# Secretion, Phosphorylation, and Cell Surface Localization of a Major Transformation-Sensitive Phosphoprotein, Identified as Osteopontin, in Normal and Transformed Cells.

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~~BIOCHEMICAL~~ STUDIES ON PP69/OSTEOPONTIN IN NORMAL AND TRANSFORMED CELLS.

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## ABSTRACT.

Phenotypic transformation is induced in non-neoplastic NRK cells by treatment with vanadium, a phosphotyrosyl-protein phosphatase inhibitor, to investigate the transformation-sensitivity of a major 69 kDa phosphoprotein (p69) secreted by normal cells. p69 is secreted in phosphorylated (pp69; pI 3.8) and nonphosphorylated (np69; pI 4.5) forms by untreated NRK cells and it is secreted only in its np form by RSV-transformed RR1022 cells. In vanadium-treated cells, whereas secretion of pp69 is decreased, the overall secretion of p69 in an np form is increased in a dose-dependent manner. However, secretion of a related, transformation-associated phosphoprotein, pp62, is not induced. This study indicates that p69 could be an extracellular matrix adhesion protein under phosphorylation control; a) np69 co-immunoprecipitates with fibronectin under nondenaturing conditions; b) by means of cell surface radioiodination and immunoprecipitation, pp69 is detected on the cell surface; and c) p69 is identified here as 2ar/osteopontin which is a mouse/rat adhesive protein containing a functional cell recognition RGD sequence.

## RÉSUMÉ.

La transformation phénotypic est indiute dans les cellules non-néoplastiques NRK par traitment au vanadium, inhibiteur des phosphatases agissant sur les protéines contenant phosphotyrosine, pour investiguer la sensibilité à la transformation d'une protéine de poids moléculaire 69 kDa (p69). p69 est sécrétée sous forme phosphorylée (pp69 pI 3.8) et sous forme non-phosphorylée (np69, pI 4.5) par les cellules NRK, et sous la forme np69 seulement par les cellules transformées par rétrovirus RSV, RR1022. Dans les cellules traitées au vanadium, la sécrétion de p69 sous forme pp69 est réduite et sous forme np69 est élevée. Cependant, la sécrétion d'une protéine associée à la transformation, pp62, n'a pas été induite. Cette étude indique que p69 peut fonctionner comme protéine adhésive de la matrice extracellulaire, dont la fonction est controlée par phosphorylation: i) la np69 co-immunoprécipite avec la fibronectine, ii) pp69 est détectable sur la surface cellulaire par radioiodination et immunoprécipitation et, iii) p69 est identifiée ici comme 2ar/ostéopontine qui est une protéine adhésive de souris/rats et qui possède une séquence Arg-Gly-Asp de reconnaissance cellulaire fonctionnelle.

## PREFACE AND ACKNOWLEDGEMENTS.

This thesis is assembled according to the guidelines of the Faculty of Graduate Studies and Research. It comprises a brief introduction to the subject and eight chapters. Relevant literature is reviewed in Chap. I–IV. Chapter V defines the objectives of this study. The experimental procedures are described in chap. VI. The results are presented in Chap. VII and discussed in Chap. VIII. In accordance with the Biology Department regulations I shall quote that: "This thesis carries a credit weight of 39 credits, from a total of 45 credits required for the Master's degree. Graduate credits are a measure of the time assigned to a given task in the graduate program. They are based on the consideration that a term of full-time graduate work is equivalent to 12 to 16 credits, depending on the intensity of the program".

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**ABBREVIATIONS.**

| | |
|---|---|
| A–BHRP | Avidin–biotinylated horseradish peroxidase. |
| ALP | Alkaline phosphatase. |
| APS | Ammonium persulfate |
| BSA | Bovine serum albumin. |
| CEF | Chicken embryo fibroblasts. |
| cpm | Counts per minute |
| DMEM | Dulbecco-modified Eagle's medium |
| ECM | Extracellular matrix. |
| EDTA | Ethylene diamine tetra-acetic acid. |
| EGF | Epidermal growth factor; –r, EGF receptor. |
| FGF | Fibroblast growth factor. |
| Fn | Fibronectin. |
| IEF | Iso-electric focusing |
| kDa | Kilodaltion. |
| KNRK | Kirsten sarcoma virus-transformed NRK [cells]. |
| MSPs | Major secreted phosphoproteins. |
| NCS | Newborn calf serum. |
| NEM | *N*-Ethylmaleimide |
| NRK | Normal rat kidney [cells]. |
| PBS | Phosphate-buffered saline. |
| PDGF | Platelet-derived Growth Factor. |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PMSF | Phenyl methyl sulfonyl fluoride. |
| PTK | Protein-tyrosine kinase. |
| PTPase | Phosphotyrosyl-protein phosphatase. |
| PT-NRK | Partially-transformed NRK [high passage NRK cells]. |
| P–Ser | Phosphoserine. |
| P–Thr | Phosphothreonine. |
| P–Tyr | Phosphotyrosine. |
| p69 | 69 kDa protein; pp69, phosphorylated–; np69, non-phosphorylated–. |
| RA | Retinoic acid. |
| RIPA | Radioimmune precipitation assay |
| RGD | Tripeptide argenine-glycine-aspartic acid. |
| SDS | Sodium dodecyl sulfate; –PAGE, SDS–polyacrylamide gel electrophoresis. |
| TBS | Tris-buffered saline; –T, TBS + Tween 20. |
| TCA | Trichloroacetic acid. |
| TEMED | *N, N, N', N'*-Tetramethylethylenediamine. |
| TGF | Transforming growth factor; –α, –type alpha; –β, –type beta. |
| TPA | Tetradecanoyl phorbol acetate. |
| V–NRK | Vanadium-treated NRK [cells] |

## INTRODUCTION.

Transformed animal cells cultured *in vitro* exhibit a number of phenotypic properties that both distinguish them from their nontransformed counterparts and correlate to different extents to their tumor-forming ability *in vivo*. Among these, growth in agar suspension cultures, called anchorage independent growth is singled as the property that correlates best with cellular tumorigenicity (Freedman and Shin, 1974). This property is not unique to irreversibly transformed cells, but can be induced in nontransformed cells by diverse growth factors, notably transforming growth factors (TGFs). Examination of growth factor actions indicate that whereas some growth factors induce anchorage independent growth by generating mitogenic signals, type-β TGF does so by enhancing extracellular matrix (ECM) formation (Ignotz and Massagué, 1986). The ECM, therefore, plays an important role in normal cell anchorage independent growth.

Cell transformation and development of tumors arise from deregulated control of cell growth as a result of genetic lesions in oncogenes which are involved in the control of cell growth (Bishop, 1985). One class of transforming genes encodes for protein-tyrosine kinases (PTKs) which are believed to control or constitute a separate transmembrane signal transduction pathway(s) (Hunter and Cooper, 1985; Macara, 1985). It is believed that cell transformation by this class of oncogenes results from abnormal or high levels of phosphorylation of cellular proteins. During the search for the mechanism of action of this class of oncogene proteins, phosphotyrosyl-protein phosphatases (PTPases) have been identified which counteract –at least at the biochemical level– the actions of protein-tyrosine kinases (Foulkes, 1983). It is now hypothesized that inactivation of this class of phosphatases will result in the same phenotype resulting from abnormal function of tyrosine kinases.

Some investigations have approached the problem of neoplastic transformation by searching molecular markers specific for malignant cells as they might provide an insight on the biochemical

lesion behind the transformed phenotype (Celis et al., 1987). Some of these were identified as major secreted phosphoproteins (MSPs). Thus, oncogenically transformed fibroblastic and epithelial cells of rodent and human origin, regardless of the transforming agent, have been shown to secrete a major phosphoprotein, pp62, commonly referred to as transformation-specific protein, which is absent in nontransformed cells, or present at markedly elevated levels in transformed cells relative to their non-transformed counterparts (Senger et al., 1979; 1985). In addition, studies in this laboratory have shown that a rat cell line transformed by Schmidt Ruppin avian sarcoma virus (RR1022), and a vole cell line transformed by Rous sarcoma virus (SR-IT) secrete a major phosphoprotein with a relative molecular weight of approximately 62-kDa, termed pp62 (Chackalaparampil et al., 1985). Interestingly, RR1022 and SR-IT cells which reversibly acquire a non-transformed phenotype after retinoic acid treatment, instead of secreting pp62, they secrete an immunologically-related 69-kDa phosphoprotein (pp69) indistinguishable from pp69 secreted by nontransformed NRK and Rat-1 cells. Although the mechanism of size shift of major secreted phosphoprotein is not known, pp62 and pp69, which could be a precursor or a modified form of one or the other, are now considered specific markers for transformed and non-transformed cells, respectively. In support for this, NRK cells transformed by a temperature-sensitive (*ts*) mutant of Rous sarcoma virus (RSV), LA23, secrete pp62 at the permissive temperature and pp69 at the non-permissive temperature (Mukherjee et al., unpublished data).

