitive yield which is possible with solid phase sequencing allowed 47 amino acids to be sequenced and provided an overlap between the two CNBr fragments of granulin-1 (shown in Fig. 5A). The low yield of aspartic acid found at cycles 5, 12 and 37 observed during solid-phase sequencing was due to the fact that granulin-1 was covalently linked to the aryl amine membrane via the free carboxyl groups of these amino acid residues. The complete sequence for granulin-1 was confirmed by determining its molecular weight (Table 1). The peptide has a determined molecular mass of 6276.8 daltons which is consistent with the predicted mass of 6277.1 daltons, based upon the primary sequence and assuming that all cysteines are in the form of six intramolecular disulfide bridges. Granulin-1, isolated from the head kidney, was not sequenced but its amino acid composition, its determined molecular mass of 6276.4 daltons (see Table 1), and its chromatographic behaviour (see Fig. 2A and B) suggest that it is identical structurally to the spleen form of granulin-1, and is designated as GRN1 in Figure 2B and Table 1.

As shown, granulin-2 and -3 purified from head kidney had different physicochemical characteristics from granulin-1. Similar strategies were employed to sequence these two related peptides. Tables 3 and 4 give the sequencing data for granulin-

2 and -3 respectively, and show yields of PTH-amino acid at each Edman degradation cycle. CNBr and Asp-N fragmentation products were generated to obtain the complete structures for granulin-2 and -3 and are also presented in Tables 3 and 4, respectively. Figure 5A shows the complete sequences for both peptides obtained through solid phase sequencing of peptides and peptide fragments. The full structural analysis depicts complete sequences of 57 residues which have been confirmed by the determined molecular masses found in Table 1. The calculated masses took into account the primary structures found in Tables 3 and 4, and incorporating six intramolecular disulfide and 56 peptide bonds. The use of Sequelon Aryl Amine membranes with gas-phase microsequencing resulted in an extensive carry over of the arginine during sequencing. This effect is most likely due to poor extraction of the phenylisothiocarbamyl derivative of arginine from the membrane using the gas phase protocol. While the arginines in positions 43 and 47 of granulin-2 were not assigned during solid phase sequencing, they were subsequently confirmed by the sequencing of the Asp-N fragments shown in Table 3 and Figure 5A. The carboxyl-terminal arginine and the tryptophan residue 24 of carp granulin-2 have been assigned to these positions based upon the calculated and observed masses and the sequence homology to granulin-1 and -3.

5.3 Establishment of a radioimmunoassay for carp granulin-1: Granulin-1 was the most abundant form of granulin peptide found in the spleen and was chosen as the candidate peptide to establish a radioimmunoassay (RIA) for the study of tissue distribution using RP-HPLC and immunocytochemistry. Our polyclonal antibody raised in New Zealand white rabbits, increased in titer over a 14 week period. Preimmunization serum showed no binding of ^{125}I granulin-1 up to a 1:10 dilution of the serum. Sera collected at 2, 6, 10 and 14 weeks demonstrated increasing titer of antibody as assessed by binding of ^{125}I granulin-1, up to a maximum showing 30% binding at a serum dilution of 1:50000. Both rabbits had a similar increase in titer over the 14 week period (data not shown). A standard competition curve for the radioimmunoassay system is shown in Figure 3. The 50% binding intercept is 657 pg/ml granulin-1. The sensitivity limit for the RIA is 10-20 pg/ml granulin-1. No cross reactivity was detected for granulin-2 or -3 up

to a concentration of 10 ng/ml (data not shown).

A preliminary study of the tissue distribution of granulin-1 in carp and goldfish has been undertaken. Figure 4A is a representative analytical RP-HPLC chromatogram of an extract of carp spleen. This study revealed a single peak of immunoreactivity which coincided with the elution position of granulin-1. Complete purification of granulin-1 using the RIA as the basis for detection has been used successfully. The peak of immunoreactivity coeluted with the expected position of granulin-1 at each step of the purification (data not shown). No peaks of apparent higher molecular weight were detected using our RIA. The analytical RP-HPLC chromatogram for the carp head kidney (Fig. 4B) and the goldfish spleen (Fig. 4C) show a similar peak of immunoreactivity and again no higher molecular weight forms were detected. The total granulin-1 immunoreactivity detected in the spleen and head kidney of carp was 23.5 and 14.6 µg/g (wet weight), respectively. The same apparent form of immunoreactive granulin-1 was found in extracts of a variety of other carp tissues including heart (1.2 µg/g), skin (0.7 µg/g), gills (0.6 µg/g) and gut (0.8 µg/g).

Discussion

Studies on peptides originating from the hematopoietic system of teleost fish are not numerous. We report the isolation and structural characterization of three novel peptides from acidic extracts of carp spleen and head kidney. From the primary sequences shown in Tables 2-4 and Figure 5A, it is clear that these carp peptides are part of the granulin family. These 57 residue polypeptides are characterized by a novel motif consisting of four pairs of cysteine residues flanked towards the amino- and carboxyl-termini by four single cysteine residues. In comparing the three carp structures, there is an overall identity of 75%. Granulin-2 and -3 show the highest degree of identity (89.5%). The sequence variations occur primarily in the amino-terminal region of the carp granulins. Some of these conservative changes most likely have little effect upon overall structure or function. Mass determinations using ion-spray-mass spectrometry (IS-MS) corroborated these carp granulin structures (Table 1). The determined molecular masses for each peptide are consistent with that predicted from the primary structure information

assuming that all cysteine residues are cross-linked to form six disulfide bridges. However, the masses do vary between granulin-1, -2 and -3, partly because of the different number of arginines present in each peptide. This results in different predicted overall charges for granulin-1, -2 and -3 which have 1, 6 and 2 positive charges, respectively.

The cloned sequences for the mammalian granulin (8) and epithelin (9) precursor predict a total of seven different granulin- or epithelin-like structures organized in tandem repeat within the same precursor molecule. Since the carp structures are very similar to each other but generated in different relative yields within the spleen and head kidney, there could be a tissue specific expression of different granulin-related genes. Since this teleost fish species is tetraploid (20) we might expect several related structures arising from at least two different genes, each encoding multiple structures similar to those predicted for the sequences of human and rat progranulin (8,9).

Initial studies suggested that the granulins originate from macrophage-like cells judging from the morphology of the cells that predominate in the dispersed spleen and head kidney tissue. Granulin-1, the first peptide to be isolated from the spleen, was chosen as a model for distribution and localization studies. Our polyclonal antibody is specific for granulin-1 and does not cross react significantly with granulin-2 and -3. The epitope recognized by the antibody has not been determined, but most likely resides in the amino-terminal portion of granulin-1 since most of the sequence variations relative to granulin-2 and -3 occur in this region. Due to its specificity, our antibody proved useful in studying the tissue distribution of granulin-1. A single immunoreactive peak of granulin-1 was observed in extracts of all tissues studied (Fig. 4). High molecular weight forms of granulin-1 were not observed when tissue extracts were examined by RP-HPLC (Fig. 4). The antibody may not-cross react with the granulin precursor which might be expected to be present in the tissue extracts. Alternatively, carp pro-granulin may be efficiently processed in spleen and head kidney tissues and, therefore, the precursor would be absent from tissue extracts. The granulin-1 antibody is being used to study the histochemical localization of granulin-1. Immunocytochemistry indicates that granulin-1 is primarily of macrophage origin both in the spleen and head kidney and in peripheral

tissues (**Chapter V**)[2]. The widespread distribution of immunoreactivity is consistent with the localization of macrophages within these tissues.

Since epithelin 1 and 2 have been shown to possess growth modulatory effects (7), it is likely that carp granulins also have similar properties with respect to the proliferation of fish epithelial cells. Preliminary investigation of the properties of granulin-1, -2 and -3 have demonstrated that these carp peptides have a distinctive pleiotropic effect on different cell types (**Appendix I**)[3]. The carp granulins promoted [^{3}H]thymidine uptake by a human epithelial cell line (A-431) and a fish epithelial cell line (fat head minnow skin cells) whereas they inhibited thymidine uptake by a fish embryo cell line (salmon, CHSE-214). Interestingly, the rat epithelins 1 and 2 were found to inhibit proliferation of A-431 cells in culture (7) which is opposite to the effect we observed for carp granulins on this cell line.

Approximately four days after experimentally wounding carp, skin healing is characterized by an increase in mitogenic activity and a marked increase in populations of macrophages close to the site of injury (21). The localization of granulin-1 to skin macrophages in carp (**Chapter V**) suggests that carp granulins may play a role in wound repair either by inhibiting or by stimulating different epithelial cell types. There is also a possible role for granulins in optic nerve regeneration either by inhibiting oligodendritic cell growth (22) or by stimulating cells involved in optic nerve elongation (23). This mitogenic activity from carp non-neuronal cells was observed after a latency period of five to eight days post injury (22). Owing to the probable presence of carp macrophages following this experimental injury, we speculate that the carp granulins could account for this mitogenic activity.

The granulins, so-called because of their localization to the granule fraction of phagocytic leukocytes, are part of an extensive family of polypeptides sharing a novel cysteine motif (Fig. 5). Members of the family include rat and human granulins (5) and the closely related growth modulatory peptides epithelins 1 and 2 (7). The mouse, rat and human precursors encode 7 epithelin/granulin domains each bearing the following cysteine motif $X_{2\text{-}3}CX_{5\text{-}6}CX_5CCX_8CCX_6CCX_5CCX_4CX_{5\text{-}6}CX_2$ (8, 9). A search of protein and nucleic acid databases using the BLAST (24) E-Mail server offered by the National

Library of Medicine, NIH revealed the existence of several new members of this polypeptide family. The cDNAs for guinea pig and mouse acrogranin, an abundant protein found in the acrosome of the sperm head, encode structures which are highly homologous to the progranulin and proepithelin sequences of other mammalian species (25). Equine neutrophil antimicrobial peptide-1 (e-NAP-1) has been partially characterized and shown to be structurally related to the granulins and epithelins (26). Finally a cystine-rich polypeptide of unknown function purified from the brain of the locust has also been shown to have the characteristic cysteine motif of the granulin/epithelin family (27). The primary structures of the three teleost granulins together with homologous peptides from the rat and locust are shown in Figure 5. This comparison emphasizes the conservation of the cysteine motif and indicates that several other residues are common to each peptide. Comparison of these structures with those found within the rat, mouse, guinea pig, and human precursors shows that only aspartate 37 and histidine 40 are found in all forms of the granulin/epithelin family. This implicates the sequence -CCXDX_2HCC- as crucial to the function of these peptides. Proline residues are frequently found following the 2^{nd}, 6^{th}, and 10^{th} cysteines and may contribute to stabilizing the compact structure of these peptides. Similarly, glycine residues are frequently found between cysteine residues and probably facilitate peptide folding. The faithful conservation of the granulin-epithelin structure through vertebrate and most likely invertebrate evolution implies an important function for this new family of polypeptides.

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[1]**Data bank accession numbers:** Carp granulin-1, -2 and -3 have been submitted to the PIR data bank under the accession numbers A40180, B40180 and C40180, respectively.

[2]Belcourt, D.R., Okawara, Y., Fryer, J.N. and Bennett, H.P.J (1995) J. Leukoc. Biol., 57:94-100.

[3]Langille, R.M., Belcourt, D.R., Fryer, J.N.and Bennett, H.P.J., unpublished observation.

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Table 1 Amino acid compositions, yield and molecular mass determinations for carp granulin (F.GRN) -1, -2 and -3 compared to the rat granulin.

	Spleen	Head Kidney			
Amino Acid	F.GRN1	F.GRN1	F.GRN2	F.GRN3	RatGRN
Asx (B)	4.0 (4)[a]	3.9 (4)	3.0 (3)	3.1 (3)	(5)
Thr (T)	3.5 (5)	4.1 (5)	4.8 (6)	4.5 (5)	(5)
Ser (S)	2.3 (4)	2.3 (4)	2.5 (4)	2.6 (4)	(2)
Glx (Z)	1.2 (1)	1.1 (1)	1.6 (1)	1.3 (1)	(5)
Pro (P)	3.4 (3)	3.2 (3)	3.1 (3)	3.6 (3)	(3)
Gly (G)	4.6 (5)	5.2 (5)	4.8 (4)	6.3 (6)	(5)
Ala (A)	1.8 (2)	2.1 (2)	1.6 (1)	0.9 (1)	(3)
Cys (C)	11.1 (12)	10.9 (12)	9.4 (12)	11.6 (12)	(12)
Val (V)	0.7 (2)	1.2 (2)	2.4 (3)	1.5 (3)	(3)
Met (M)	0.9 (1)	0.8 (1)	0.9 (1)	1.0 (1)	(0)
Ile (I)	1.5 (3)	1.7 (3)	0.0 (0)	0.9 (1)	(1)
Leu (L)	2.0 (2)	1.9 (2)	4.6 (3)	3.1 (3)	(2)
Tyr (Y)	2.8 (3)	3.1 (3)	2.5 (3)	1.7 (2)	(1)
Phe (F)	0.9 (1)	0.9 (1)	2.1 (2)	3.1 (3)	(3)
His (H)	4.5 (5)	4.7 (5)	1.8 (2)	3.1 (3)	(2)
Lys (K)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	(3)
Arg (R)	3.0 (3)	2.9 (3)	7.4 (8)	5.1 (5)	(1)
Trp (W)[b]	≈1.0 (1)	≈1.0 (1)	≈1.0 (1)	≈1.0 (1)	(1)
Yield[c]	137 µg	60.2 µg	84 µg	207 µg	
Molecular mass (Da)					
Calculated[d]	6277.1	6277.1	6523.5	6303.2	6174.90
Observed[e]	6276.8	6276.4	6523.1	6302.9	6176.95

a) Values in parenthesis are the calculated number for each amino acid derived from the sequence information shown in Tables 2-4.

b) Tryptophan estimated as described under "Materials and Methods".

c) Yield of purified peptide from three teleost fish (*Cy. carpio*) estimated by amino acid analysis.

d) The calculated mass represents the sum of all observed amino acids in Fig. 5 taking into account 56 peptide and 6 disulfide bonds. The average monoisotopic mass for each amino acid was used in this calculation.

e) Mass obtained by ion-spray-mass spectrometry as described under "Materials and Methods".

Table 2 Yields for the phenylthiohydantoins which were obtained during microsequencing of carp granulin-1. The pyridylethylated peptide (N-term) or fragments generated by cyanogen bromide (CNBr→) and endoprotease (Asp-N→) were sequenced by gas-phase microseqencing as described under "Materials and Methods". PE-Cys, pyridylethyl-cysteine present but not quantified. [a]Solid-phase microsequencing using a Sequelon Aryl Amine membrane and a Milligen Prosequencer.