In this study, we sought to block endogenous PTPases to induce transformation in NRK cells in order to investigate further the transformation sensitivity of MSP pp69, and to determine whether or not a shift in secretion of pp69 to pp62, a reverse of the phenomenon observed in RSV-transformed cells upon RA treatment, will occur. Using vanadium as a PTPase inhibitor (Swarup et al., 1982a), this study shows that concomitant with the induction of a reversible transformed phenotype, secretion and phosphorylation of pp69 is altered in a dose-dependent manner thus confirming its transformation sensitivity. In addition, secretion of pp62 is not induced by vanadium indicating that pp62 secretion does not occur in reversibly transformed cells. Furthermore, this study gives biochemical evidence that this protein is secreted in phosphorylated and nonphosphorylated forms by nontransformed cells and that in vanadium-transformed cells the the ratio of np to pp69 increases

as the phenotypic transformation is more pronounced. This study also gives the first evidence for the localization of pp69 to the cell surface. In addition, by providing immunological and biochemical evidence, and in the light of recent developments in characterization of MSPs, this study argues that the proteins described here, the protein called 2ar/osteopontin and MSPs described by Senger and co-workers (Senger et al., 1979; 1985) are all the same protein or different isoforms of the same protein.

## I). ANCHORAGE INDEPENDENT GROWTH.

**Growth In Soft Agar as an Assay for Transformation**

Normal epithelial and fibroblastic cells cultured *in vitro* require attachment to a solid substratum for growth and survival whereas this is not an absolute requirement for transformed cells; virally-, chemically- and spontaneously-transformed cells can grow without attachment to a substratum. Growth in absence of attachement to a solid support is called anchorage-independent growth (Stoker et al., 1968). It is assayed *in vitro* by plating cells in semi-solid media such as soft agarose medium and examining their colony-forming ability (Macpherson and Montagnier, 1964). With few exceptions, colonigenicity of cells *in vitro* has proven to be the property of cells that correlates best with their tumorigenicity *in vivo* (Freedman and shin, 1974; Shin et al., 1975). Because of its simplicity and stringency, soft agar assay is widely used to assess the transformed phenotype of cultured cells as well as the transforming potential of various agents such as specific growth factors, tumor promoters, and of transfected oncogenes. For the latter studies, proper indicator cell lines like selected clones of NRK, AKR-2B, Rat-1, CEF and Balb/C 3T3 cells are used. This assay was used to identify and purify transforming growth factors (TGFs) and to determine the potency of such agents as retinoids in inducing reversible loss of transformed phenotype in target cells (Mukherjee et al., 1982). It was originallly thought that anchorage-independent growth is a property restricted to irreversibly transformed cells, but it is now evident that this property can also be reversibly induced in nontransformed target cells under specific growth conditions. The availability of agents affecting growth of normal and transformed cells in suspension made the assay particularly useful as a system to study anchorage independent growth as a biological process. Studies designed to understand anchorage independent growth *per se* and the events associated with it are carried out with the belief that elucidating its molecular mechanisms will help to identify mechanisms of cellular

transformation *in vitro* and tumor growth *in vivo*.

**Growth Factors and Transformation**

The growth of eukaryotic cells is primarily controlled by specific growth factors. The most important characteristic of transformed cells, that distinguishes them from nontransformed cells, is their partial or complete independence of exogenous supply of growth factors (Holley, 1975), which are necessary for the growth of their nontransformed counterparts. The autonomous growth of transformed cells is due to constitutive expression of one or more of the signal-transducing elements along the mitogenic pathway. One of the earliest events in the mitogenic process is the interaction of mitogenic growth factors with their receptors and the biochemical and physiological events triggered thereupon. Epidermal growth factor receptor is one of such signaling elements that is either constitutively over expressed as in the case of human epidermoid carcinoma cell line A431, or constitutively expressing high or deregulated biochemical activity such as the protein tyrosine kinase of v–erbB transforming oncogene of avian erythroblastosis virus (Kris et al., 1985) which encodes a truncated EGF receptor lacking a regulatory EGF binding domain (Downward et al., 1984). An earlier stage of the mitogenic pathway is the production of the mitogenic peptide itself, necessary for such a signal to occur. This implication led to the autocrine hypothesis (Sporn and Todaro, 1980) which postulates that reduced growth factor requirement of transformed cells could be due to auto-stimulation of transformed cell proliferation by mitogenic growth factors which they constitutively produce. A number of other findings relating growth factors or their receptors to oncogenes support the implication of the formers in cell transformation (Bradshaw and Prentis, 1987)

Early studies for example, have shown that Molony murine sarcoma virus-transformed cells, cultured in serum-free medium produce mitogenic growth factors, whose mitogenic activity is brought about by interaction with EGF receptor (DeLarco and Todaro, 1978). In addition to their strong mitogenic activity, these crude isolates induce a transformed phenotype in nonneoplastic cells characterized by transformed morphology, anchorage independent growth, growth to high densities and reorganization of the cytoskeleton. Partial purification of the crude isolates by gel filtration yielded three peaks of transforming activity of estimated molecular weights of 25, 12 and 7 kDa, collectively

called sarcoma growth factor. Subsequent studies showed that such isolates consist at least of two types of growth factors termed transforming growth factors (TGFs) α and β (TGF-α and TGF-β).

**Transforming Growth Factors**

TGF-α is a single chain polypeptide of molecular weight 5.7 kDa. The mature peptide (50 amino acids long), is produced by post-translational processing of a larger (160 amino acids) transmembrane precursor. It has sequence and structural homology with EGF and competes with EGF for binding to EGF receptor (Marquardt et al.,1984). It triggers identical biochemical and physiological effects to those of EGF, subsequent to its interaction with the EGF receptor. TGF-α is produced by tumor cells and developing embryonic tissues (Derynck, 1988). Like EGF, it has strong mitogenic activity on a number of cell types and in the presence of TGF-β it induces anchorage independent growth in nontransformed responsive cells.

TGF-β is structurally and functionally unrelated to TGF-α. The peptides purified from human placenta and platelets and from bovine kidney appear to be homodimeric and display a molecular weight of 26 kDa. However, later studies have shown that TGF-β exists in homodimeric (TGF-$[\beta 1]_2$ and TGF-$[\beta 2]_2$) or heterodimeric (TGF-β1,2) forms depending on the combination of the two disulfide-linked structural subunits β1 and β2 and that it belongs to a larger family of growth factors involved in the control of growth and differentiation (reviewed by Massagué, 1987). Its biological activity is triggered by interaction with a receptor distinct from EGF receptor although precise biochemical events that occur subsequent to such an interaction are presently unknown.

Some of the actions of TGF-β are thought to be mediated through the modulation of the number of the high or low affinity EGF cell surface receptors (Assoian et al., 1984). Unlike TGF-α, TGF-β has differential effects on cell growth; it is mitogenic for normal mesenchymal cells in monolayer culture, and in the presence of EGF or TGF-α induces anchorage independent growth. In transformed and epithelial cells, it inhibits monolayer and anchorage independent growth, therefore it is considered as a bifunctional growth factor (Roberts et al., 1985). In addition, an epithelial cell growth inhibitory factor isolated from African green monkey (BSC-1) cells has been shown to have a close biological similarity

to TGF–β (Tucker et al., 1984). The autocrine hypothesis now emphasizes the role of negative, autocrine growth inhibitory factors. According to the new revised hypothesis, autonomous growth of transformed cells could be due either to constitutive production of growth stimulatory factors or to absence of inhibitory factors, or to both (Sporn and Roberts, 1985). It has been suggested that these growth inhibitory factors act by altering the cell's ability to respond to growth stimulatory factors as illustrated by suppression of EGF-induced expression of competence genes (Takehara et al., 1987).

**Growth Factor Requirements for Anchorage Independent Growth in Normal Cells**

The finding that TGFs induce a reversible transformed phenotype has led to investigation of the growth factor requirements for anchorage independent growth of normal nonneoplastic cells. The established normal rat kidney (NRK) cell line, is widely used as an indicator cell line for transforming agents. Selected clones of this cell line, such as 49F, show strict serum requirement for growth, contact inhibition of growth and anchorage dependence. Earlier studies have shown that untreated NRK or NRK cells treated with individual growth factors do not grow in soft agar; soft agar growth of NRK cells can be induced by the simultaneous treatment with EGF (or TGF–α) and TGF–β in the presence of 10% calf serum (Anzano et al., 1982; 1983). Similarly, human platelet extracts induce soft agar growth of NRK cells and they contain of PDGF, TGF–β and EGF (Assoian et al., 1984a). However, such assays in presence of serum were inaccurate because whole blood serum contributes variable amounts of growth factors whose activities cannot be measured and have not been accounted for previously. For example, addition of PDGF is required for soft agar growth of NRK and other normal cell lines when plasma-derived serum (PDS) is used instead of whole blood serum, which is due to growth factors present in serum and absent from plasma. This is also supported by a widely observed variation in the basal levels of soft agar growth of indicator cell lines with different batches of commercially available sera.

**Anchorage Independent Growth on Normal Cells in Serum-free Media**

For more detailed analysis, serum-free and growth factor-defined media have been formulated and growth factor requirements of normal cells for anchorage independent growth reassessed.

van Zoelen et al. (1986) have assayed soft agar growth of NRK cells in presence of serum in which growth factors have been chemically inactivated by reduction and alkylation. Exogenous EGF, TGF-β, PDGF and retinoic acid (RA), another agent which affects soft agar growth (Mukherjee et al., 1982; 1983), have been assayed for their activity. It has been found that soft agar growth of NRK cells can be induced by either combination of EGF and TGF-β; EGF and RA; PDGF, TGF-β and RA; thus demonstrating that simultaneous presence of EGF and TGF-β is not mandatory for inducing anchorage independent growth. RA, like TGF-β acts as a modulator of EGF action by increasing the number of EGF receptors. This study also showed that there is no correlation between the mitogenic activity of such agents and their ability to induce or modulate anchorage independent growth. In a similar study, Rizzino et al. (1986) investigated induction and modulation soft agar growth in NRK cells by EGF, PDGF, TGF-β and fibroblast growth factor (FGF) in serum- and plasma-supplemented media and serum-free medium. Of particular interest, this study has shown that soft agar growth of NRK cells could be induced by PDGF alone in presence of serum-free or plasma-supplemented medium but not in serum containing-medium. The factor present in serum but not in PDS, which inhibits PDGF actions, is TGF-β. Such effects of TGF-β have also been observed by Anzano et al. (1986) in primary rat embryo and NIH 3T3 cells; they occur only in the absense of EGF, and the mechanism for such effects is not known. Other results in this study were essentially consistent with the study above.

van Zeolen et al. (1988) extended their studies on growth factor requirements for phenotypic transformation to another important property of transformed cells namely, loss of contact inhibition of growth. This has been assessed by measurement of DNA synthesis in quiescent, confluent cultures of NRK cells after exposure to growth factors. The type of response of NRK cells to such treatments and the correlation between growth factor requirements for acquisition of anchorage independent growth and loss of contact inhibition suggests that both processes have the same underlying mechanism and that anchorage independent growth may be a more restricted property of transformed cells.

**Role of Intrinsic Factors in Anchorage Independent Growth of Normal Cells**

Studies with different cell lines have shown that growth factors such as EGF, TGF–α, TGF–β, PDGF and FGF (and RA) all have the potential to induce or modulate anchorage independent growth in nontransformed cells, but the typical response depends on factors which are intrinsic to the cell. These unidentified intrinsic factors, which vary between and within cell lines, could be altered by establishment, immortalization, cloning, prolonged culture or transformation of a given cell line. Such alterations result in altered responses to different agents. For example, nonestablished primary rat embryo (PRE) fibroblasts have different growth factor requirements than established cell lines: PRE cells can be induced to grow in soft agar by PDGF alone whereas NRK cells require the synergism of EGF (or TGF–α) and TGF–β (and PDGF); mouse embryo AKR-2B cells grow in presence of TGF–β alone in contrast to NRK which need the addition of mitogenic factors such as EGF (Tucker et al., 1983). Requirement of and/or sensitivity to a particular growth factor can also change as a result of subcloning or prolonged *in vitro* passaging. Sub-clones of AKR2-B, NRK and Rat-1 cell lines have been isolated which show increased or varying requirements and sensitivity to different growth factors compared to the parental cell lines (De Larco and Todaro, 1978a; Moses et al. 1985; Kaplan and Ozanne.,1983); partially transformed sub-clones have been isolated from NRK cells by prolonged culture and selection in soft agar (Newman et al. 1986). One particular clone, named NRK PT-14, has retained the requirement for EGF but lost the requirement for TGF–β for growth in soft agar, which is not due to increased secretion of TGF–β. In monolayer culture, TGF–β, which was not found mitogenic to NRK cells, stimulated the growth of this PT-14 clone although it reduced EGF binding to the cells, effects which are totally opposite to the ones observed in NRK cells. Transformation also alters the response of cells to growth factors. In Fisher rat 3T3 fibroblasts, transfected with the cellular proto-oncogene *c–myc*, TGF–β synergises with PDGF to stimulate colony formation but suppresses the EGF-induced colony formation (Roberts et al., 1985). These studies indicate that normal cells can be induced to grow in soft agar by treatment with a variety of agents. All factors have the potential to affect soft agar growth but none of them is essential. The type of response to a particular growth factor depends on the cell line and the combination of other growth factors simultaneously present in the growth medium. In this context, it is interesting to note that anchorage independent growth can be induced in fibroblasts by CSF–1, an unrelated growth factor which normally acts on cells of the

hematopoietic lineage, when these fibroblasts are expressing the *c-fms* gene product (CSF-1 receptor; Roussel et al., 1987).

**Difficulties in the Study of Anchorage Independent Growth**

Although the growth factor requirements of normal cells for soft agar growth are now known in more detail, the complex events associated with it remain unknown. This is due, first, to the lack of knowledge of the biological effects of soft agar growth-inducing or modulating agents and more precisely which of those effects are relevant and necessary for the process of colony formation to occur. Second, cells subjected to the assay are not readily amenable to biochemical analysis as in monolayer culture and, the assay itself is carried out over a period of time often extending to several days making it difficult to analyse the precise timing and nature of the events which must take place. One obvious action of growth factors such as EGF and PDGF is to provide a mitogenic signal allowing a cell to initiate and progress through the cell division cycle, yet this is complicated by the fact that the mitogenicity of a given agent does not usually correlate with its soft agar growth inducing ability. EGF for instance is strongly mitogenic for most cultured cells but not sufficient to induce soft agar growth alone; conversely, TGF-β is not mitogenic and sometimes acts as a growth inhibitor for a number of cell lines yet is required for anchorage independent growth in certain cell lines and assay conditions. But, because most cells require the synergistic action of more than one growth factor or agent, it appears that more than a mitogenic signal is required for nontransformed cell growth in suspension. However, the cascade of events which occur subsequent to interaction of a growth factor with its receptor, as in the case of TGF-β, are not known.

**Action of TGF-β on Extracellular Matrix Components**

One of the most significant actions of TGF-β is its recently discovered stimulatory effect on ECM formation. By hypodermal injection of TGF-β into mice, Roberts et al. (1986) have shown that injected TGF-β induces the production of collagens and its local deposition near the site of injection. Studies on cultured cells from various origins, normal and transformed, demonstrate that enhanced formation

of ECM is a specific and early response to TGF–β. These studies also show that TGF–β enhances ECM formation by increasing the synthesis of its major components fibronectin and collagen and by reducing the rate of matrix protein degradation. TGF–β increases the synthesis of fibronectin and collagen and their integration into ECM of human, rat, mouse and chicken cells (Ignotz and Massagué, 1986) and rat calvarial bone cells (Wrana et al., 1988). This increase involves transcriptional activation as is the case of NRK cells where the mRNA for collagen type I, III and V are increased. Subsequent studies (Ignotz and Massagué, 1987; Roberts, C. J., et al.,1988) have shown that TGF–β increases fibronectin incorporation into insoluble ECM by enhancing fibronectin receptor synthesis and its levels on the cell surface. This effect is accomplished by both elevation of receptor subunits mRNA and receptor subunit maturation. TGF–β also stabilizes pre-formed ECM by protecting its components from proteolytic degradation. This is achieved by enhancing the synthesis of protease inhibitors such as plasminogen activator inhibitor, as is the case in human lung fibroblasts (Laiho et al., 1986), or by reducing the production of serine proteases (Laiho et al., 1986), thiol proteases (Chiang and Nielson-Hamilton, 1986) and metalloproteases (Martisian et al., 1986).

**Cell Adhesion**

Cell adhesion to substratum or to adjacent cells is important for cells to carry out normal physiological functions required for survival and growth. Cell-cell adhesion is mediated by cell adhesion molecules (CAMs) and the calcium-dependent cell adhesion molecules (Cadherins) which are cell surface glycoproteins insuring selective homophilic and heterophilic adhesion of adjacent cells (Edelman, 1985; Takeichi, 1987; 1988). Adhesion to the extracellular environment is mediated by substratum adhesion molecules (SAMs) which form the ECM, an extensive insoluble network of proteins and carbohydrates. Expression of both CAMs and SAMs is coordinately regulated during embryonic development (Edelman, 1985; Thiery et al., 1985).

**Composition of the Extracellular Matrix**

The major components of ECM are fibronectin, laminin, collagens and glucosaminoglycans usually attached to core proteins (proteoglycans), and among them fibronectin is the best studied

(Ruoslahti, 1988). Fibronectin is defined as a large extracellular multifunctional glycoprotein. Its broad biological activities observed *in vitro* include cell-substrate adhesion as manifested in mediation of cell attachement to surfaces coated with collagen, gelatin, fibrin, and glass or plastic support; cell-cell adhesion; cell migration by stimulating cell motility, haptotaxis and chemotaxis; and maintenance of cell morphology and alignment of fibroblasts into regular arrays; activities which are thought to reflect fundamental roles of fibronectin in embryonic development and morphogenesis, *in vivo*.