		Yield (pmoles)			
Residue	A.A.	N-term	CNBr	Asp-N	N-term[a]
1	Val	293	128	349	390
2	Ile	293	64	333	268
3	His	30	45	98	330
4	Cys	PE-Cys	PE-Cys	PE-Cys	PE-Cys
5	Asp	127	77		6.9
6	Ala	78	77		327
7	Ala	223	106		340
8	Thr	30	23		120
9	Ile	72	48		189
10	Cys	PE-Cys	PE-Cys		PE-Cys
11	Pro	168	31		152
12	Asp	65	37		3.8
13	Gly	69	19		96
14	Thr	35	8		63
15	Thr	46	10		88
16	Cys	PE-Cys	PE-Cys		PE-Cys
17	Cys	PE-Cys	PE-Cys		PE-Cys
18	Leu	23	9		100
19	Ser	8	3		62
20	Pro	33	6		47
21	Tyr	48	9		61
22	Gly	26	9		47
23	Val	40	4		51
24	Trp	5	4		10
25	Tyr	22			54
26	Cys	PE-Cys			PE-Cys
27	Cys	PE-Cys			PE-Cys
28	Pro	21			33
29	Phe	9			57
30	Ser				50
31	Met				41
32	Gly		CNBr→98		27
33	Gln		85		24
34	Cys		PE-Cys		PE-Cys
35	Cys		PE-Cys		PE-Cys
36	Arg		83		14
37	Asp		98		2
38	Gly		40		16
39	Ile		58		12
40	His		21		12
41	Cys		PE-Cys		PE-Cys
42	Cys		PE-Cys		PE-Cys
43	Arg		30		6
44	His		22		11
45	Gly		22		8
46	Tyr		21		10
47	His		18		6
48	Cys		PE-Cys		
49	Asp		27	Asp-N→218	
50	Ser		22	91	
51	Thr		9	130	
52	Ser		17	63	
53	Thr		17	95	
54	His		5	130	
55	Cys		PE-Cys	PE-Cys	
56	Leu		5	172	
57	Arg			73	

Table 3 Yields for the phenylthiohydantoins which were obtained during microsequencing of carp granulin-2. GRN-2, cyanogen bromide (CNBr→), and endoprotease (Asp-N→) fragments were sequenced as described under "Materials and Methods". PE-Cys, pyridylethyl-cysteine present but not quantified. [a]Solid-phase sequencing using a Sequelon Aryl Amine membrane and an Applied Biosystems Sequenator.

Residue	A.A.	Yield (pmoles) N-term[a]	CNBr	Asp-N
1	Val	78	35	
2	Val	101	90	
3	Tyr	69	44	
4	Cys	PE-Cys	PE-Cys	
5	Asn	57	31	
6	Ala	93	48	
7	Arg	23	34	
8	Thr	29	23	
9	Thr	32	21	
10	Cys	PE-Cys	PE-Cys	
11	Pro	32	28	
12	Ser	11	10	
13	Arg	17	24	
14	Thr	15	7	
15	Thr	13	8	
16	Cys	PE-Cys	PE-Cys	
17	Cys	PE-Cys	PE-Cys	
18	Arg	15	21	
19	Ser	7	11	
20	Pro	19	8	
21	Phe	22	9	
22	Gly	25	14	
23	Val	15	7	
24	Trp			
25	Tyr	6	3	
26	Cys	PE-Cys	PE-Cys	
27	Cys	PE-Cys	PE-Cys	
28	Pro	10	7	
29	Phe	8		
30	Leu	10		
31	Met	5		
32	Gly	8	CNBr→158	
33	Gln	6	158	
34	Cys		PE-Cys	
35	Cys		PE-Cys	
36	Arg		31	
37	Asp		21	Asp-N→80
38	Gly		116	39
39	Arg		39	25
40	His		7	13
41	Cys		PE-Cys	PE-Cys
42	Cys		PE-Cys	PE-Cys
43	Arg		28	21
44	His		14	10
45	Gly		83	27
46	Tyr		55	11
47	Arg			6
48	Cys		PE-Cys	PE-Cys
49	Asp		16	Asp-N→80
50	Ser		11	38
51	Thr		19	50
52	Ser		13	11
53	Thr		8	19
54	Leu		29	18
55	Cys		PE-Cys	
56	Leu		16	
57	Arg			

Table 4 Yields for the phenylthiohydrantoins which were obtained during microsequencing of carp granulin-3. GRN-3 and endoprotease (Asp-N→) fragments were sequenced as described under "Materials and Methods". PE-Cys, pyridylethyl-cysteine present but not quantified. [a]Solid-phase sequencing using a Sequelon Aryl Amine membrane and an Applied Biosystems Sequenator.

Residue	A.A.	Yield (pmoles) N-term[a]	Asp-N
1	Val	225	
2	Val	487	
3	Phe	308	
4	Cys	PE-Cys	
5	Asp	110	
6	Ala	384	
7	Gly	346	
8	Ile	318	
9	Thr	251	
10	Cys	PE-Cys	
12	Ser	87	
13	Gly	294	
14	Thr	113	
15	Thr	232	
16	Cys	PE-Cys	
17	Cys	PE-Cys	
18	Arg	56	
19	Ser	73	
20	Pro	192	
21	Phe	95	
22	Gly	199	
23	Val	186	
24	Trp	59	
25	Tyr	157	
26	Cys	PE-Cys	
27	Cys	PE-Cys	
28	Pro	137	
29	Phe	112	
30	Leu	165	
31	Met	128	
32	Gly	142	
33	Gln	100	
34	Cys	PE-Cys	
35	Cys	PE-Cys	
36	Arg	58	
37	Asp	35	Asp-N→182
38	Gly	83	89
39	Arg	22	177
40	His	17	61
41	Cys	PE-Cys	PE-Cys
42	Cys	PE-Cys	PE-Cys
43	Arg		90
44	His		62
45	Gly	37	114
46	Tyr	47	68
47	His		48
48	Cys		PE-Cys
49	Asp		Asp-N→115
50	Ser		19
51	Thr		12
52	Ser		10
53	Thr		38
54	Leu		81
55	Cys		PE-Cys
56	Leu		54
57	Arg		36

Figure 1. Reversed-phase HPLC purification of carp granulin-1 extracted from three spleens. Panel A: The tissue was subjected to reversed-phase extraction and loaded onto a C_{18} μBondapak column which was eluted over 1 hour at 1.5 ml/min with a linear gradient of 8 to 48% acetonitrile containing 0.1% TFA throughout. Material contained within fractions 23 to 30 (indicated by an arrow) was found to be rich in cystine and subjected to further purification. Panel B: The material of interest was reloaded onto the same HPLC column which was eluted over 1 hour at 1.5 ml/min with a linear gradient of 20 to 40% acetonitrile over 1 hour containing 0.13% HFBA throughout. Material contained within fractions 35 to 39 (designated ▬) was subjected to further purification. Panel C: Final RP-HPLC purification of carp granulin-1 (designated GRN1) was achieved using a gradient of 12 to 28% acetonitrile over 1 hour containing 0.1% TFA.

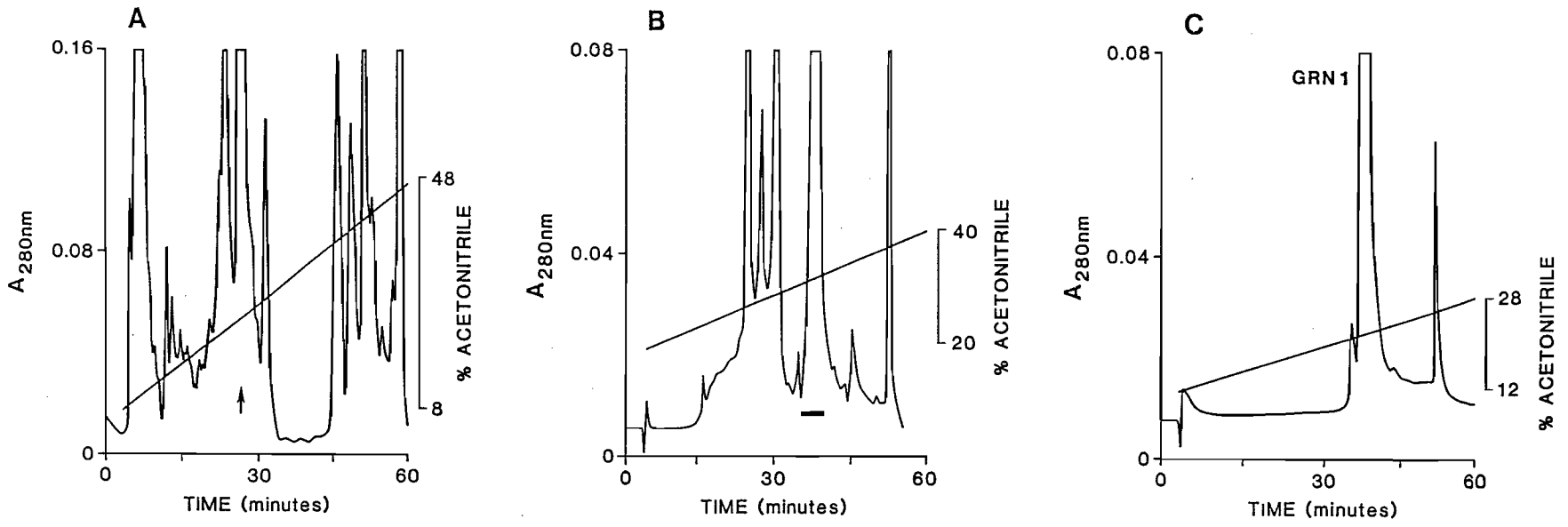

A
0.16
0.08
0
A_{280nm}
48
8
% ACETONITRILE
0
30
60
TIME (minutes)
B
0.08
0.04
0
A_{280nm}
40
20
% ACETONITRILE
0
30
60
TIME (minutes)
C
0.08
0.04
0
A_{280nm}
GRN1
28
12
% ACETONITRILE
0
30
60
TIME (minutes)

Figure 2. Reversed-phase HPLC purification of carp granulin-1, -2 and -3 extracted from three head kidneys. Panel A: The tissue was subjected to reversed-phase extraction and loaded onto a C_{18} μBondapak column which was eluted over 1 hour at 1.5 ml/min with a linear gradient of 0 to 50% acetonitrile containing 0.1% TFA throughout. Material contained within fractions 20 to 26 (indicated by an arrow) was found by amino acid analysis to be rich in cystine and subjected to further purification. Panel B: The material of interest was reloaded onto the same HPLC column which was eluted over 1 hour at 1.5 ml/min with a linear gradient of 20 to 40% acetonitrile over 1 hour containing 0.13% HFBA throughout. Granulin-1 (designated GRN1) within fractions 36 to 40 was purified seperately from the material contained within fractions 41 to 48 which contained granulin-2 and -3 (designated ▬). Panel C: Final RP-HPLC purification of granulin-2 and -3 (designated GRN2 and GRN3) was undertaken using a gradient of 15 to 35% acetonitrile over 1 hour containing 0.1% TFA.

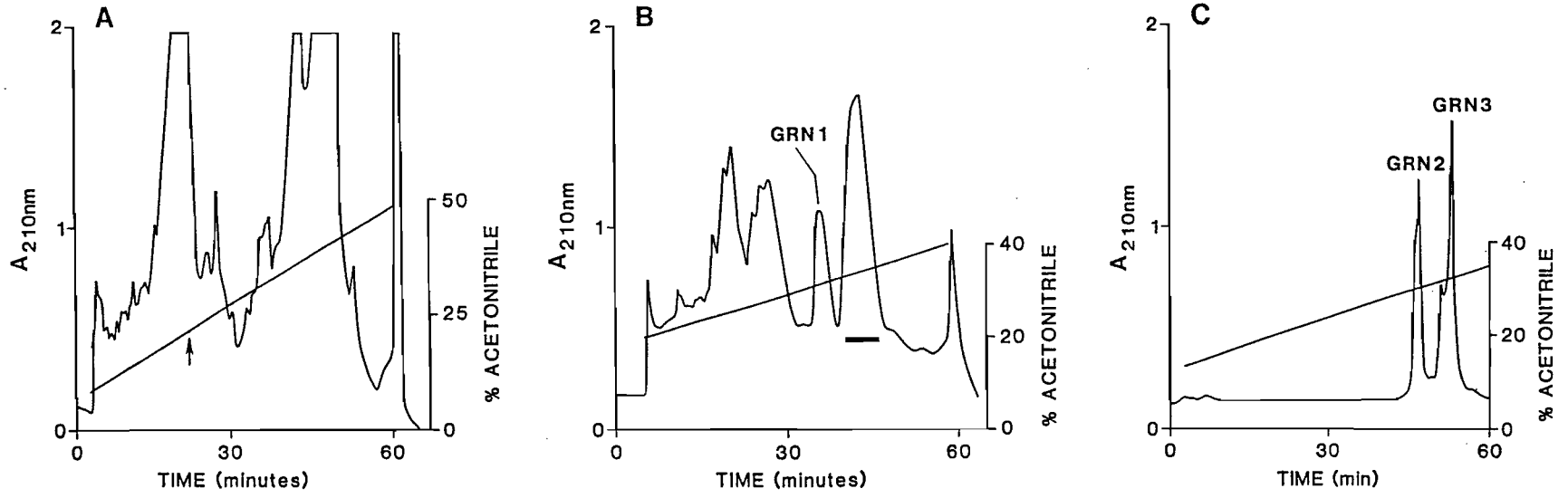

A
B
C
GRN1
GRN2
GRN3
A_{210nm}
% ACETONITRILE
TIME (minutes)
TIME (min)
0
1
2
30
60
25
40
50
20

Figure 3 Radioimmunoassay standard curve for carp granulin-1. B/B_o, Bound/free ratio. Results of individual experiments ± standard error is presented, (n=6). EC_{50} = 657 pg/ml; minimal detectable level of carp granulin-1 is 10-20 pg/ml.