**Characteristics of Adhesive Functions of Fibronectin**

The different fibronectin functions are carried out by specialized structural domains (Yamada et al., 1985). By means of proteolytic dissection and functional analysis, each of the collagen, heparin, fibrin and cell binding domains have been isolated with their activities preserved. Of these domains, the cell binding domain has received much attention because it has been found that it alone can mediate cell attachement and spreading to inert substrate. The precise cell attachement site, also termed cell recognition sequence, has also been identified which consists of the amino acid sequence Arg-Gly-Asp(-Ser). Subsequent studies have shown that this sequence plays a key role in the function of other adhesion proteins as well (reviewed by Ruoslahti and Pierschbacher, 1987) These now include fibronectin, vitronectin, thrombospondin, osteopontin and collagens (nectins)

Of particular interest to the adhesive properties of fibronectin, was the finding that fibronectin mediates cell adhesion in a dualistic manner. It readily promotes cell attachement and spreading when used to coat the cell culture substratum, but this activity is increasingly inhibited when soluble fibronectin is added in increasing concentrations to the culture medium (Yamada and Kennedy, 1984). Similar effects were also observed using the cell binding domain of fibronectin or peptides containing the sequence Arg-Gly-Asp (Yamada and Kennedy, 1984, 1985; Pierschbacher and Ruoslahti, 1984). This dualistic nature of the fibronectin adhesive function suggested that fibronectin mediates cell adhesion through binding to a specific and saturable cell surface receptor. In addition to the identification of the cell adhesion sequences, antibodies which also block cell adhesion have also been produced. These latter and ECM components have been used to purify cell surface receptors

for cell matrix proteins (integrins), some of which recognize specifically the tripeptide Arg-Gly-Asp (Reviewed by Hynes, 1987). The identification of these receptors and cell recognition sequences should allow direct analysis of functional roles of ECM proteins and their receptors in cell attachement, spreading and motility.

**Role of Extracellular Matrix in Anchorage Independent Growth**

Ignotz and Massagué (1986) have examined the possibility that ECM mediates the TGF-β-induced anchorage independent growth of nontransformed cells. They have shown that fibronectin induces anchorage independent growth of nontransformed cells in a manner which mimics TGF-β in the additional requirement of mitogenic growth factors (EGF, PDGF) and that the tripeptide Arg-Gly-Asp inhibits both TGF-β- and fibronectin-induced soft agar growth presumably by competitively inhibiting fibronectin binding to its cell surface receptor. They have, therefore, proposed that TGF-β and fibronectin induce anchorage independent growth by providing an extensive matrix to which the cells adhere and grow into colonies. However, the behavior of transformed cells is contradictory. It has been shown that anchorage independent growth of transformed cells is inhibited by TGF-β (Roberts et al., 1985) although transformed cells, like SV-40 transformed cells also respond to TGF-β treatment by increasing the synthesis of their cell matrix proteins. This suggests that the soft agar growth of nontransformed cells is mechanistically different from that of transformed cells in that it depends on the elaboration or exogenous supply of ECM while that of transformed cells does not.

**Mechanistic Differences in Anchorage Independent Growth of Normal and Transformed Cells**

The idea that soft agar growth of transformed cells is independent of ECM formation is compatible with the fact that most transformed cells lack their own ECM and experimental evidence also indicates that this is the case Humphries et al. (1986) have indicated that soft agar growth of highly malignant melanoma cells, in contrast to that of nontransformed cells, is not inhibited by Arg-Gly-Asp-containing peptides. This insensitivity is not due to nonrecognition of the peptides, or lack of cell surface receptors on melanoma cells since these peptides as well as the cell recognition sequence of laminin inhibit their metastatic and invasive activities *in vivo* and *in vitro* (Humphries et al., 1986; Iwamoto et

al., 1987; Gehlsen et al., 1988), processes which at their various stages depend on cell attachement to ECM components of the invaded tissue (Nicolson, 1984; Liotta, 1986). Although addition of fibronectin to transformed cell cultures causes the cells to assume a nontransformed phenotype, inhibition of anchorage independent growth of transformed cells also does not appear to depend on elaboration of ECM since retinoic acid inhibits soft agar growth of Rous sarcoma virus-transformed cells without restoring the normal levels of fibronectin (Mukherjee et al., 1982). It appears therefore, that in addition to the requirement for a mitogenic signal provided by growth factor-receptor interaction, the interaction nectin-integrin interaction is also required for growth of normal cells in suspension; and such an interaction could generate a signal, transmitted through the cytoskeleton with which integrins interact (Horwitz et al., 1986), to the nucleus where it will affect gene expression. It is possible that this signal is not required for growth in suspension of transformed cells which have a relaxed cell cycle control (Heldin and Westermark, 1984). Alternatively, this signal could be constitutively turned on, by modification of any of the signaling elements, as is the case of fibronectin receptor complex which is phosphorylated in Rous sarcoma virus-transformed chicken cells (Hirst et al., 1986), but whether other nectin receptors are also phosphorylated or whether this phosphorylation has any functional consequences is not known.

A study by Menko and Boettiger (1987) gives evidence that nectin-integrin interaction generates a signal which is a control point in myogenic differentiation. They have shown that treatment of chicken embryo myoblasts with a monoclonal antibody, CSAT, which prevents fibronectin and laminin from biding to integrin by blocking β–integrin subunit, reversibly blocks myoblast differentiation into contractile myotubes. Because soluble, but not immobilized, fibronectin also inhibits myogenic differentiation, it appears that nectin-integrin provides a positional signal required for differentiation. The authors further suggested that insoluble ECM creates a permissive, but not an instructive environment for growth or differentiation; this could be achieved by altering accessibility, sensitivity or responsiveness of cells to soluble growth and differentiation factors as shown in other studies (Hedin et al., 1988). Whether TGF–β-induced or exogenously-supplied matrix components induce anchorage independent growth in nontransformed cells by generating such signals or creating permissive environment requires further investigation.

## II). PROTEIN-TYROSINE PHOSPHORYLATION AND CELL TRANSFORMATION.

### Protein-tyrosine Kinases and Cell Transformation

Post-translational modification of proteins, by phosphorylation, is a major mechanism by which protein function is regulated (Rubin and Rosen, 1975; Krebs, 1986). Protein phosphorylation occurs on serine, threonine and tyrosine amino acid residues. A number of serine and threonine kinases and their substrates have been extensively characterized, and in several cases serine and threonine phosphorylation is found to be a key switch in the control of many aspects of cellular metabolism (Krebs, 1986). Protein-tyrosine phosphorylation has been discovered more recently and although its physiological significance is less understood, it has received much more attention because it is believed to be involved in the control of cell growth and transformation (Cooper and Hunter, 1983). This stems from the finding that protein-tyrosine kinase (PTK) activity is the only identified primary biochemical activity of a number of retroviral oncogene-encoded proteins and cell surface receptors for growth factors (Hunter and Cooper, 1985; Yarden and Ullrich, 1988). This activity is easily demonstrated since most PTK s retain their activity when isolated in *in vitro* kinase assays. This is achieved by incubation of the immunoprecipitated kinase with the exogenous substrates, in the presence of [$\gamma$-$^{32}$P]-ATP, and the subsequent detection and quantification of the radioactivity incorporated into either the kinase itself or the exogenous substrates. Protein tyrosine kinases are of two types (Hunter and Cooper, 1985). The growth factor receptor type of PTK (Yarden and Ullrich, 1988)includes the best characterized EGF receptor (EGF-r) and the receptors for insulin (I-r), PDGF (PDGF -r) insulin-like growth factor-1 (IGF–1-r) and CSF–1 (*c-fms*). The nonreceptor type of PTK is typified by the oncogene product of Rous sarcoma virus, pp60$^{v-src}$, and includes the products of *abl, fes/fps, yes and fgr* viral oncogenes (Bishop, 1985). Their cellular proto-oncogene products also display a tyrosine kinase activity, and in the case of pp60$^{c-src}$, the activation of its kinase activity is

thought to be of primary importance in the polyoma virus middle T antigen-mediated transformation. Both types of PTK are found in most vertebrate cells and exhibit structural homology, to each other and to other kinases with different amino acid specificities, particularly in the catalytic domains (Hanks et al., 1988). The growth factor receptor-type of PTKs display an extracellular ligand-binding domain, a transmembrane region and a cytoplasmic domain where the catalytic site is located (Yarden and Ullrich, 1988). The nonreceptor-type of PTK are cytoplasmic or associated with the cytoplasmic side of cellular membranes to which some of them are anchored by covalently attached fatty acid. The receptor-type of PTK activity is positively regulated by ligand binding. For example, the interaction of EGF with the extracellular domain of its receptor triggers both receptor autophosphorylation and the phosphorylation of other soluble or membrane-associated cellular proteins *in vivo* and exogenous artificial substrates *in vitro* (Bishop, 1985). The regulation of PTK activity of nonreceptor-type of kinases is less well understood, but it appears that it is regulated by phosphorylation (see below).

The present working hypothesis proposes that the PTK class of transforming proteins brings about initiation and maintenance of the transformed phenotype by abnormal or constitutive phosphorylation, on tyrosine, of critical protein substrates which are key regulatory elements of cellular proliferation such as proteins whose functions are relevant to transmembrane signal transduction. Evidence supporting this hypothesis is that protein-tyrosine phosphorylation constitutes an integral part of the cellular response to a number of growth factor-receptor interactions (Hunter and Cooper, 1985); the phosphotyrosine content in cellular proteins is higher in transformed and growth factor-stimulated cells than in normal or unstimulated cells (Sefton et al., 1980); *ts*–RSV mutants for kinase activity are thermo-dependent for transformation; $pp60^{c-src}$ acquires a transforming potential when a 19 amino acid fragment from its carboxyterminus, which suppresses its kinase activity, is replaced with an arbitrary peptide or the $pp60^{v-src}$ counterpart. Similarly, EGF receptor with large deletions in the extracellular domain and chimearic EGFr-*v–erb*B receptors, whose kinase activities are independent of EGF regulation, have transforming potential (Schlessinger, 1986). Although the long term consequences of deregulated expression of retroviral oncogene-encoded PTKs or ligand stimulation of PTK activity in growth factor receptors are known, the exact mechanism of action of these proteins is still unknown.

**Detection of P-Tyr-containing Proteins**

Because of the low level of phosphotyrosine (P-Tyr) in proteins compared to phosphoserine and phosphothreonine, various approaches have been used to detect P-Tyr-containing proteins phosphorylated *in vivo* or *in vitro*. Early investigations have taken advantage of the discriminative effect of alkali on phosphodiester linkages; alkali hydrolyses P-Ser and P-Thr but not P-Tyr. Technically, this is achieved by autoradiographic visualization of gels, on which $^{32}$P-labeled proteins have been resolved, before and after incubation in alkali (1 N KOH) at 55 °C (Feuerstein and Cooper, 1983). A more sensitive approach is by phosphoamino acid analysis after acid hydrolysis of proteins eluted form individual gel bands (Cooper et al., 1983). This technique, in combination with phosphopeptide mapping is particularly useful in determining which peptide is phosphorylated on which amino acid residue, information which is important for understanding the regulation of enzymatic activity, by phosphorylation, such as in the case of pp60$^{src}$ (Cooper et al., 1986). Because of the limitations set by the sensitivity and the limited applications of these methods and the growing needs for identification and characterization of more PTK substrates, the immunological approach has been undertaken. Phosphotyrosine and its structural analogues (*p*-azobenzylphosphonate and *N*-bromoacetyl-*O*-phosphotyramine) coupled to carrier proteins such as bovine serum albumin, immunoglobulins, keyhole limpet hemocyanin, or poly-L-lysine have been used as immunogens to raise polyclonal as well as monoclonal antibodies for P-Tyr-containing proteins (Ross et al., 1981; Frakelton et al., 1983; EK and Heldin, 1984; White et al.; 1987). It has also been observed that immunization using naturally tyrosine-phosphorylated proteins also causes the production of specific anti-P-Tyr antibodies (Wang,1985). In every case, the final step in the preparation of these antibodies involves affinity purification by P–Tyr or P–Tyr analogue affinity chromatography. These antibodies are, in general, highly specific to P–Tyr and do not react with P–Ser or P–Thr. The use of these antibodies allows the detection of phosphoproteins not previously detected by then available methods (Ek and Heldin, 1984), and the study of the localization of P-Tyr-proteins at the structural level (Marchisio et al., 1988) and their spatiotemporal distribution during embryonic development

(Takata and Singer, 1988, Maher and Pasquale, 1988). When used as high density immunosorbents, they are particularly useful in large scale affinity purification of P-Tyr-proteins, making them directly available for biochemical and functional analysis (Bell et al., 1987; Wahl et al., 1988).

**Substrates for PTKs**

With the realization that protein-tyrosine kinase is the transforming principle of many acute oncogenic retroviruses and that protein-tyrosine phosphorylation is also a primary response to binding of many growth factors to their receptors, a great deal of intensive research has been carried out to identify physiological PTK substrates. Although a large number of protein substrates have been identified in earlier investigations, none of them appeared to be directly involved in any aspect of the transformed phenotype. With the use of various analytic approaches, data on potentially important candidates, some of which is summarized below, begins to be accumulated.

**Protein tyrosine kinases.** An important feature of PTKs is that they are themselves phosphorylated on multiple tyrosine residues *in vivo* and *in vitro* (Cooper and Hunter, 1983) and the importance of phosphorylation of these proteins is obvious if it is to have a functional regulatory role in the physiological actions of these proteins. Phosphorylation of some tyrosine residues in PTKs is indeed one of the few, if not the only presently known cases where tyrosine phosphorylation has a functional consequence. PTKs of the *src* family are constitutively phosphorylated on tyrosine in transformed and normal cells, whereas the phosphorylation of the receptor-type of PTKs is triggered by ligand binding. Both types of PTK are phosphorylated on a number of additional serine and threonine residues.

The major tyrosine phosphorylation site in $pp60^{v-src}$ is $Tyr^{416}$, which is phosphorylated to a lower stoichiometry in $pp60^{c-src}$ and is also the major phosphorylation site *in vitro*. Phosphorylation of $Tyr^{416}$ is thought to have a positive regulatory role on kinase activity, since it is correlated with increased *in vitro* v–*src* kinase specific activity; by contrast, $pp60^{c-src}$ is underphosphorylated on $Tyr^{416}$ and has a lower specific activity. $pp60^{c-src}$ is also phosphorylated on $Tyr^{527}$ near the carboxyterminus which is absent from $pp60^{v-src}$(Cooper et al., 1986). Phosphorylation of $Tyr^{527}$ suppresses both the kinase and transforming activities of$pp60^{c-src}$. It has been shown that

dephosphorylation of $Tyr^{527}$ by phosphatase treatment, or binding of antibody to the carboxyterminus containing $Tyr^{527}$ stimulates $pp60^{c-src}$ kinase activity (Cooper and King, 1986). Similarly, the $pp60^{c-src}$ complexed with polyoma virus middle T antigen is not phosphorylated on this site and has elevated tyrosine kinase activity (Cartwright et al., 1986) and substitution of $Tyr^{527}$ with a nonphosphoacceptor amino acid renders $pp60^{c-src}$ oncogenic with high kinase activity (Kmiecik and Shalloway, 1987).

A number of other tyrosine and serine residues are phosphorylated *in vivo* under various conditions (Hunter and Cooper, 1985). Particularly, a recent study has shown that $pp60^{c-src}$ kinase is activated, by phosphorylation on a novel threonine site in the N-terminus, during mitosis of synchronized fibroblasts, and that this activation is independent of the phosphorylation of $Tyr^{416}$ or $Tyr^{527}$ (Chackalaparampil and Shalloway, 1988), thus giving direct evidence to suggest that the *src* family of kinases may regulate mitotic events. In support for this evidence is the observed cell cycle-dependent protein-tyrosine phosphorylation of some cellular proteins (Morla and Wang, 1986).

EGF-r is also phosphorylated on serine, threonine and tyrosine *in vivo*. Its kinase activity is primarily regulated by ligand binding. EGF stimulation leads to rapid receptor autophosphorylation on three tyrosine residues residing near the carboxyterminus of the molecule. However, although the autophosphorylation of at least $Tyr^{1137}$ coincides with increased kinase activity, the role of tyrosine phosphorylation in the EGF receptor is not clear (Yarden and Ullrich, 1988). EGF-r activities are rather controlled by phosphorylation on $Thr^{654}$, by calcium and phospholipid-dependent kinase C (PKC), which occurs in response to stimulation by other growth factors (PDGF) or tumor promoters (phorbol esters). Phosphorylation on this site causes a decrease in kinase activity, a reduction in high affinity cell surface receptors, and promoted ligand-independent receptor internalization (Sibley et al., 1988; Yarden and Ullrich, 1988).

**Cytoskeletal proteins.** Cellular morphology, cell-cell interaction, and cell-substratum adhesion is mediated by adhesion plaques or focal contacts. These are highly specialized regions of the plasma membrane where cellular matrix components attach and cytoskeleton microfilament bundles (stress fibers) terminate and anchor. The proteins contained in these structures include actin, talin,

vinculin, α–actinin and integrin. The loss of cytoskeletal integrity and the appearance of rounded morphology is a common property among cells transformed by oncogenes specifying protein-tyrosine kinases (PTK), and by the use of temperature-sensitive mutants of RSV (*ts*-RSV), Boschek et al. (1981) have shown that the induction of this property is an early event following reactivation of $pp60^{v-src}$ kinase. Evidence also indicates that $pp60^{v-src}$ induces morphological transformation by phosphorylating cytoskeletal elements located in adhesion plaques. In addition to being attached to cellular membranes, $pp60^{v-src}$ is particularly concentrated in adhesion plaques (Henderson and Rohrschneider, 1987) and its association with these structures, where its critical substrates are located, is essential for transformation (Kamps et al., 1986). By using affinity-purified anti-azobenzyl phosphonate antibodies, Marchisio et al. (1984; 1988) have shown that phosphotyrosine-containing proteins are specifically located at adhesion plaques, at the level of cell-cell contacts and filopodial protrusions in RSV-transformed cells. Moreover, a reduction in number or structural abnormality of adhesion plaques and loss of spatial codistribution of fibronectin with its receptor is usually a consequence of RSV-caused transformation (Nigg et al., 1986).