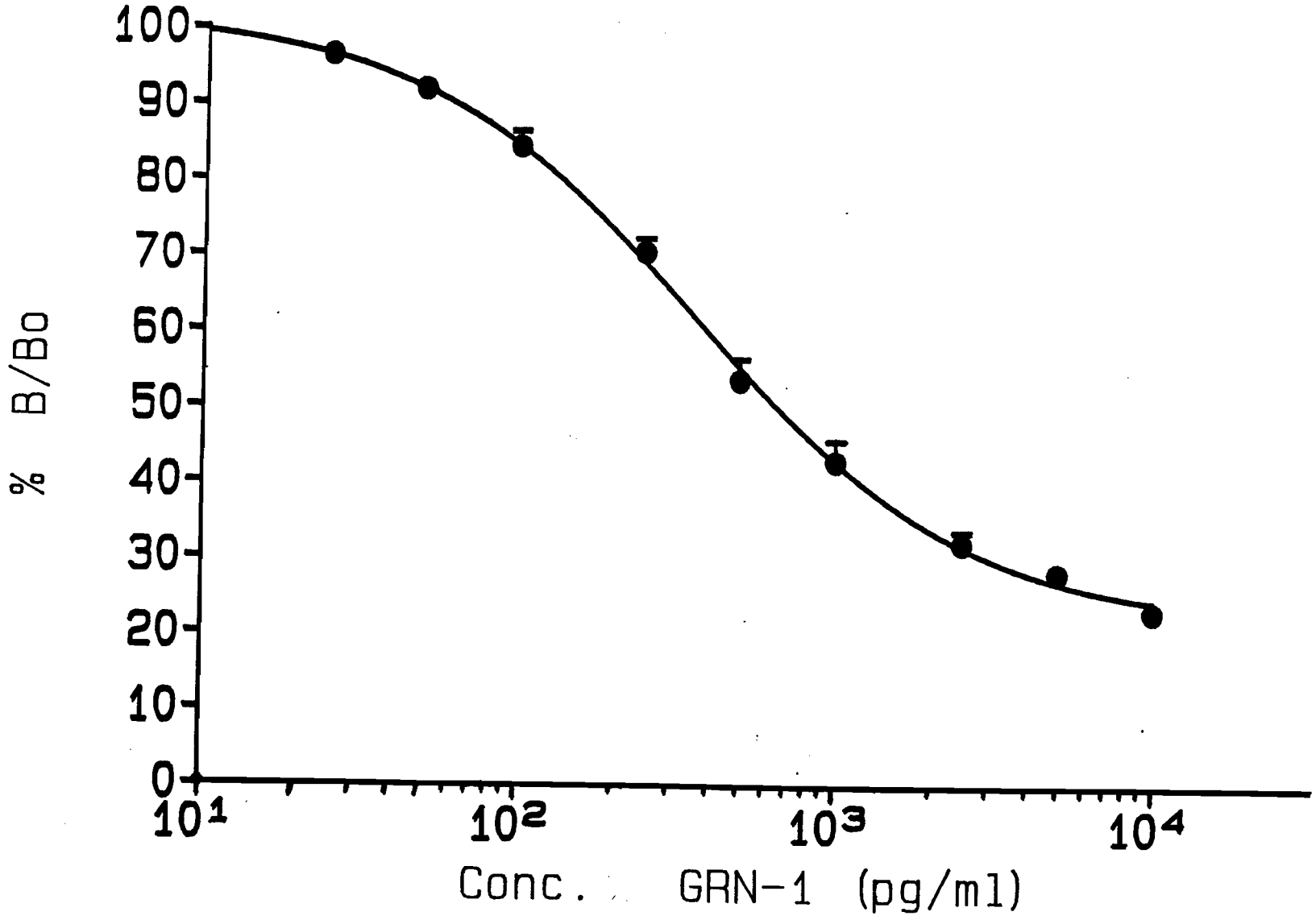

100
90
80
70
60
50
40
30
20
10
0
% B/Bo
10¹
10²
10³
10⁴
Conc. GRN-1 (pg/ml)

Figure 4 Immunoreactive detection of carp granulin-1 in spleen and head kidney of carp and spleen of goldfish. Carp and goldfish tissues were extracted and loaded onto a C_{18} μBondapak column as described in the Materials and Methods Section which was eluted over 1 hour at 1.5 ml/min with a linear gradient of 8 to 48% acetonitrile containing 0.1% TFA throughout. Panel A) carp spleen, Panel B) carp head kidney, Panel C) goldfish spleen. HPLC fractions were assayed in duplicate in the RIA for granulin-1 as described in the Materials and Methods section and results are expressed as μg of immunoreactive peptide per gram of tissue (wet weight).

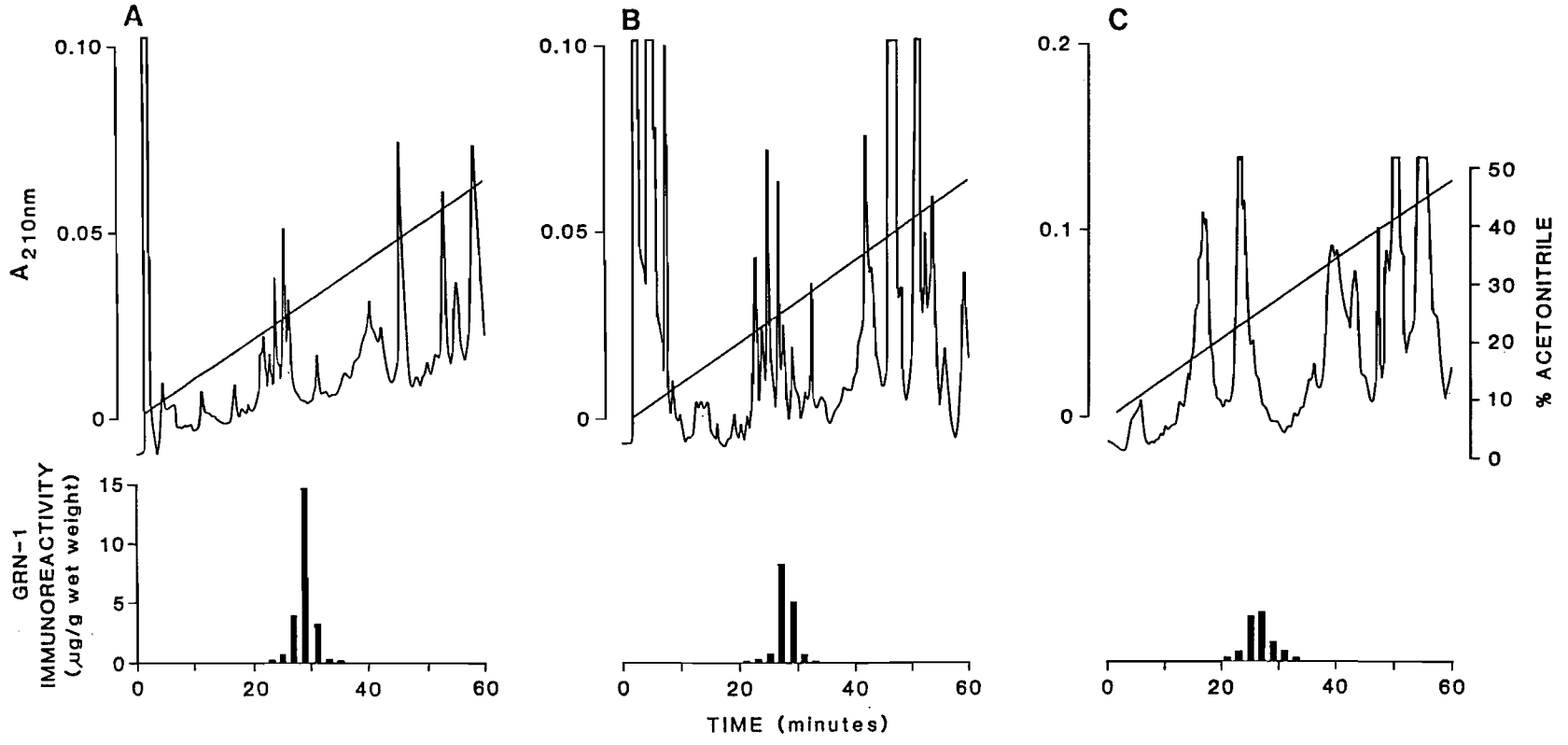

A
B
C
0.10
0.05
0
0.10
0.05
0
0.2
0.1
0
A 210nm
50
40
30
20
10
0
% ACETONITRILE
GRN-1
IMMUNOREACTIVITY
(µg/g wet weight)
15
10
5
0
0
20
40
60
0
20
40
60
0
20
40
60
TIME (minutes)

Figure 5 Primary sequences of carp granulin-1, -2 and -3[1] and their CNBr and AspN fragments, compared with the primary sequences of members of the granulin/ epithelin family and the locust peptide (PMP-D1). Sequencing was accomplished by automated Edman degradation as described in the Materials and Methods Section (see Tables 2, 3 and 4 for repetitive yields). Horizontal line (—) represents amino acids which have been positively identified. Bar (▮) represents a chemical or enzymatic cleavage site. Peptides were sequenced by conventional gas-phase microsequencing or by [a]solid phase sequencing using Sequelon Aryl Amine membranes. The consensus sequence shows amino acid residues common to the granulin/epithelin family.

A. Primary sequences of carp granulins (C.GRN):

B. Members of the granulin/epithelin family:

Rat GRN/EPN-1 EVKC-DLEVSCPDGYTCCRLNTGAWGCCPFTKAVCCEDHIHCCPAGFQCHTETGTCEL

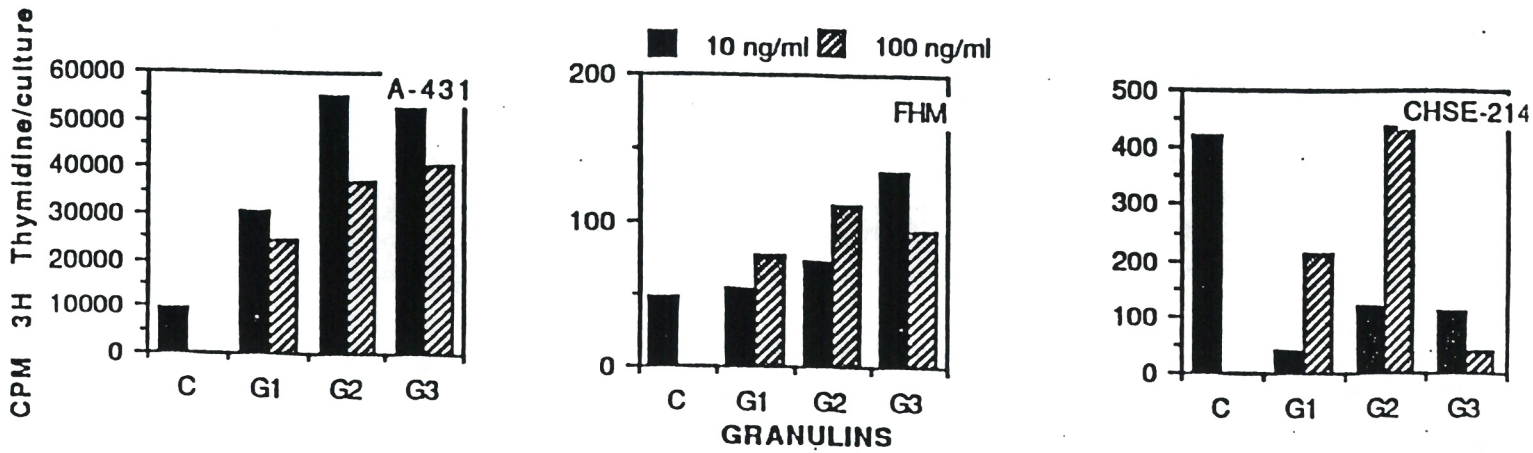
Rat EPN-2 VVCPDAKTQCPDDSTCCELPTGKYGCCPMPNAICCSDHLHCCPQDTVCDLIQSKCIS

LOCUST PMP-D1 SC--TEKTCPGTETCCTTPQGEEGCCPYKEGVCCLDGIHCCPSGTVCDEDHRRCIQ

Consensus --C------CP--- TCC---- G--- CCP ----- CC- D--HCC -----C ------ C--

Appendix I

Results of preliminary investigations of the effects of granulins on human A-431 and piscine cell lines. Cell lines A-431 (derived from human epidermis), FHM (derived from fat head minnow skin) and CHSE-214 (derived from salmon embryos) were obtained from the American Tissue Culture Collection and immediately, thawed and plated out at 2×10^5 cells/dish in DMEN, MEM or MEM ± 20 mM HEPES respectively (all media included 10% FCS and antibiotics). A-431 and FHM cells were incubated in 5% CO_2 at 37°C while CHSE-214 cells were incubated at 21°C in room air. The cultures were left overnight and the media changed the following morning to remove the cryoprotectants. After a further 12 hours, 10 or 100 ng of granulins-1 (G1), -2 (G2) or -3 (G3) were added to the medium. A-431 and FHM cells were allowed to grow for a further 24 hours. During the last 6 hours of culture, 2 µCi (A-431) or 10 µCi (FHM; very slow growing cells) of ^{3}H-thymidine was added to the medium. CHSE-214 cells were grown for a further day prior to the addition of 2 µCi ^{3}H-thymidine and the cells were exposed to this radioisotope for an additional 24 hours due to the lower incubation temperature. Granulins increased the uptake of ^{3}H-thymidine by the epithelial cells (A-431 and FHM) but reduced it in the salmon embryo cells (CHSE-214) in all but one case. The increase in proliferation was most dramatic with granulins-2 and -3 raising uptake 5 fold in A-431 cells and 2.5-3 fold in FHM cells. Uptake of ^{3}H-thymidine was reduced in CHSE-214 cells by 2 to 4 fold in all but one case.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10 ng/ml
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200
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0
FHM
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G1
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GRANULINS
500
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100
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CHAPTER V

IMMUNOCYTOCHEMICAL LOCALIZATION OF GRANULIN-1 TO MONONUCLEAR PHAGOCYTIC CELLS OF *CYPRINUS CARPIO* AND *CARASSIUS AURATUS*.

Preface

The preceding Chapters III and IV presented evidence for the existence of a novel class of peptides found in leukocytes of rats, human and the carp. We have stated that these novel peptides, designated granulins, were presumably found in granules of isolated leukocytes. However, no direct immunocytochemical localization had been performed in rats and humans due to the low abundance of the mammalian granulins. Determination of the cell type which stores growth modulatory polypeptides like the granulins is of fundamental importance in determining their *in vivo* biological activities. The high abundance of the carp granulins enabled us to isolate enough granulin-1 to generate rabbit polyclonal antibodies (see Chapter IV) and study tissue distribution of granulin-1 in carp and goldfish tissues. This chapter presents evidences that demonstrates direct immunolocalization of granulin-1 to granules in the cytoplasm of mononuclear phagocytic cells found in melanomacrophages of the two hematopoietic tissues of the teleost fish, namely the spleen and head kidney. This version of the manuscript shows light-micrographs in colour and these include some controls not shown in the published paper.