Among the proteins concentrated in adhesion plaques, vinculin, talin and integrin receptor complex have been found to be phosphorylated on tyrosine in cells transformed by the PTK family of oncogenic proteins. Vinculin was the first cytoskeletal protein to be identified as a PTK substrate (Sefton et al., 1981) and there has been much speculation about the significance of its phosphorylation (Hynes, 1982; Cooper and Hunter, 1983). However, subsequent studies using morphological mutants of RSV which transform cells but do not produce the rounded morphology characteristic of wild type RSV transformants, indicate that tyrosine phosphorylation in vinculin is, by itself, not enough to produce a transformed morphology (Critchley and Wyke, 1986). In addition, a nontransforming mutant of RSV produces a cytoplasmic, nonmyristylated, $pp60^{v-src}$ which has been found to phosphorylate vinculin in the absence of cell transformation (Kamps et al., 1986). Finally, like most of the identified PTK substrates, vinculin is also and abundant protein and only a small proportion of its intracellular pool (1%) is phosphorylated on tyrosine (Sefton et al., 1981) making its phosphorylation less likely to be critical for transformation.

Talin, a protein present in adhesion plaques has also been recently shown to be phosphorylated

on tyrosine in RSV-transformed cells (Pasquale et al.,1986; Declue and Martin, 1987). Although talin-tyrosine phosphorylation has been studied in less detail, the fact that it is phosphorylated in rounded and nonrounded cells indicates that its phosphorylation is also insufficient to produce morphological transformation.

The third component of the adhesion plaques which serves as a substrate for PTK is the fibronectin receptor. This consists of a complex of three proteins of M.W. 160, 140 and 120 kDa. Hirst et al. (1986) have shown that in RSV-transformed cells the 140 and 120 kDa proteins are phosphorylated on tyrosine (and to a lesser extent on serine). This phosphorylation, undetectable in nontransformed cells, is temperature-dependent in cells transformed by *ts*-RSV kinase mutants and accompanies a reorganization of the receptor complex as its distribution becomes diffuse in RSV-transformed cells. Unlike vinculin (Antler et al., 1986), the phosphorylation of fibronectin receptor appears to be a general phenomenon in PTK (v–*fps*, v–*yes* and v–*erb*B)-transformed cells. Integrins are believed to be a molecular link between the ECM and the cytoskeleton (Tamkun et al., 1986) with which they interact through talin (Horwitz et al., 1986). It is possible that integrin complex phosphorylation abolishes such interactions, which could explain both the loss of cell surface fibronectin and the disorganization of the cytoskeleton observed in most transformed cells (Chen et al., 1986). Morphological mutants of RSV, which cause cell transformation without grossly altering cell morphology and cell surface fibronectin, will serve as a good system for the examination of this possibility.

**Cytoskeleton-associated proteins.** The first known PTK substrate is a protein with heterogeneous molecular weights of 34-39 kDa, designated as p36 (Cooper and Hunter, 1983). It is easily detected as a phosphotyrosine-containing protein in most PTK-transformed cells as well as normal cells treated with EGF and PDGF, but not in normal, untreated cells or cells transformed by other agents. It is a basic and abundant protein found in cells of various animal species where it is present in phosphorylated and nonphosphorylated forms (Isacke et al., 1986). It is associated with plasma membranes at the level of cell-cell junctions and it binds membranes, phospholipid and actin in a

calcium-dependent manner. A 35 kDa protein (p35), immunologically distinct from p36, is also phosphorylated on tyrosine in response to EGF treatment and also binds membranes, actin and phospholipid in the presence of calcium. Both p35 and p36 are thought to link membranes (phospholipid) to the cytoskeleton (actin), and are termed calpactin I and II. Peptide mapping, immunological and cDNA cloning studies have shown that p35 and p36 are homologous or identical to the the inhibitors of phospholipase $A_2$ known as lipocortin I and II, respectively (Pepinsky and Sinclair, 1986; Brugge, 1986). The role of these proteins in PTK actions is not known, although it appears that the phosphorylation of p36 is not sufficient for transformation (Kamps et al., 1986).

The eucaryotic calcium binding protein, calmodulin, which is also a cytoskeleton-associated protein, has been found to be phosphorylated on two tyrosine residues in RSV-transformed cells but not in nontransformed cells. Phosphorylation of tyrosine in calmodulin, which is also phosphorylated on serine, results in a structural alteration in the calcium-calmodulin complex (Fukami et al., 1986). Phosphorylation of calmodulin could be important for PTK action if it is to alter the calcium-binding properties of calmodulin.

**Other protein substrates.** The association of PTKs with plasma membranes suggest that they function in the transmission of signals generated by external stimuli, a function which is obvious in the case of receptor-type of PTKs. There is also evidence that they do so by regulating phosphatidylinositol (PI) turnover. For example, treatment of cells with PDGF leads to increased PI turnover and the rate of PI metabolism is increased in PTK-transformed cells (Hunter and Cooper, 1985). In addition PI kinase activity has been detected in $pp68^{v\text{-}ros}$ immunoprecipitates, and $pp60^{v\text{-}src}$ also has PI, PIP, and 1, 2-DG kinase activity *in vitro* but it is not clear whether this activity is intrinsic to these PTKs or is due to an associated lipid kinase (Sugimoto et al., 1984; Macara et al , 1984). Whitman et al. (1985) have reported the detection of inositol lipid kinase associated with polyoma middle T (mT) antigen preparation from mT-transformed cells. More recently, Courtneidge and Heber (1987) have shown that in addition to the previously detected mT and $pp60^{c\text{-}src}$ complexes, an 81 kDa protein (p81) is also present in either anti-mT or anti-$pp60^{c\text{-}src}$ immunoprecipitates of mT-transformed cell lysates. The p81 complexed to mT and $pp60^{c\text{-}src}$ is phosphorylated on tyrosine *in vitro* and *in vivo*, and concomitant with its presence in the

Kaplan et al. (1987) have detected an 85 kDa protein in anti phosphotyrosine immunoprecipitates of mT-transformed or PDGF -treated 3T3 cells. This protein is also phosphorylated on tyrosine and coelutes with a PI kinase activity in various purification steps. Both p81 and the 85 kDa proteins, which could be similar or identical, were proposed to be the putative PI kinases associated with the immunoprecipitates *in vitro*. Because both are phosphorylated on tyrosine, and because PI phosphorylation is a first step in the formation of PI derivatives (most notably $PIP_2$), the phosphorylation of these proteins could be an important step where PTKs control PI turnover. PI turnover results in the formation of 1,2-diacylglycerol (DAG) and 1,4,5-trisphosphate ($IP_3$), two intracellular second messengers, which result from the the hydrolysis of phosphatidylinositol 1,4,5-trisphosphate ($PIP_2$) by phospholipase C. A recent study has suggested that phospholipase C could also represent a step where PTKs control PI turnover. Wahl et al. (1988) have shown that a phospholipase C activity, specific for $PIP_2$, is increased 10-fold in phosphotyrosine immunoprecipitates of EGF-stimulated A431 cells. This study suggests that a component of the phospholipase activity (either the phospholipase itself or a protein associated with it) is phosphorylated on tyrosine subsequent to ligand activation of receptor-type of PTK, and that it could have an effect on $PIP_2$ hydrolysis and events subsequent to it.

**Phosphotyrosyl-protein Phosphatases and Cell Transformation**

Unlike the protein-tyrosine kinases which have been shown to be the protein products of viral oncogenes and their cellular counterparts, the existence of protein-tyrosine phosphatases (PTPases) has only been inferred from *in vitro* studies on protein tyrosine phosphorylation. During these early studies, it has been realized that phosphates incorporated into proteins in the form of phosphotyrosine turn over rapidly. It has been found that phosphotyrosine in proteins of *ts*–RSV-transformed cells decreases rapidly after shifting to a temperature restrictive for transformation and $pp60^{v-src}$ kinase activity. Similarly, A431 cell membrane proteins, which are phosphorylated rapidly in response to EGF treatment, gradually become dephosphorylated. These initial observations suggested the existence of phosphatases which act specifically to dephosphorylate

suggested the existence of phosphatases which act specifically to dephosphorylate phosphotyrosine in proteins and prompted several workers to purify these putative phosphatases (Foulkes, 1983).

**Assay for Phosphotyrosyl-protein Phosphatase activity**

The existence of the PTPase activity is demonstrated solely on the basis of the ability of crude or fractionated tissue or cell extracts to dephosphorylate P–Tyr-containing protein substrates. The protein substrates include histones, IgG, caseins, bovine serum albumin, $pp60^{v-src}$ and EGFr and among them, only these latter two appear to be natural substrates for PTPases, since other substrates are not phosphorylated on tyrosine *in vivo*. These substrates are usually first phosphorylated on tyrosine *in vitro* using $pp60^{v-src}$ or purified EGFr in the presence of [$\gamma^{32}$P]ATP. These $^{32}$P-labeled substrates are incubated with phosphatase preparations and the phosphatase activity is assessed by counting the acid-soluble radioactivity released from the substrates or by quantifying the residual radioactivity associated with the substrates by SDS-PAGE and autoradiography. Because of its structural similarity to phosphotyrosine, the chromogenic non-protein substrate *p*–nitrophenyl phosphate is also used in some studies.

**Purification of PTPases**

PTPases have been partially purified from cell and tissue extracts of various origins. In every extract studied, there appears to be more than one PTPase activity which can be physically separated by chromatography. Nelson and Branton (1984) have used $^{32}$P-labeled IgG phosphorylated by $pp60^{v-src}$ as a substrate to monitor the PTPase activity in cultured chick embryo fibroblasts. They have found that 70% of the activity is associated with the soluble fraction and the remaining 30% is associated with particulate material. Fractionation of the soluble fraction by DEAE-cellulose and carboxymethyl-cellulose yielded three peaks of activity, termed PTI-III which identify proteins of molecular weights of 55, 50, and 95 kDa, respectively, as estimated by gel filtration. Similarly, Foulkes et al. (1983) have fractionated the PTPase activity from chicken brain into three peaks of estimated molecular weights of 30-100 ($T_1$), 43 ($T_2$) and 95 kDa ($T_3$) which probably correspond to the PTI-III

the PTPase activity is 10-fold higher in the particulate than in the soluble fraction of TCRC-2 cell homogenates. Fractionation of the detergent solubilized particulate yielded a peak of PTPase activity which co-eluted with P–Ser-protein phosphatase activity, and had a *p*-nitrophenyl phosphatase activity. Both P–Ser-protein phosphatase and PTPase activities showed differential sensitivity towards phosphatase inhibitors and particularly, vanadate selectively inhibited the PTPase activity.

**Relationship with Acid and Alkaline Phosphatases**

Both acid and alkaline phosphatases, which are operationally-defined based on their *p*H optima for activity, exhibit PTPase activity. Leis and Kaplan (1982) showed that plasma membranes from human astrocytoma cells contain an acid phosphatase which shows an increased specificity for phosphotyrosine. It hydrolyses free phosphotyrosine but not phosphoserine or phosphothreonine. It also dephosophorylates P–Tyr-histone at a higher rate and a lower $K_m$ than P–Ser-histone and the P–Tyr-histone phosphatase activity is selectively inhibited by vanadate. In addition, the acid phosphatase of human prostate has been shown to have a specific PTPase activity (Lin and Clinton, 1986). Swarup et al.(1981) have shown that alkaline phosphatases from various sources preferentially dephosphorylate P–Tyr-histones and A431 cell membrane proteins at a higher rate than P–Ser-histones indicating that alkaline phosphatases also have PTPase activity. Chan and Stinson (1986) have also shown that purified alkaline phosphatase and the endogenous alkaline phosphatase of human liver plasma membranes dephosphorylate membrane proteins and that this activity is inhibited by vanadate.

**Characteristics of PTPases**

A common property of all known PTPases is their selective and potent inhibition by vanadate *in vivo* and *in vitro*. Swarup et al. (1982; 1982a) were the first to show that vanadate inhibits the dephosphorylation of P–Tyr-histones and A431 plasma membrane proteins (which are phosphorylated primarily on tyrosine) but does not inhibit the dephosphorylation of P–Ser-histones. Because it is a potent and selective PTPase inhibitor, vanadate is now being used in various experimental protocols to enhance the detection of P-Tyr-containing proteins in normal and

experimental protocols to enhance the detection of P-Tyr-containing proteins in normal and transformed cells and PTPases are being identified as such partly on the basis of being inhibited by vanadate (Kalrlund, 1985; Bell et al., 1987; Yonemoto et al., 1987). Zinc, in micromolar concentrations (5-100μM) is also an inhibitor of PTPases (Brautigan et al., 1981; Gallis et al., 1981) but it is somewhat less selective and less effective than vanadate. P–Tyr and its analogue *p*NPP are competitive inhibitors in the micromolar and in the millimolar range, respectively, reflecting the differential affinities of PTPases. Phosphoserine and phosphothreonine have not been reported to have an inhibitory effect. EDTA and fluoride, which are potent inhibitors of other phosphatases have little or no effect on PTPase activity

**Regulation of Protein-tyrosine Phosphorylation by PTPases**

Because purified PTPases show distinctive phosphoamino acid specificity, selective sensitivity to phosphatase inhibitors and could be separated chromatographically, they are now believed to constitute a distinct class of protein phosphatases, different from P–Ser- and P–Thr-protein phosphatases (Foulkes, 1983; Sparks and Brautigan, 1986). Therefore, the phosphotyrosine levels in proteins do not reflect only the activity of protein-tyrosine kinases, but are the result of the combined action of kinases and phosphatases. PTPases may regulate the levels of P-Tyr in proteins either by acting directly to dephosphorylate PTK substrates or indirectly through modulation of PTKs whose activity is regulated by tyrosine phosphorylation. For example, inhibition of endogenous PTPases by treatment of cells with vanadate results in 3-40–fold increase in the cellular content of phosphotyrosine in proteins (Klarlund, 1985; Yonemoto et al., 1987; DeSeau et al., 1987; Kato et al., 1987). In at least one study, evidence is given to indicate that the increase in phosphorylation of liver plasma membrane proteins is due to inhibition of a vanadate-sensitive alkaline phosphatase (Chan and Stinson, 1986). Evidence that PTPases regulate PTK activities comes from studies on catalytic properties of $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ immunoprecipitated from vanadate-treated normal and RSV-transformed cells. In both cases inhibition of phosphatase activity resulted in elevated states of phosphorylation of *src* proteins accompanied by increased activity of the viral *src* kinase activity *in*

*vitro* (Brown and Gordon, 1984; Collet et al., 1984) and decreased activity of the cellular *src* kinase (Ryder and Gordon, 1987). In addition, dephosphorylation of $Tyr^{527}$ in $pp60^{c-src}$ by phosphatase treatment *in vitro* resulted in a 10-20–fold increase in $pp60^{c-src}$ kinase activity (Cooper et al., 1986). There is also evidence that PTPases regulate PTK activity differently in normal and transformed cells. DeSeau et al. (1987) have shown that inhibition of endogenous PTPase leads to increased kinase activity of $pp60^{c-src}$ isolated from normal colon mucosal cells but that of colon carcinoma is not affected. In human prostate tissue, the major phosphatase is an acid phosphatase which has an activity specifically directed against phosphotyrosine. Lin et al. (1986) have used an experimental system whereby the activity of this phosphatase can be controlled; it is increased by addition of exogenous acid phosphatase to the cell extracts and suppressed by treatment of cells with dihydrotestosterone. They have shown that, in two cell lines derived from prostate tumor, the *in vitro* PTK activity is inversely related to the PTPase activity, giving further evidence for the role of PTPases in the regulation of PTKs.

**Regulation of Cell Growth and Transformation by PTPases**

Because phosphorylation of tyrosine is thought to be a key event in the control of cell growth, the finding that the levels of P–Tyr in proteins is regulated by both PTKs and PTPases suggests that protein tyrosine phosphatases are as much involved in control of cell growth as protein tyrosine kinases. Since PTKs are associated with the stimulation of cell growth and induction of cell transformation it is proposed that PTPases would act as negative regulators of growth and would have anti-oncogenic effects (Weinstein, 1987). Little study has been carried out to prove these points because PTPases are not yet accessible to biochemical and genetic analysis and, like in other anti-oncogenes, mutations would be recessive; therefore, their presumptive actions, if any, would be difficult to detect. Nevertheless, human malignant prostate tissue has been reported to have low acid (phosphotyrosyl) protein phosphatase activity, although it is not known whether this is the primary lesion behind its malignant transformation (Lin and Clinton, 1986). Lin et al (1986) have also noted that one prostate tumor-derived cell line (LNCaP) has higher PTPase and lower PTK activity and grows considerably more slowly than another prostate tumor-derived cell line (DU145). Inhibition of PTPase

that treatment of NRK1 cells with vanadate results in a transformed phenotype characterized by transformed morphology, growth in semi-solid medium, loss of density-dependent inhibition of growth and increased uptake of 2-deoxyglucose. Although the inhibition of PTPases is evident, as shown by a 40-fold increase in P–Tyr-containing proteins in these treated cells, the effects of vanadate cannot be fully attributed to PTPase inhibition, since vanadate acts on other cellular functions which could contribute to the observed phenotype (see below). Fibroblasts derived from patients with infantile hypophosphatasia have low (tissue non-specicfic) alkaline phosphatase activity (1-4% of controls) using the fluorogenic substrate 4-methylumbelliferyl-phosphate (4-MU-P) as a substrate yet they exhibit normal growth properties compared to control fibroblasts (Whyte and Vrabel, 1987). This study was presented to give evidence against a role for this class of phosphatases in the control of cell growth. Unfortunately, this study did not examine whether or not this phosphatase is a PTPase, and the use of 4-MU-P will not allow such a conclusion. The likely role of PTPases in control of cell growth awaits further study.