Abstract

Recently, a new class of low molecular weight cystine-rich regulatory growth factors, designated granulins, have been isolated from the hematopoietic tissues of a teleost fish (*Cyprinus carpio*) and structurally characterized (1). Granulin-1, the predominant form found in carp spleen was used to raise polyclonal antibodies in rabbits and to establish a radioimmunoassay for this peptide. This permitted preliminary tissue distribution studies of granulin-1 to be undertaken in carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*). We now report immunolocalization studies of granulin-1 in carp and goldfish tissues. Granulin-1 immunoreactivity was found in the melanomacrophage centers of the spleen and head kidney which also showed abundant endogenous acid phosphatase activity. Carp tissues anatomically involved in the first line of defense against infection including skin, gills, gut and also heart, showed intense granulin-1 immunoreactive staining within presumptive macrophage cells scattered throughout these tissues. Granulin-1 immunoreactive macrophages prepared from goldfish spleen and head kidney were found to adhere to glass slides, to actively phagocytose carbon particles, and were shown to contain granulin-1 immunoreactivity as well as abundant endogenous peroxidase activity. This study demonstrates that granulin-1 is synthesized and stored in macrophages/ monocytes of spleen, head kidney and peripheral tissues of teleost fish.

Introduction

With the exception of the pituitary hormones, growth hormones, prolactin and thyroid stimulating hormone, there are relatively few studies of proteinaceous factors with growth modulatory activities in lower vertebrates such as the teleost fish. We have previously described the primary sequences of three related granulins isolated from acidic extracts of the spleen and head kidney of carp (Cyprinus carpio) (1). These peptides are 57 residues in length, having 12 cysteines arranged in a novel structural array of four pairs flanked at amino- and carboxyl-termini by two singletons (see Fig. 1*A*). The three homologous carp granulin structures differ primarily within the amino- terminal region. They also vary with respect to their overall basic charge. Forms of rat and human granulins have also been structurally characterized (2). Peptides with related structures have also been identified in extracts of horse neutrophil granules (3) and locust brain (4). The structures of rat and human granulin A are compared with those of carp granulins-1, -2 and -3 in Fig. 1*B*. The extensive homology between the fish and mammalian structures is evident especially with respect to the position of cysteine residues. The cDNA encoding human preprogranulin has been cloned and sequenced (5) and the structure and chromosomal localization of the human granulin gene has been determined (6). Structural analysis indicates that the granulins are homologous to a new family of growth modulatory peptides known as epithelins that have pleiotropic effects on a number of epithelial cell types in culture. The rat epithelins isolated from kidney extracts have been shown to have growth modulatory activities when incubated with epithelial cells in culture (7). An epithelial cell surface receptor for epithelin has been partially characterized (8). The cDNA encoding preproepithelins from human, mouse and rat have also been cloned and sequenced (9). Both pre-progranulins and pre-proepithelins are encoded by a single gene and according to Northern blot analysis and in situ hybridization are found to be expressed in many different tissues and cell lines (5, 9, 10).

Factors related to the granulin/epithelin family have been characterized. A protein of unknown function corresponding structurally to progranulin has been purified from the mammalian sperm head (11, 12). It was named acrogranin to indicate its location within the acrosomal compartment. A potent autocrine growth factor structurally identical to

progranulin and acrogranin has been purified from culture medium conditioned by a highly tumorigenic mouse PC cell line (13). Structural analysis of transforming growth factor e (TGFe) purified from bovine kidney suggests that this mitogen for epithelial and fibroblastic cells is also part of the granulin/epithelin family (14). Finally extensive homology exists between the granulin motif (Fig. 1) and a cysteine-rich non-catalytic domain of a plant thiol protease (15). The significance of this structural similarity is not clear but it does emphasize the phylogenetically ancient nature of the granulin/ epithelin motif.

The work completed on the isolation and structural characterization of carp granulins indicated that these peptides are synthesized in cells possibly located within melanomacrophage centers of the spleen and head kidney. Numerous cell types are found in these tissues and constitute major components of the immune system, functioning as clearing units for invading organisms, antigen presenting centers and possibly acting as germinal centers for lymphocytes (16-20). Using antibodies raised against carp granulin-1, we demonstrate in the present study the presence of immunoreactive peptide within macrophage-like cells of carp and goldfish tissues.

Materials and Methods

4.1 Animals: Live carp were obtained from Waldman Fish Market, Montreal, Canada. Goldfish housed at the University of Ottawa were kept in a flow through tank supplied with aerated, dechlorinated fresh tap water at 15°C and fed daily.

4.2 Tissue preparation for immunocytochemistry: The spleen, head kidney (anterior kidney), skin (epidermis and dermis) from a scaleless head region, gills, heart and intestine were dissected from carp and goldfish and fixed using Bouins fixative containing 30% acetic acid for 2 hours. The tissues were washed in 50 mM phosphate-buffered saline (PBS)[1], pH 7.4, and post-fixed with Bouins fixative for a further 24 hours. Tissues were dehydrated in a graded series of ethyl alcohol, cleared with xylene and embedded in Tissue Prep (Fisher, Scientific, Montreal, Canada). Tissue sections of 6 or 7 μm were mounted on 3-aminopropyltriethoxysilane-coated slides (21).

4.3 Bouin's fixation of cryostat sections of goldfish tissue: Difficulties arose in obtaining specific immunostaining of Bouin-fixed spleen and head kidney, tissues from which we had originally isolated granulin-1. As an alternative, sections (5 µm) of goldfish spleen were cut in a cryostat (Bright Instrument Company, Cambridge, UK) at -25°C, thaw mounted onto glass slides and fixed using Bouin's fixative for 10 minutes.

4.4 Macrophage isolation: Macrophages from spleen and head kidney of goldfish were isolated essentially as previously described (22). Briefly, tissues were dispersed in Leibovitz L-15 medium (Gibco, Grand Island, NY) supplemented with 100 I.U. penicillin-streptomycin (Gibco) by mincing into small pieces with a razor blade and aspirating in and out of an 18 gauge needle attached to a 10 ml syringe. Cell aggregates were allowed to settle and discarded. Suspended cells were washed three times by repeated dispersal in L-15 medium followed by centrifugation at 1000 rpm for 10 minutes. The cells were 95% viable as indicated by trypan blue exclusion. Cell number was adjusted to 5 x 10^4 cells/ml by the addition of L-15 medium. For culture, 2.5 x 10^4 cells in 500 µl were seeded onto 14 mm glass coverslips, placed in 6 well culture dishes (Linbro, Flow Labs, McLean, VA) and incubated at 10°C for 24 hours. Adherent cells were washed and incubated for 18 hours at 4°C with fresh L-15 medium alone or L-15 medium containing 0.2% (v/v) India ink. The cells were then washed three times with 50 mM Tris HCl buffered saline (TBS), pH 7.5 unless otherwise indicated, followed by fixation in 2% paraformaldehyde (w/v) / 0.5% picric acid (w/v). To facilitate visualization of phagocytosed carbon particles and cell structure, slides were counterstained using Wrights stain.

4.5 Hematopoietic tissue imprinting: Spleen and head kidney smears were prepared by cutting approximately 1 mm cubes of tissues and smearing them between two glass slides. Smears were immediately immersed in cold 2% paraformaldehyde / 0.5% picric acid and left overnight at 4°C.

4.6 Electrophoresis: Whole antisera and immunoglobulins (IgG) fragments were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) as described previously (23). The electrophoresis was performed on a Bio-Rad mini-gel system (Bio-Rad Labs., Richmond, CA) under non-reducing conditions in order to maintain the integrity of the IgG antigen binding domain fragments [$F(ab')_2$]. The gels were then fixed in 50% methanol / 30% acetic acid (v/v) and stained with Coomasie blue in a 10% methanol / 5% acetic acid solution (v/v).

4.7 Isolation, fragmentation and biotinylation of the rabbit anti-granulin-1 polyclonal antibody: A rabbit polyclonal antibody to granulin-1 was obtained as described previously (1). Briefly, purified granulin-1 isolated from the spleens of 12 carp was conjugated to keyhole limpet hemocyanin and injected in two New Zealand white rabbits. The primary injection consisted of conjugated granulin-1 (500 µg/ml per rabbit) while secondary and subsequent injections consisted of 250 µg/ml of granulin-1 conjugate.

Serum containing the anti-granulin-1 antibody was used to generate purified IgG using a previously described method (24). Briefly, 4 ml of serum was diluted to 8 ml with 0.1 M TBS, pH 7.5 and passed over a 1 ml bed of Protein A agarose (Bethesda Research Laboratories, Gaithersburg, MD). The agarose was step eluted with 0.1 M sodium citrate buffer of decreasing pH (pHs 6, 4.5 and 3). Fractions of 1 ml were collected into 300 µl of 1 M TBS, pH 8, to bring the pH to 7.5. The protein content of each eluate was determined using a Bio-Rad protein determination kit. The IgG fraction which eluted at pH 3 was subjected to SDS-PAGE as described (23) and tested for its affinity for ^{125}I granulin-1 as described previously (1).

This purified IgG fraction was fragmented using pepsin to generate $F(ab')_2$ fragments as described previously (25). The $F(ab')_2$ fragment was separated from the IgG constant domain (Fc) fragment using a 2cm X 1m column of Sephadex G-100 (Pharmacia LKB Biotech. Inc., Piscataway, NJ) and 50 mM TBS, pH 7.5. Fractions were assessed for protein content and those containing protein were subjected to SDS-PAGE.

Biotinylation of $F(ab')_2$ fragments was performed using a previously described method (26) and as specified by Enzo Biochem, Inc. (New York, NY). The $F(ab')_2$

fragments were dialysed overnight against 0.2 M sodium bicarbonate, pH 8.2. Enzotin (N-biotinyl-w-amino caproic acid-N-hydroxy succinimide ester) was added to a final concentration of 0.2 µg/ml in 1 mg/ml solution of $F(ab')_2$ fragment in 0.1 M sodium bicarbonate, pH 8.2. The reaction was allowed to proceed for 4 hours at 22°C, followed by the addition of 30 µl of a solution of 20 mg/ml L-lysine (Sigma) to inactivate the biotin linker. The $F(ab')_2$ fragment was dialysed overnight in a 100 mM phosphate buffer, pH 7.4, and stored at 4°C.

4.8 Immunocytochemistry: Rehydrated, Bouin-fixed sections or cryostat sections were equilibrated in 50 mM TBS, pH 7.5. Non specific binding was reduced by washing sections for 1 hour in 50 mM TBS containing 0.6% carrageenin (w/v) (Sigma), 0.1% Triton X-100 (v/v) (Sigma) and either 2% bovine serum albumin (BSA) (w/v) or 2% carp serum (v/v). Slides were washed three times with 50 mM TBS and incubated overnight at room temperature with rabbit anti-granulin-1 antiserum (1:400 or 1:800 dilution). Control slides were incubated with antiserum preabsorbed for 1 hour with 4 µg/ml granulin-1. The slides were washed three times in 50 mM TBS and the secondary antibody consisting of a 1:100 (v/v) dilution of biotinylated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL) was placed on the tissue sections for 1 hour. After three washes in 50 mM TBS, a 1:100 (v/v) solution of streptavidin-horseradish peroxidase (Amersham) was placed on the slides for 1 hour. Visualization was accomplished by incubating the slides with 3,3'-diaminobenzidine (10µg/50ml) and H_2O_2 (4.5µl/50ml) in 50 mM TBS for 10 to 12 minutes whereupon a brown precipitate appeared on the sections. Slides were washed extensively in water, dehydrated with ethanol, cleared with xylene and mounted with Permount (Sigma).

Immunocytochemical localization of granulin-1 in smears and isolated macrophages from spleen and head kidney was performed using purified biotinylated anti-granulin-1 $F(ab')_2$ fragments and the avidin-ß-galactosidase enzyme detection system (Pierce Co., Rockford, IL). Slides or coverslips with adherent macrophages were blocked with 3% crystalline grade BSA (w/v) and 0.1% Triton-X 100 (v/v) in 50 mM TBS, pH 7.5 for 1 hour to lower non-specific binding. The slides were incubated with purified

Biotinylated $F(ab')_2$ fragment for 2 hours in the same blocking buffer and then washed three times with 50 mM TBS and incubated for an additional hour with 100 µl of avidin-ß-galactosidase diluted 1:100 (v/v) in 50 mM TBS containing 0.3% BSA (w/v). The slides were washed three times with 50 mM TBS and incubated with 4 µg/ml 5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal, Pierce) in 50 mM TBS at 22°C for 18 hours. This resulted in a blue precipitate on the sections. Control slides were incubated with biotinylated $F(ab')_2$ fragment preabsorbed for 1 hour with 4 µg/ml granulin-1.

4.9 Endogenous acid phosphatase: Acid phosphatase is a lysosomal enzyme with a pH optimum of 3.8 to 6 usually associated with the lysosomes of phagocytic cells. An azo coupling method (27) was used to detect this endogenous acid phosphatase activity. Briefly, cryostat sections of goldfish spleen were fixed for 10 minutes in Bouin's fixative and equilibrated in 50 mM TBS followed by addition of 500 µl of a naphthol AS-MS phosphate (100 µg/ml) and pararosaniline (600 µg/ml) solution in 0.1 M acetate buffer, pH 5.0 for 15 minutes to 1 hour until a red precipitate appeared. The slides were washed with water, counterstained with Fast green, dehydrated and mounted as described above for the immunocytochemistry.

Results

5.1 Granulin-1 antiserum and generation of a biotinylated $F(ab')_2$ fragment: A rabbit polyclonal antibody for carp granulin-1 was used with routine immunoperoxidase staining procedures. To reduce non-specific immunostaining, biotinylated anti-granulin-1 $F(ab')_2$ fragments were prepared from purified IgG. Purification of the polyclonal antibody using Protein A Sepharose was monitored using SDS-PAGE which was also used to confirm that pepsin digestion had cleaved the IgG, to generate $F(ab')_2$ and Fc fragments. The $F(ab')_2$ fragment purified by Sephadex G-100 gel permeation chromatography was shown by SDS-PAGE to be a homogeneous band at 125 kDa and to contain no contaminating Fc fragments at 26 kDa. Anti-granulin-1 biotinylated $F(ab')_2$ fragments retained their ability to bind ^{125}I granulin-1 (data not shown).

5.2 Immunocytochemical localization of granulin-1 in Bouins fixed, paraffin embedded tissues: We were unable to observe positive immunostaining in the spleen and head kidney from carp or goldfish in tissues prepared with Bouins fixative and paraffin embedding. These negative results were contradictory in view of our previous work showing that these tissues are a major source of bioactive and immunoreactive granulin-1 (1). Positive staining was obtained using spleen cryostat sections (see later). Bouins fixed paraffin sections demonstrated positive immunocytochemical staining only in a number of carp tissues such as skin, gills, heart and gut. Typical observations for the epidermis of carp is presented in Figure 2, demonstrating granulin-1 immunoreactivity contained within irregularly shaped cells resembling macrophages or monocytes. The cell diameter was estimated at 10 to 15 μm. Adjacent sections of skin epidermis dissected from the carp cranium devoid of scales is shown in Fig. 2*A* and *B*. Figure 2*A* is a control slide where the anti-granulin-1 polyclonal antibody was preabsorbed with granulin-1. Figure 2*B* represents the granulin-1 immunoreactivity in two morphologically distinct cell types. The first type is irregular in shape (Fig. 2*C*) and scattered within this tissue (arrow, Fig. 2*B*) with some cells being closely associated with club cells and unicellular mucous cells (Fig. 2*B*, arrow head). A second elongated cell type (▌, Fig. 2*B* and Fig. 2*D*) has morphological characteristics of cells apparently moving through the tissue with an oval shaped eccentric nucleus positioned at one pole of the cell. The distribution of granulin-1 immunoreactivity in the interstitial connective tissue of a gill arch is presented in Figure 3*B* and *C* which demonstrates intense immunoperoxidase staining in elongated and rounded cells. The possibility that the rounded cells are elongated cells cut in cross section should not be excluded. Their size and shape are consistent with the macrophage/monocyte cell type found in the skin (Fig 2 and ref.28). These cells are located in blood vessels within the gill or seen infiltrated within the gill tissue itself (Fig. 3*C*). Granulin-1 immunoreactivity is also detected within the myocardium of the sinous venosus of the heart either interdispersed between the muscle fibres (Fig. 4*A*, *B*, *C* and *D*). Granulin-1 immunoreactivity in cells of the intestine of the carp was observed within cells also of macrophage morphology located within the intestinal villi (Fig. 4*E*, *F* and

G). Preincubation of the granulin-1 antiserum with purified granulin-1 totally abolished immunostaining for all tissues examined in the control slides (Fig. 2*A*, 3*A*, 4*A* and 4*G*). We did not observe granulin-1 immunoreactivity in Bouins fixed paraffin sections of any of the goldfish tissues we studied, including spleen, head kidney, skin, heart, gill or intestine.

5.3 Granulin-1 immunoreactivity and endogenous acid phosphatase activity in frozen sections of goldfish spleen: Cryostat sections of goldfish spleen demonstrating positive granulin-1 immunoreactivity within melanomacrophage centers are shown in Figure 5. Staining was observed in cells which formed large clusters (Fig. 5*B* and *C*) of various sizes and shapes with no consistent organization. The cell morphology is essentially as described for the cells found in the carp skin (Fig. 2) and gills (Fig. 3). The immunopositive cells forming the clusters appear to be in close contact with a collagenous fibrous matrix which stains with fast green. A control slide, Figure 5*A*, demonstrates the elimination of immunoperoxidase staining of the granulin-1 antibody pre-incubated with excess granulin-1. However, a slight coloration appears on the control slides presumably resulting from melanomacrophage pigmentation or background immunostaining.

The detection of acid phosphatase activity in the melanomacrophage centers is shown in Figure 6*A* and *B*. The phosphatase positive histochemical reaction on the slide revealed the melanomacrophage centers with a dark red precipitate. Counterstaining with fast green confirmed the collagenous nature of the matrix onto which the cells seem to be tightly attached. Based upon the presence of endogenous acid phosphatase activity and morphology, cells found in these centers are presumed to be macrophages.

5.4 ß-galactosidase immunostaining of head kidney and spleen tissue smears: The purpose of this study was to observe immunostaining of whole cells and to discriminate between different cell sizes which had been found in the cryostat sections of spleen and head kidney. These investigations were done with biotinylated $F(ab')_2$ fragments specific for granulin-1. The appearance of immunoreactive cells found in goldfish and carp head

kidney and spleen tissue smears are shown in Figure 7 which demonstrates cells of different sizes with nuclei which are oval and bilobed, representing 20 to 50% of the cell population. Staining is observed exclusively in the cytoplasm which appears as granular precipitates of immunocomplex with precipitated X-Gal end product (blue on the slide). The staining of cells in the tissue smears suggested that the peptide is of cytoplasmic origin and contained within cells of a characteristic mononuclear phagocytic morphology. The spleen and head kidney smears for the goldfish are represented in Figure 7*A* and *C* respectively. Whereas the carp spleen and head kidney cells are shown in Figure 7*B* and *D* respectively and seem to be less numerous but have a greater intensity of staining. Presumptive lumphocytes also stain positively for granulin-1 in the carp spleen shown in Figure 7*E*. Staining of these smears with Wrights stain revealed numerous other cell types including lymphocytes, erythrocytes and various leukocytes which did not stain for granulin-1 immunoreactivity.

5.5 Immunocytochemical localization of granulin-1 within mononuclear phagocytic cells isolated from the goldfish head kidney: Isolated cells were prepared from goldfish head kidney which adhered to the glass slides and displayed a similar morphology to macrophage/monocytes as described by other investigators (17). We have shown the macrophages from head kidney in these studies since there is a significantly greater number of cells which attach to the glass slides as compared to the spleen macrophages. Consistent with our observation, the single cell suspensions of the carp spleen were difficult to obtain by cell dispersal. The immunocytochemical localization of granulin-1 in these macrophages is shown in Figure 8*A*, whereas Figure 8*B* represents the control slide. Using the biotinylated $(Fab')_2$ fragments and the ß-galactosidase enzyme system, an immunocomplex precipitate (blue on the slide) was confined to the cytoplasm of the adherent cells and not found in the nuclei. In order to establish the macrophage nature of these adherent cells, we assessed their ability to take up carbon particles by incubating the cells with diluted India ink. The results are shown in Figure 8*C* and *D* and demonstrate the uptake of carbon particles into the cytoplasm of the adherent cells.

Staining for immunoperoxidase activity using 3,3' diaminobenzidine and H_2O_2 revealed that the adherent macrophages also contain abundant endogenous peroxidase activity (Fig. 8*E*).

Discussion

The primary sequence of the three related carp granulins isolated from the spleen and head kidney have been determined and shown to be homologous to that of rat and human granulin A (see Fig. 1) (1). Carp granulin-1 is the predominant granulin purified from the spleen (Fig. 1*A*). A rabbit polyclonal antibody to this peptide was raised and was employed successfully to establish a radioimmunoassay, which was in turn used to undertake tissue distribution studies (1). This antibody does not cross-react significantly with granulin-2 or -3 purified from the carp head kidney (shown in Fig. 1*A*). When granulin-2 or -3 were tested in the granulin-1 radioimmunoassay, no cross-reactivity was observed at concentrations up to 10 ng/ml. However, the homologous granulin-1 peptide purified and partly characterized from goldfish spleen cross-reacted in the granulin-1 radioimmunoassay (1). The present study demonstrates the immunocytochemical localization of granulin-1 as an integral cellular component of the hematopoietic tissues found within carp and goldfish tissues. The immunoreactive cells have also been shown to be mononuclear phagocytic cells based on phagocytosis and specific endogenous enzymes systems.

Results obtained for carp skin (Fig. 2), gills (Fig. 3), heart and gut (Fig. 4) using Bouin-fixed paraffin sections demonstrated positive granulin-1 immunocytochemical staining in cells scattered within these tissues. No staining was observed in serial sections using antiserum preabsorbed with granulin-1 (Fig. 2, 3 and 4). These cells had a morphology similar to that of macrophages reported by other investigators (17, 29) and displayed the ability to phagocytose colloidal carbon in vitro. The elongated cell type containing immunoreactive granulin-1 has been observed at the sites of injury in experimentally wounded carp. These cells appear to be in transit to and from the site of injury and also have the ability to phagocytose colloidal carbon particles, suggesting that these cells are macrophages (29).

Carp granulin-1 has been found by radioimmunoassay to be in abundance in acid extracts of the spleen and head kidney of the carp. However, granulin-1 immunoreactivity was not observed in the Bouin-fixed paraffin sections of either spleen or head kidney of the carp or goldfish. This discrepancy raises concerns regarding the method of preparing the tissue samples for immunocytochemistry. The negative immunocytochemical results can possibly be explained by a rapid degranulation of the macrophage/monocytes prior to penetration of the Bouin's fixative into the tissue. This issue was resolved by using cryostat sections of goldfish tissues that demonstrated positive immunocytochemical staining for granulin-1 in mononuclear phagocytic cells residing in melanomacrophage centers of spleen (Fig. 5). These cells are arranged as irregular clusters distributed unevenly throughout both the spleen and head kidney, are 10 to 15 μm in diameter (Fig. 5*C*) and appear to be closely associated with a collagenous matrix. We have shown that cells within these melanomacrophage centers stain for endogenous acid phosphatase activity (Fig. 6), suggesting that the cells within the centers have enzyme systems usually associated with lysosomes of mononuclear phagocytic cells. Studies undertaken by other investigators have also indicated that cells found within these centers are macrophages and play important roles in host defense (16-20), especially as clearing units which can remove foreign particulates by phagocytosis.

The spleen and head kidney smears gave most surprising results in terms of morphology of the cells found in the melanomacrophage centers (Fig. 7). Examination of tissue smears from the head kidney revealed cells of numerous sizes staining positively for granulin-1 within granules found in the cytoplasm. The morphological characteristics of these cells indicated that they belong to the mononuclear phagocytic cell lineage.

A series of experiments using isolated goldfish head kidney cells demonstrated that granulin-1 is localized to mononuclear phagocytes. We have shown that granulin-1 is immunolocalized to a cell type which can adhere to glass slides (Fig. 8*A*) and can phagocytose carbon particles (Fig. 8*C* and *D*). The ability of these isolated cells to take up carbon particles in this way is in good agreement with similar characteristics shown for macrophage/monocytes isolated from trout head kidney (22) and a monocyte-like cell line from channel catfish (28). These previous studies demonstrated that macrophage-like

cells could adhere to glass slides and phagocytose latex beads. Furthermore, the cells isolated in the present study showed endogenous peroxidase activity (Fig. 8*E*), indicating that, together with all other findings mentioned above, these cells are mononuclear phagocytic cells.

The immunocytochemical localization of carp granulin-1 to macrophages suggests numerous possible physiological roles for this peptide in teleost fish. Studies have shown an increase in the number of macrophages during healing that are responsible for clearing cell debris at the site of injury in experimentally wounded carp (29). This increase in macrophage number coincided with a marked remodelling of cell mass involving growth and differentiation at the site of injury (29). Biological studies of epithelins 1 and 2 (homologues of rat granulin), isolated from rat kidney extracts, demonstrated pleiotropic growth modulatory activities when incubated with epithelial cells in vitro (7). We have undertaken preliminary studies to characterize the biological activity of carp granulin-1, -2 and -3 that have confirmed pleiotropic growth effects characteristic of this family of peptides . Carp granulins promoted ^{3}H-thymidine uptake by a human epithelial cell line (A-431) and a fish epithelial cell line (fat head minnow skin cells). Also, inhibition of thymidine uptake was observed when granulin -1, -2 and -3 were incubated with a fish embryonic cell line (salmon; CHSE-214) (see Appendix I, Chapter IV)[1]. It is interesting that carp granulins and rat epithelins have the opposite effect on A431 cells in vitro, suggesting the presence of more than one receptor for this class of peptides. The immunocytochemical localization of carp granulin-1 to macrophages and the growth modulatory activity of this family of peptides suggest that carp granulins could have profound effects by either stimulating or inhibiting growth of different epithelial cell types at the site of injury.

Owing to the probable involvement of macrophages in optic nerve regeneration in carp either by inhibiting oligodendritic cell growth (30) or by stimulating cells involved in optic nerve elongation (31), it is possible that pleiotropic growth factors like the granulins have significant roles to play in these physiological processes. Our immunocytochemistry study has not included granulin-1 localization at the sites of injury but will be subject of future studies aimed at elucidating the biological function of

granulins in teleost fish physiology. Experiments published to date concerning the biosynthetic fate of the granulins and related structures indicate that they are found in two contexts. Results from our laboratory including the current study have demonstrated that granulins are found only in the fully processed form within the granule fraction of phagocytic leukocytes (1,2). Other studies indicate that the unmodified precursor is produced and may be stored as in the case of acrogranin found in the sperm head (11) or secreted as in the case of the autocrine growth factor derived from a mouse PC cell line (13). Whether these proteins require proteolytic processing after secretion for biological activity to be expressed remains to be determined.

Our immunological studies have shown localization of granulin-1 to mononuclear phagocytes residing in the melanomacrophage centers of spleen and head kidney and to macrophages found in peripheral tissues important in the first line of defence against bacterial infection. The biological functions of teleost granulins can now be addressed in the context of macrophage involvement in numerous biological processes.

Acknowledgments

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Figure 1. Primary structure of carp granulin-1, -2 and -3 compared to rat and human granulin A. Primary structures are taken from reference 1 which describes the isolation and structural characterization of carp granulins. All amino acids are identical for carp granulin-1, -2 and -3 with the exception of those underlined. The rat and human sequences are taken from reference 2. The conserved amino acids shown were deduced by comparing the structures of the carp granulins to those of rat and human granulin A. An amino acid was considered to be conserved if it was present in both rat and human granulin A and in at least two out of the three carp granulins.

A) **Primary sequences of carp granulins:**

```
                    10                  20                  30                  40                  50
GRN1   VIHCDAATICPDGTTCCLSPYGVWYCCPFSMGQCCRDGIHCCRHGYHCDSTSTHCLR
GRN2   VVYCNARTTCPSRTTCCRSPFGVWYCCPFLMGQCCRDGRHCCRHGYRCDSTSTLCLR
GRN3   VVFCDAGITCPSGTTCCRSPFGVWYCCPFLMGQCCRDGRHCCRHGYHCDSTSTLCLR
```

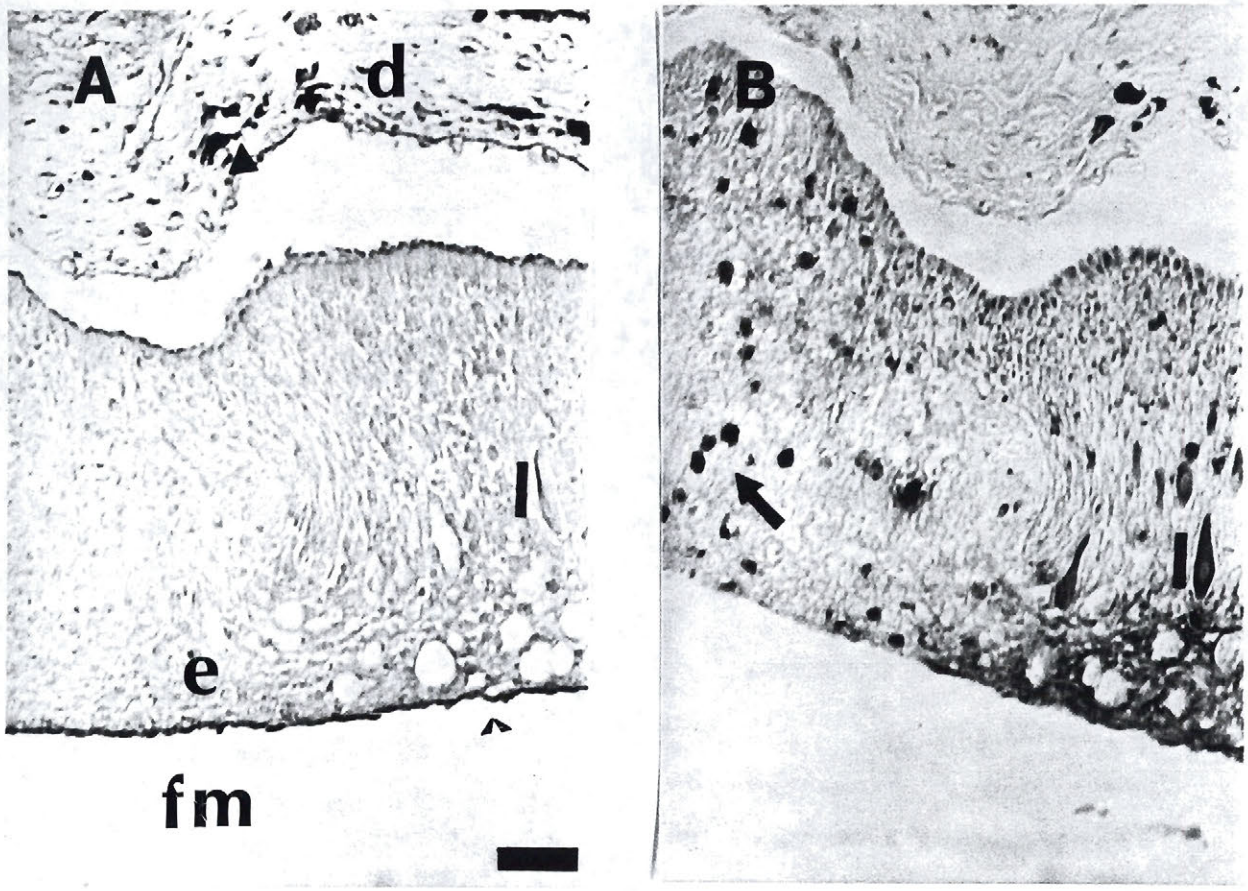
B) **Rat and Human granulin A:**

```
R.GRN  EVKCDLEVSCPDGYTCCRLNTGAWGCCPFTKAVCCEDHIHCCPAGFQCHTETGTCEL
H.GRN  DVKCDMEVSCPDGYTCCRLQSGAWGCCPFYQAVCCEDHIHCCPAGFTCDTQKGTCE
```

Conserved amino acids

```
---CD----CP-G-TCCR----G-W-CCPF----CC-D--HCC--G--CD-----C-
```

Figure 2. Immunocytochemical localization of carp granulin-1 in carp epidermis. Bouin-fixed tissue sections were prepared as described in the Materials and Methods. Panels *A* through *D* represent granulin-1 immunoreactivity found in skin epidermis from a scaleless head region of carp. Adjacent tissue sections were incubated with 1/800 dilution of anti-granulin-1 antibody: A) Control slide with anti-granulin-1 antibody preincubated with 4 µg/ml carp granulin-1. e, epidermis; d, dermis; fm, free margin of epidermis; >, club cells or mucous cells, ▲ represents melanin inclusions present before peroxydase staining. The bar (▌) represents an elongated cell type. B) The bar (▌) represents the same cell as in panel A, showing positive granulin-1 immunostaining. The arrow (→) represents scattered macrophage-like cells showing positive granulin-1 immunostaining. C) and D) are higher magnifications of the immunopositive cells for granulin-1 indicating a cytoplasmic immunostaining. Bars represent 50 µm for A and B and 10 µm for C and D.


A
d
l
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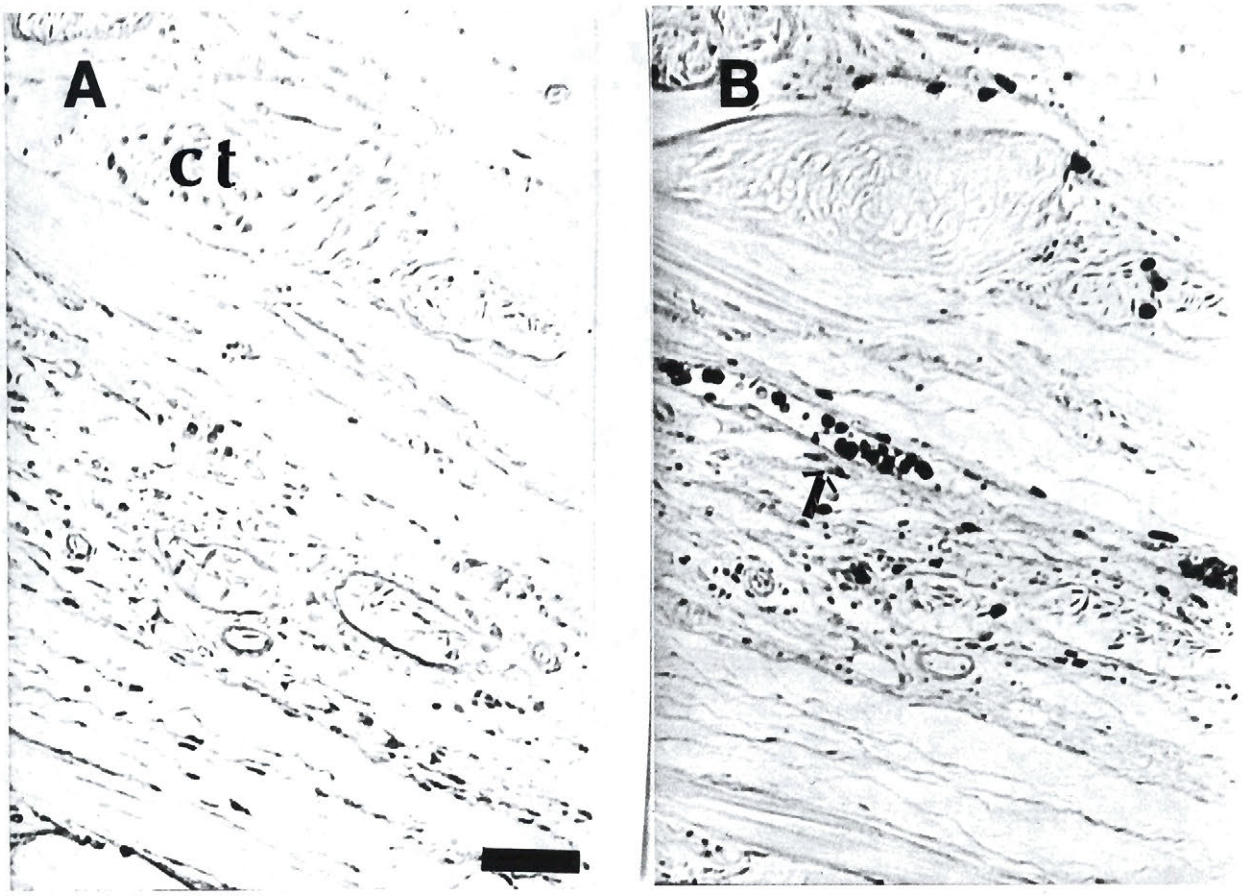
D

Figure 3. Immunocytochemical localization of carp granulin-1 in carp gills. Bouin-fixed tissue sections were prepared as described in the Materials and Methods. A) Control slide showing no granulin-1 immunoreactivity using 1/800 anti-granulin-1 antibody and 4 µg/ml granulin-1. ct, cartilage. B) Arrow (→) represents positive granulin-1 immunoreactivity in scattered macrophage-like cells within gill tissue and small blood vessels. C) The arrow (→) represents higher magnification of macrophage-like cells. Bars represent 100 µm in A and B and 10 µm in C.


A
ct
B

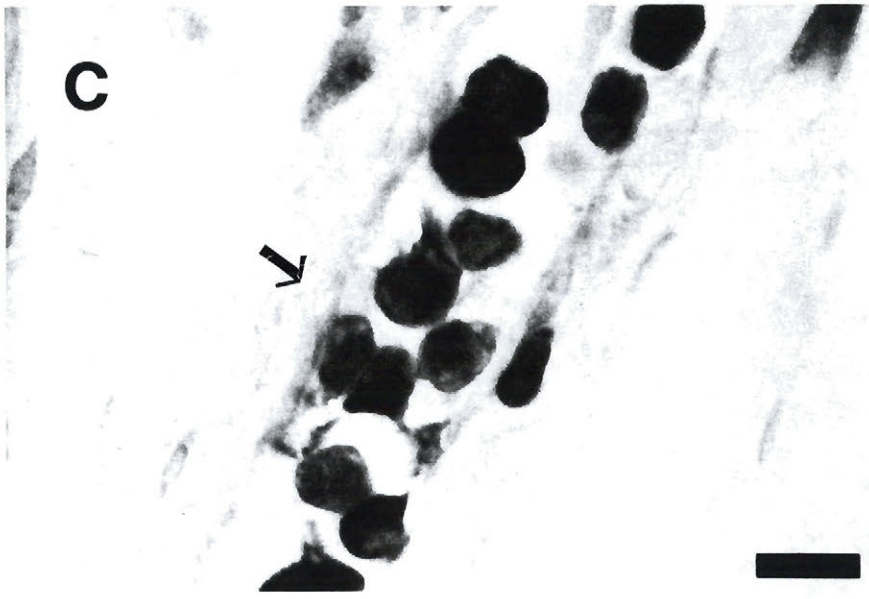

C

Figure 4. Immunocytochemical localization of carp granulin-1 in heart and intestine of carp. Bouin-fixed tissue sections were prepared as described in the Materials and Methods. Panels A through D represent granulin-1 immunoreactivity found in the heart. A) is a control slide showing immunoreactivity absorption with 4 µg/ml granulin-1. B) represents scattered macrophage-like cells between muscle fibres (mf) of the sinous venosus, also represented in D) at a higher magnification. C) represents granulin-1 immunopositive cells within a heart muscle fibre. Panels E through G demonstrate granulin-1 immunoreactivity in cells of macrophage morphology within intestinal villi (iv). E) and F) demonstrate positive granulin-1 immunostaining. G) is a control slide showing no granulin-1 immunoreactivity. Bars represent 50 µm in A, B, E and G and 10 µm in C, D and F.

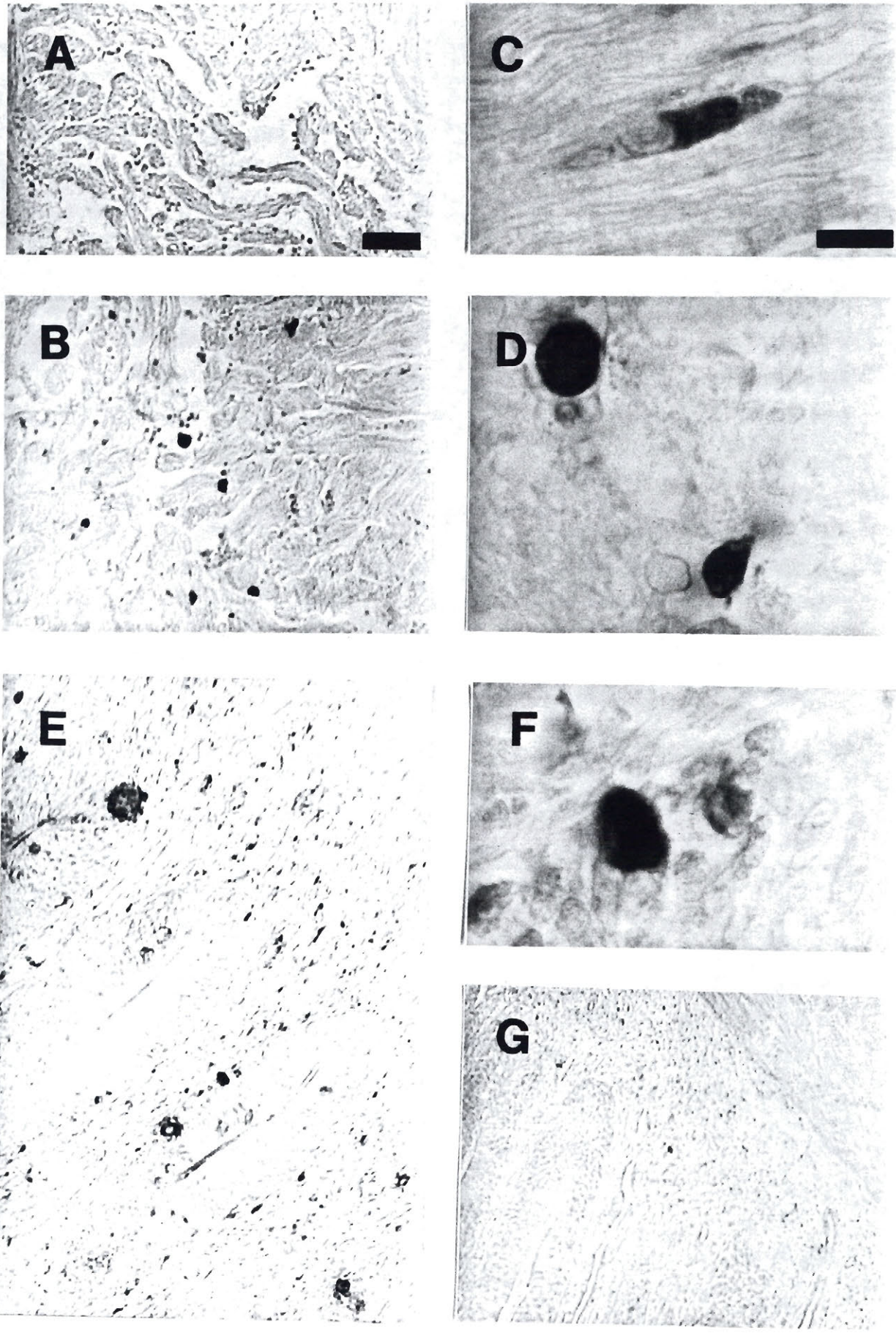

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Figure 5 Immunocytochemical localization of granulin-1 in melanomacrophage centers within goldfish spleen. Cryostat sections were prepared as described in the Materials and Methods. A) A control section incubated with carp anti-granulin-1 antibody (1/800 dilution) preabsorbed with granulin-1 (4 µg/ml). B) and C) immunoreactive granulin-1 localized in melanomacrophage centers. Notice the bundled cells tightly associated with the matrix surrounding the clusters. Bars represent 100 µm in A and B and 25 µm in C.

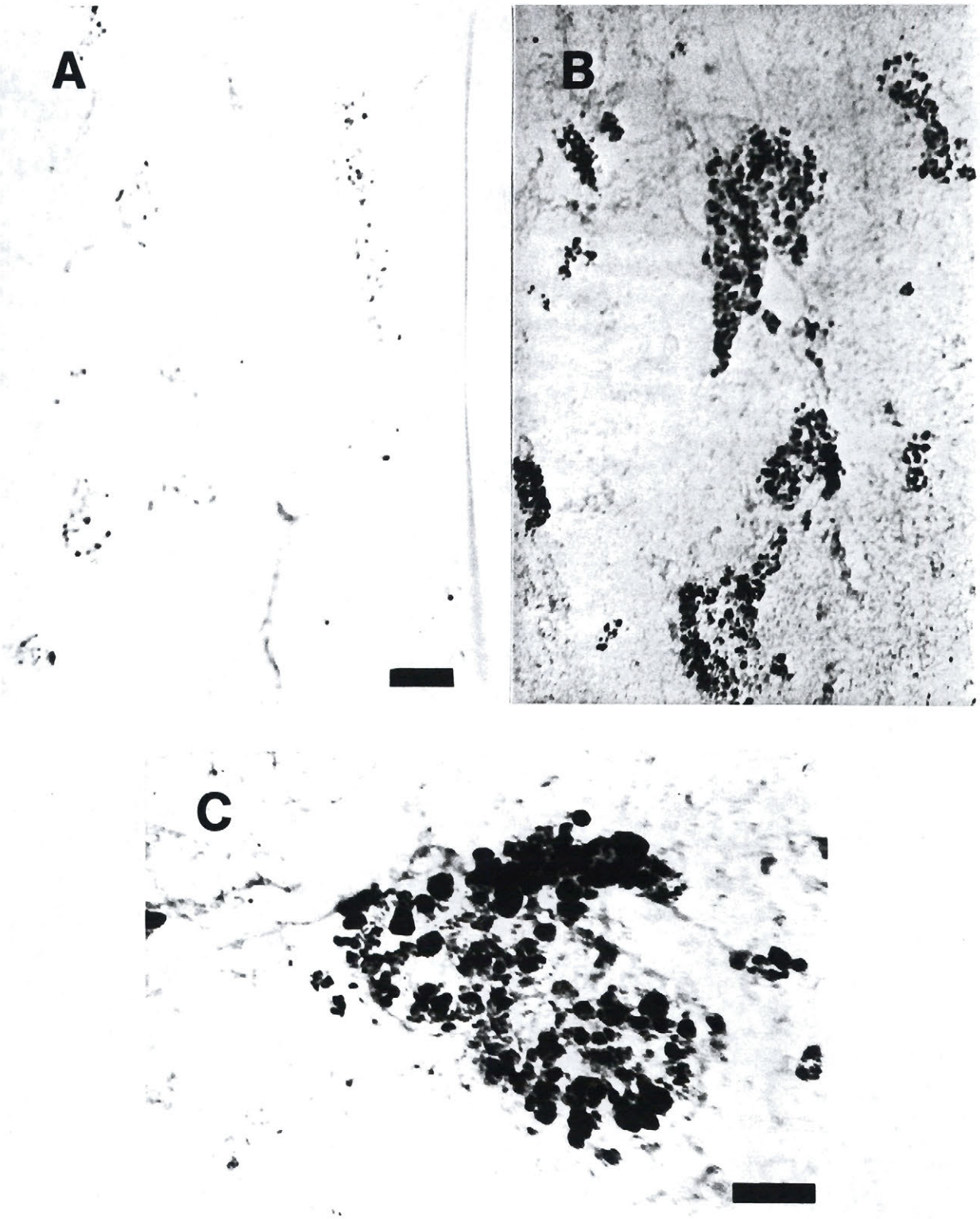
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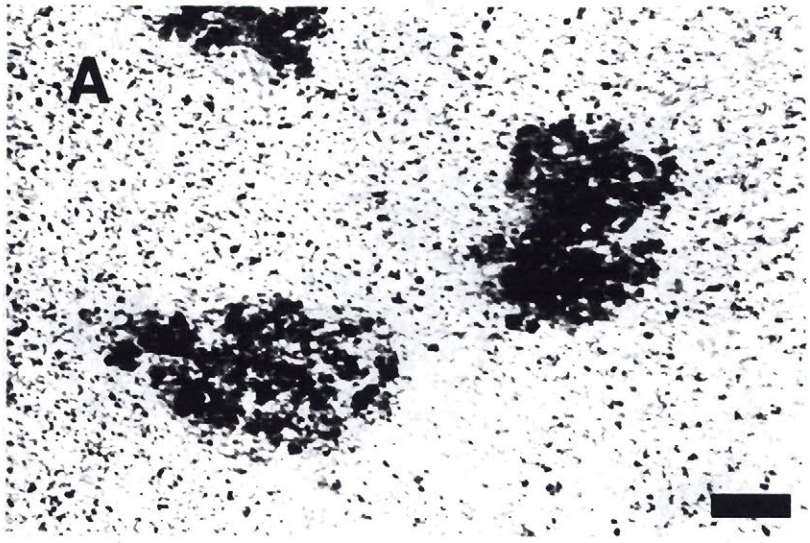
Figure 6 Endogenous acid phosphatase enzyme activity in melanomacrophage centers within goldfish spleen. Cryostat sections were prepared as described in the Materials and Methods. The endogenous acid phosphatase activity was determined with a simultaneous azodye coupling procedure and counter stained with fast green as described in the Materials and Methods. A) and B) demonstrate the staining of clusters of macrophage-like cells in red closely associated with a collagen matrix in green. Bars represent 50 μm in A and 25 μm in B.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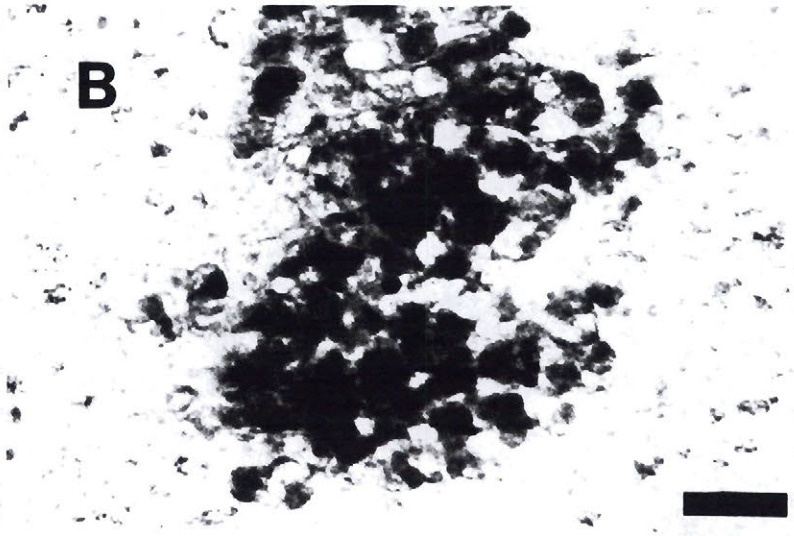
B

Figure 7 Immunocytochemical localization of granulin-1 in spleen and head kidney of both the carp and goldfish. Smears were generated by smearing tissues between two glass slides, fixed and prepared for immunocytochemistry as described in the Materials and Methods. Immunocytochemical localization was obtained using a purified biotinylated anti-granulin-1 $(Fab')_2$ fragment of the polyclonal anti-granulin-1 in conjunction with an avidin linked ß-galactosidase enzyme detection system. Positive immunostaining is shown by a blue colour caused by the precipitation of X gal detection system. Numerous phagocytic/monocytic-like cells are found in groups in smears of A) goldfish spleen, B) carp spleen, C) goldfish head kidney and D) carp head kidney. E) represents a smaller immunopositive cell type in carp spleen, possibly lymphocytes. Bar represents a scale of 10 µm.

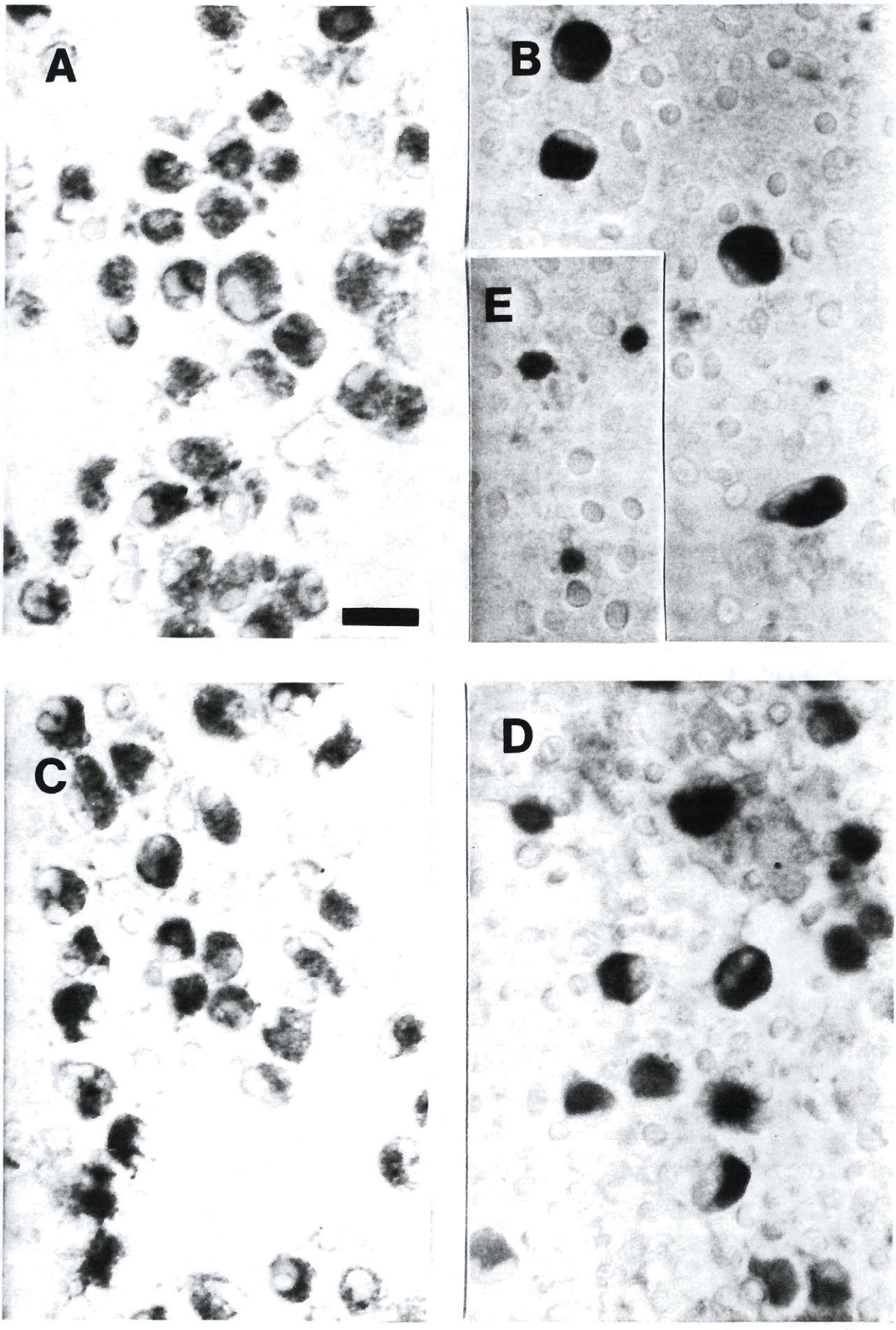
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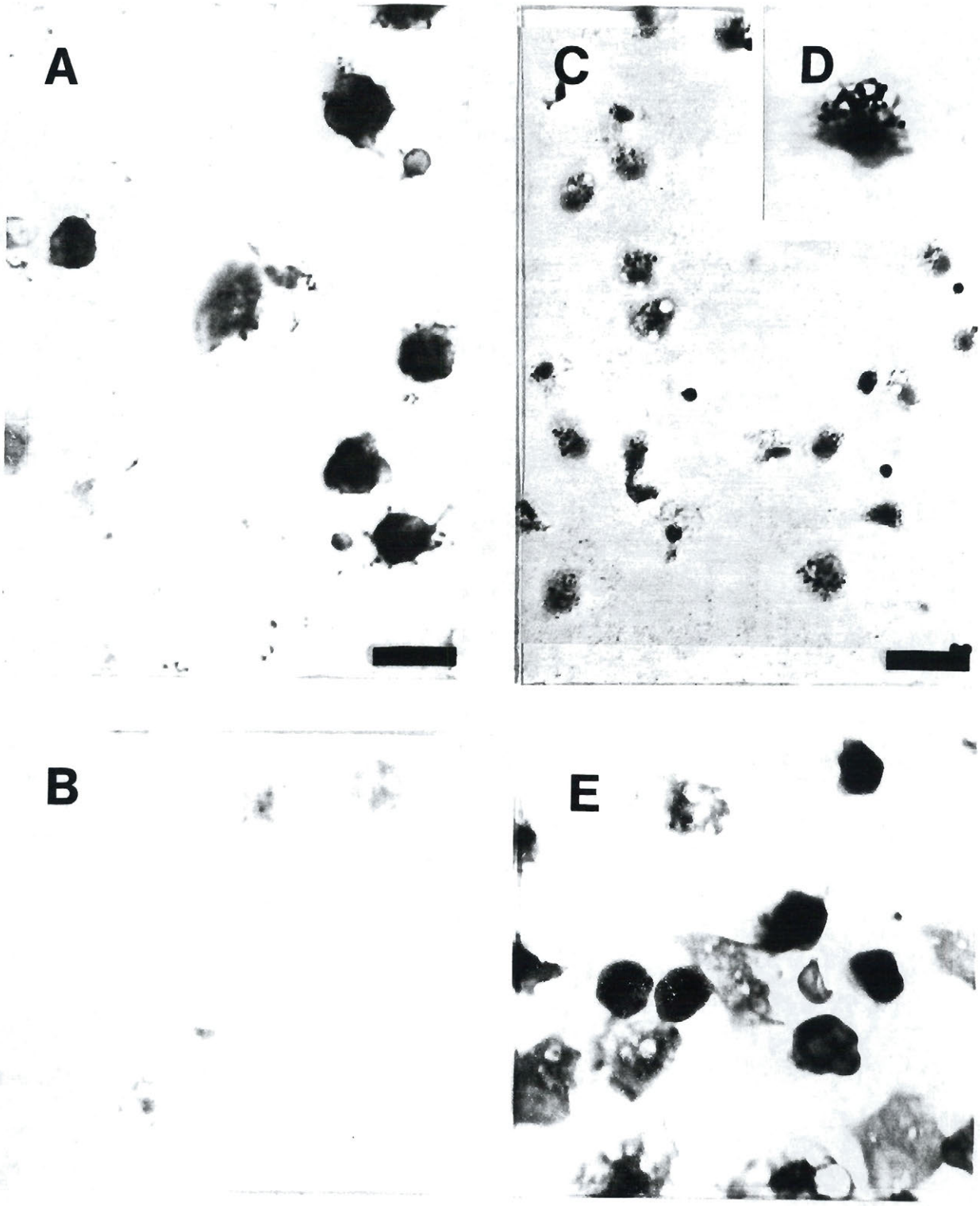
Figure 8 Immunocytochemical localization of granulin-1, the carbon uptake and the endogenous peroxidase enzyme activity in primary cultures of head kidney macrophages. Dispersed head kidney cells were seeded on glass slides for 24 hours then fixed or reincubated with India ink as described in the Material and Methods. A) Positive granulin-1 immunostaining shown in blue within adherent macrophages. Detection was done as described in Figure 7 with the Biotin anti-granulin $(Fab')_2$ fragment linked to the avidin ß-galactosidase system. B) Control slide. Biothinylated anti-granulin-1 $(Fab')_2$ fragment was preabsorbed with granulin-1 (4 µg/ml). C) and D) demonstrate the carbon uptake of isolated macrophages from head kidney. Numerous carbon particles from the India ink are found phagocytosed. E) represents the endogenous peroxidase activity of these adherent macrophages. Fixed cells were incubated with diamino-benzidine and peroxide as described in the Materials and Methods.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CHAPTER VI

GENERAL DISCUSSION

Preface

In the process of attempting to identify corticostatin-like polypeptides in teleost fish, we characterized a novel cystine-rich polypeptide from extracts of the heart and spleen of goldfish. Based on initial sequence information, it was apparent that we were studying a novel peptide with intriguing structural features. Because of the low levels and the difficulty in sequencing the complete goldfish peptide, we investigated the possibility that the same peptide could be isolated from carp, rat and human hematopoietic tissues. During these studies, a known family of cystine-rich polypeptides, the corticostatin/ defensins, was structurally and biologically characterized from the bone marrow of the rat. This work was presented in Chapter II and relates interesting physicochemical properties of the corticostatins. Chapters III and IV of my dissertation discussed the isolation and structural characterization of homologous granulin peptides found in carp, rat and humans. Chapter V showed the immunolocalization of carp granulin in carp and goldfish tissues. In this concluding chapter, I will summarize the implications of the work carried out so far in determining the various functions of the corticostatin and granulin families and make suggestions concerning future lines of investigation.

2 Corticostatins

Work from the Endocrine laboratory has previously demonstrated that corticostatins acted as competitive inhibitors of the ACTH-stimulated steroid secretion by rat adrenocortical cells *in vitro* (39). Corticostatins, initially isolated from neonatal and adult rabbit lungs, are homologous structures to the defensin family of peptides and share a common cysteine-rich motif characteristic of this peptide family (40).

During attempts to isolate granulin from rat bone marrow, the major cystine-rich fraction was found to contain members of the rat corticostatin/defensin family of peptides. Chapter II discussed the purification of seven structures related to these corticostatin/ defensin peptides. Characterization of these peptides was an essential part of my project since it resulted in the co-purification of rat granulin. We reported the determination of the primary sequences of five of these structures which were confirmed by ion-spray mass spectrometry and amino acid analysis. Biological characterization of these peptides in an *in vitro* rat adrenal assay confirmed that the more basic peptides were corticostatic while the three others failed to inhibit ACTH-induced steroidogenesis. We also deduced from the sequence data and the relative corticostatic potencies of corticostatins R-4 and R-5 that the substitution of an arginine in position 7 for serine decreased the corticostatic effect dramatically and that a basic amino acid (i.e. arginine) in this position is important for biological activity. Based on our findings, we concluded that basic charge alone is insufficient to convey corticostatic activity but that the positioning of arginine in positions 6 to 8 in the primary sequence is crucial for corticostatic activity. Finally, we concluded that the rat corticostatin (R-4) is a good representative of all the different activities attributed to this class of cystine-rich polypeptide, based on its corticostatic, cytotoxic, antimicrobial properties (41) and activity as an L-type Ca^{2+} channel agonist (42).

3 Granulins

The teleost granulins purified from hematopoietic tissues of carp and goldfish were characterized structurally and biologically. These prototypic forms of a novel class of growth modulatory polypeptides are typically 57 residues in length having 12 cysteines arranged in a novel structural array of four pairs of cysteines flanked at both the amino-

and carboxyl-termini by two singletons. The fortuitous discovery of these prototypic granulins is reminescent of the discovery of epidermal growth factor (EGF) by Cohen (3). His observations led to the isolation and structural characterization of EGF which was found to be highly expressed in the mouse salivary gland. Although teleost fish hematopoietic tissues (i.e. spleen and head kidney) remain to date the richest source of granulin, these peptides have been characterized independently in other biological systems. The rat and human granulins have been isolated and partially characterized (presented in Chapter III). It is evident when comparing teleost and mammalian granulins, that they are part of the same polypeptide family. The most striking feature is the conserved cysteine-rich backbone structure represented by the following:

$$CX_{5-6}CX_5CCX_8CCX_6CCX_5CCX_5CX_{5-6}C$$

Aside from this strict conservation of cysteine positioning, there exists a large degree of primary structure variation between the cysteine residues. The different biological systems in which this structural motif has been independently investigated reveal even more clearly the variant nature of these polypeptides. Epithelins-1 and -2, isolated from rat kidney tissue, were found to inhibit the growth of A431 cells in culture. Epithelin-1 also stimulated the *in vitro* proliferation of mouse keratinocyte cells in culture while epithelin-2 antagonized the growth promoting actions of epithelin-1 (22). Peptides were also isolated from equine neutrophils (eNAP-1) (36) and the brain of the insect *Locusta migratorias* (PMP-D1) (37) which contain within their structures, the conserved cysteine motif. Also, extensive similarity to the granulin cysteine motif is found in the non-catalytic domain of two plant thiolproteases (oryzains α and ß) (38). These structures certainly emphasize the evolutionary conservation and likely importance of the epithelin/granulin motif. The cloning of the cDNA for the preprogranulin/epithelin from several mammalian species has been completed and it has become apparent that these peptides are synthesized as part of a larger polypeptide precursor (19, 23). The predicted structures contain tandem repeats of seven and one half copies of the granulin motif. From this precursor, the peptides are generated by post translational events. The characteristics of these processing events remain to be investigated. The granulin/epithelin precursor has been purified from the mammalian sperm head (34). This

protein of unknown function, termed acrogranin, to indicate its location within the acrosomal compartment of the developing spermatids also contains the seven and one half cysteine-rich motif and apparently is structurally identical to progranulin/epithelin (19, 23). The mouse acrogranin exon/intron organization is very similar to the one found for the human preprogranulin gene (21, 35). Furthermore, a potent autocrine growth factor identical to the progranulin/epithelin/acrogranin family has been purified from conditioned medium derived from a transformed and highly tumorigenic mouse PC cell line (25). It has potent mitogenic effects on fibroblastic cell lines and can, through an autostimulatory mechanism, promote growth of the producer cell namely PC cells (25).

Transforming growth factor e (TGFe) is also part of this cystine-rich growth modulatory peptide family. Isolated from SW-13 cells and bovine kidney, TGFe causes autocrine anchorage independent growth of the SW-13 cell line (26). TGFe has been well characterized biologically and shown to have mitogenic effects on numerous normal fibroblastic and epithelial cells (29). Its physicochemical characteristics are however different from either the individual peptides or the complete precursor previously discussed. It seems that the post translation processing in these cells or tissues is different, giving rise to a protein with an intermediate molecular weight. Based on the amino-terminal sequence and its molecular weight, it appears that TGFe is most likely the carboxyl-terminal half of the progranulin/epithelin precursor starting at the granulin A structure found in the mid-region of progranulin (31). From the information acquired thus far, it seems likely that the processing of progranulin/epithelin will be tissue specific thus allowing for an array of different biological activities to be expressed depending on how it is processed.

Throughout my thesis, I have emphasized the importance of teleost granulins. The high concentration of granulins found in the hematopoietic tissues of teleost fish, made it feasible to characterize structurally and biologically several members of this naturally occuring polypeptide family. I have characterized carp granulins-1, -2 and -3 and partly sequenced goldfish granulin which is apparently the equivalent to carp granulin-1. Through a reinvestigation of the granulin content of carp spleen and head kidney using large scale preparative HPLC, seven new granulin structures, other than granulin-1, have

recently been identified (Bennett, H.P.J., unpublished observations). The partial structures of two of these new forms designated Carp A and B are shown below. Based on the overall nature of the progranulin structure, it seems likely that carp granulins are generated from at least two distinct precursor molecules. These new structures are markedly different from granulin-2 and -3 which appear to have arisen through a gene duplication event. The structures of other granulins purified from trout spleen, whose partial sequences are shown below, also suggest that multiple genes encode these peptides in teleost fish (Bennett, H.P.J., unpublished observations).

Goldfish	V I H C D S S T I C P D G T . . .
Carp GRN1	V I H C D A A T I C P D G T T C C L S P Y G V W Y C C P F S M G Q C C . . .
Carp GRN2	V V Y C N A R T T C P S R T T C C R S P F G V W Y C C P F L M G Q C C . . .
Carp GRN3	V V F C D A G I T C P S G T T C C R S P F G V W Y C C P F L M G Q C C . . .
Carp A	D V P C I D T V A C A D G T T C C K T Q E G G W X C C P L P G A V C C . . .
Carp B	D V P C X D T A A C P D G S T C C K T K E G E W X C C P L P Q A V C C . . .
Trout A	D V P C D E S T A C L D G T T C C K T Q E G W G A C C K . . .
Trout B	V T C D P T – V C P D N T T C C K T A S G G . . .

This accumulation of structural information suggests two distinct families of teleost granulin-like peptides, one which is related to carp granulin-1, including granulins from goldfish and carp granulins-2 and -3, and the other group comprising trout granulin-A and -B and the recently discovered carp granulin-A and -B. The latter group seems to be more closely related to the mammalian granulins based on their primary sequences. Teleost fish granulins are an interesting model because data on structure/function relationships can be quickly accumulated on very closely related structures which can be isolated in relative abundance. Furthermore, it is feasible to isolate milligram quantities of carp granulin-1; this was demonstrated in my establishment of a polyclonal antibody to carp granulin-1. These quantities of peptide will be important for structural studies such as nuclear magnetic resonance and x-ray crystallography of this peptide family. Such experimental approaches will be required to elucidate the complex disulfide bridging structure of the granulin peptide family.

The biological activities of the granulin/epithelin family of peptides suggest certain parallels with regards to the pleiotropic function of growth factors in general. Biological activities identified for teleost granulins, although preliminary, demonstrate this type of pleiotropism (see Appendix I, Chapter IV). Studies using A-431 cells (derived from human epidermis) indicated that granulin-1, -2 and -3 have proliferative effects in stimulating DNA synthesis *in vitro*. This result seems at variance with evidence suggesting that epithelins-1 and -2 inhibit the growth of A-431 cells (23), although it is consistent with the proposed pleiotropic properties of these peptides and could suggest that there exists several receptor sub-types with different specificities. We cannot exclude the possibility that the same receptor could be affected differently by different granulin/epithelin peptides resulting in activation of different transduction pathways within the same cell type. Also in these preliminary studies, carp granulins were shown to have effects on other cell types namely stimulation of DNA synthesis by fat head minnow skin cells (FHM) and by decreasing thymidine incorporation in a fish embryo cell line (CHSE-214) in culture. In other preliminary studies, carp granulin-1 has been shown to stimulate thymidine incorporation (i.e. increase DNA synthesis) into Madin Darby canine kidney (MDCK), a kidney epithelial cell line (Congote, F.C. and Bennett, H.P.J., unpublished observations). Stimulation of DNA synthesis of MDCK cell varies according to cell density. Low density plating does not respond well to granulin treatment whereas cultures that approach confluency seem to be sensitized MDCK cells to granulin treatment. Similar effects have been reported for TGFe when incubated with SW-13 cells (26). The potency of granulin-1 in the MDCK cell bioassay seemed to be 10-20 fold less than that found for EGF but the maximum response in terms of thymidine uptake was found to be similar for both peptides. The characterization of a binding protein for epithelins-1 and -2 (24) would suggest that it should be possible to characterize one or more receptors for teleost granulins. Since tyrosine kinase activities are usually associated with growth factors receptors(1, 2), it seems likely that the granulin receptor also acts in conjonction with a tyrosine kinase activity.

Although it was clearly important to structurally and biologically characterize teleost granulin, it was equally important to study the cell type of origin and to determine

its subcellular localization. Using a polyclonal antibody, we have demonstated (Chapter V) by immunocytochemistry that granulin-1 is localized in mononuclear phagocytic cells mainly residing in the major hematopoietic organs (i.e. spleen and head kidney) of teleost fish. By comparing different immunocytochemical techniques, we have shown that storage is most likely within cytoplasmic vesicles. In studying other characteristics, namely phagocytosis and lysosomal enzyme activity usually associated with phagocytic cells, we have confirmed that granulin-1 is found within mononuclear phagocytic cells. However, these data do not necessarily imply that granulin-2 and -3 or other granulins recently found in carp spleen and head kidney will have similar tissue localization and cellular origins. Studies carried out thus far on mammalian granulins have shown granulin synthesis in a number of cell types of both hematopoietic and epithelial origin.

In conclusion, we have identified a novel class of growth modulatory proteins, the granulins. These proteins are synthesized by mononuclear phagocytic cells in teleost fish. They have a novel cysteine motif which is highly conserved throughout evolution. Based on preliminary *in vitro* biological characterization, we have demonstrated that they have characteristics consistent with their proposed role as growth factors for epithelial cells.

CHAPTER VII

CLAIMS TO ORIGINAL RESEARCH

The following experimental results and conclusions represent original contributions to science:

1. Performed structural and biological characterization of rat corticostatin/defensins isolated from rat bone marrow.

2. Performed isolation and structural characterization of rat granulin from rat bone marrow.

3. Developed methodologies for the purification of carp granulin-1, -2 and -3 from spleen and head kidney.

4. Performed structural characterization of the prototypic forms of granulins, carp granulin-1, -2 and -3.

5. Developed methodologies to acquire a rabbit polyclonal antibody to carp granulin-1 and a radioimmunoassay for the detection of carp granulin-1 in tissue extracts fractionated by HPLC.

6. Developed methodologies for the immunocytochemical localization of granulin-1 in carp and goldfish tissues.

7. Established that carp granulin-1 is primarily localized in melanomacrophage centres in the spleen and head kidney and in macrophage/monocytes in peripheral tissues.

8. Established that the immunopositive cells are macrophages by their ability to adhere to glass slides, phagocytose carbon particles and contain specific enzyme markers (i.e. peroxidase and acid phosphatase).

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