Recently, the major phosphotyrosyl-protein phosphatase form human placenta (PTPase 1B) has been partially sequenced and it shows amino acid sequence homology and structural similarity to leukocyte common antigen, a transmembrane protein, also called CD45 (Charbonneau et al., 1988). PTPase 1B does not share homology with P–Ser/P–Thr phosphatases, supporting the distinctiveness of PTPases from other phosphatases. Because CD45 has an extracellular domain, and its overall structure is reminiscent of growth factor receptors, notably EGF–r, it has been suggested that its putative PTPase activity could also be regulated by ligand binding and that a third signal-transducing mechanism, operationally controlled by PTKs and PTPases, may exist.

## III). ACTIONS OF VANADIUM ON BIOLOGICAL SYSTEMS.

### Chemical Properties of Vanadium

Vanadium is a transition element which can assume different anionic and cationic valence states. It is an essential nutrient for animals where it is found in trace amounts and affects many organ functions. Vanadium has a complicated chemistry but in the conditions of low concentration and neutral pH used in this study only a few of its chemical properties are relevant (Rubinson, 1981). Extracellular vanadium exists in the pentavalent vanadate ($V^V$) form and intracellular vanadium in the tetravalent vanadyl ($V^{IV}$) form. Upon entry into cells, vanadate is reduced by glutathione (GH) into vanadyl, in a reaction which is probably enzymatically catalyzed by GH oxidase (Cantley and Aisen, 1979). Benabe et al. (1987) have found that after 4 hr, 90% of intracellular vanadate is converted to vanadyl in red blood cells (RBC). *In vitro*, reduction of vanadate also takes place in the presence of GH or NADH. Similarly, extracellular vanadyl is oxidized to vanadate by atmospheric oxygen (Rubinson, 1981). In micromolar concentrations and neutral pH both vanadyl and vanadate would be in the monomeric form. Extracellular vanadate and intracellular vanadyl are thought to be bound to macromolecules as well as smaller molecules such as the complex formed with $PO_4$, ATP and ADP (Nechay et al., 1986). In RBC, vanadyl is bound to a soluble protein which, by gel filtration and immunoprecipitation, was identified as hemoglobin (Cantley and Aisen, 1979). Vanadium enters cells by the anion transport system, thus may compete with phosphate, and its intracellular concentration can reach 10-fold higher levels than the extracellular concentration (Ramasarma and Crane, 1981).

### Actions of Vanadium on Enzymatic Reactions

Vanadium ions affect many enzymatic reactions mainly those involving phosphotransferases.

Most data on effects of vanadium on enzyme activities has been accumulated after the discovery that vanadate is the inhibitor of (Na/K)-ATPase present as a contaminant in some commercial preparations of ATP (Cantley et al., 1977). It is now known that vanadate inhibits all members of ion transport ATPases (reviewed in Ramasarma and Crane, 1981). These are transmembrane enzymes which assume two (E1 and E2) conformations and are defined as such by the presence of an aspartyl group in their active site of phosphorylation. In the case of the $Na^+/K^+$-ATPase, inhibition involves formation of a stable inactive complex of vanadate with the active site of the enzyme in E2 conformation (Nechay et al., 1986). Like $P_i$ and ATP, binding of vanadium to this site, hence inhibition of enzyme activity, requires the presence of the divalent cation $Mg^{2+}$. *In vivo*, inhibition of Na/K ATPase by vanadate takes place intracellularly and the concentration of vanadate required to achieve such inhibition is generally higher than the concentration needed to inhibit the enzyme *in vitro*. It has been suggested that this is due to conversion of vanadate into vanadyl which does not have inhibitory effects on the ATPase *in vivo*. In fact, it has been found that in micromolar concentrations, both vanadate and vanadyl activate rather than inhibit Na/K-ATPase in human fibroblasts (Smith, 1983). Vanadate was also found to stimulate Na/K-ATPase in Friend erythroleukemia cells (English et al., 1983) and in nonmuscle cardiac cells (Werdan et al., 1982). It has been suggested that vanadate inhibition of sodium ATPase *in vivo* may be restricted to the RBC-type of enzyme.

Other enzymes affected by vanadate *in vitro* are alkaline and acidic phosphatases which have phosphotyrosine protein phosphatase activities mentioned in the previous section, adenylate kinase, and several enzymes of the glycolytic pathway. Most vanadate inhibitory effects have been explained by its structural analogy to the transition state of phosphate. The glycolytic enzyme diphosphoglutarate dehydrogenase, however, is not inhibited by a transition state analogue mechanism but rather by changes in the redox state of sulfhydryl groups in the enzyme (Benabe et al., 1987).

Little studies have been carried out to investigate the *in vitro* effects of vanadyl on enzymatic reactions because of its sensitivity to atmospheric oxygen. Nevertheless, North and Post (1984) have studied the effects of millimolar concentrations of vanadyl on (Na/K)-ATPase *in vitro* in anaerobic conditions to avoid oxidation of vanadyl and the resulting inhibition by vanadate. This study

has shown that vanadyl is also a potent inhibitor of this ATPase and that the extent of inhibition depends on the purity of enzyme preparations.

**Insulin Mimetic Effects of Vanadium**

One of the most studied *in vivo* actions of vanadate is its insulin mimetic effects on glucose uptake and metabolism. Because of this biological action, vanadate is widely used as a specific probe to investigate mechanisms of insulin actions. Vanadate increases the rate of glucose uptake in adipocytes and skeletal muscle in a manner similar to that of insulin by activating the insulin-sensitive facilitated transport system for glucose (Dubyak and Kleinzeller, 1980; Clark et al., 1985). The effects of insulin and vanadate are additive in that vanadate does not enhance the rate of glucose uptake in adipocytes preincubated with insulin and simultaneous presence of vanadate and insulin does not further increase maximum level of stimulation. Glycogen synthase is also activated by vanadate in a similar manner than insulin (Tamura et al., 1984). Vanadate effect is independent of Na/K ATPase, whose inhibition also has insulin mimetic effects, but occurs as vanadate is converted into vanadyl which does not inhibit Na/K ATPase *in vivo* (Dubyak and Kleinzeller, 1980).

Tamura et al. (1984) found that vanadate, in addition to stimulating glycogen synthase in a dose- and time-dependent manner in rat adipocytes, it also enhances tyrosine phosphorylation of the 95 kDa insulin receptor β-subunit, by activation of insulin receptor PTK activity towards the receptor itself and exogenous substrates. Stimulation of the receptor PTK activity was proposed as a main mechanism by which vanadate mimics insulin. However, it has been argued that insulin mimetic actions of vanadate do not depend on its effects on insulin receptor phosphorylation or kinase activity (Green, 1986). In this study, it has been found that insulin receptor depletion, either by trypsin treatment or ligand-induced receptor internalization, abolishes the effects of insulin on hexose uptake, but the vanadate effects are unaltered. Vanadate also mimics one important biological function of insulin which is receptor down-regulation. However insulin and vanadate, which induce internalization of 40% and 60% of cell surface receptors, respectively, act differently; insulin-induced receptor down-regulation is accompanied with receptor degradation, and results in a decrease in total

cell insulin receptors. On the other hand, vanadate in addition to inhibiting degradation of internalized receptors, it also increases the total number of cryptic receptors, accumulated in intracellular compartments (Torossian et al., 1988). Because vanadate, in this case, did not increase the phosphorylation of the insulin receptor or its kinase activity, it has been suggested that vanadate acts independently of insulin receptor or at a post-receptor level. It has recently been shown that exogenously added diacylglycerol (DAG) or phospholipase C (PLC) stimulate glucose uptake in adipocytes and that DAG can mediate the effects of insulin (Strålfors, 1988). Vanadate also stimulates PLC which produces DAG from $PIP_2$ (see below), therefore, it is possible that insulin-mimetic effects of vanadate are also mediated by DAG.

**Vanadium and Transmembrane signal Transduction**

The present model offered to explain how mitogens (competence factors) bring about initiation of cell proliferation proposes that these effectors trigger a cascade of events which lead to DNA synthesis. These events, which include activation of PLC; breakdown of $PIP_2$ to generate diacylglycerol and inositol phosphates; increase in cytoplasmic calcium; and activation of Na/H exchange leading to cytoplasmic alkalinization, act in concert to induce gene expression (*myc*, *fos*) and commit cells to initiate replication (Macara, 1985). It has been found that vanadium elicits most of these immediate and rapid events and has been suggested to act as a competence factor (Macara, 1985). In quiescent human HSWP cells, vanadate treatment stimulates formation of inositol phosphates, mobilization of intracellular calcium and Na/H exchange (Jamieson et al., 1988). The action of vanadate is similar to that of competence growth factors in that it is abolished by TPA pretreatment which blocks a step before the inositol phosphates release, which in this case is the inactivation of PLC by protein kinase C. It has been suggested that vanadate acts by either inhibiting the dephosphorylation of a growth factor receptor or its substrates or by stabilizing a growth factor receptor/PLC-coupling G protein in the stimulatory ($G_s$) conformation (Jamieson et al., 1988). Formation of inositol lipids has also been observed in resting chinese hamster lung fibroblasts as a response to vanadate treatment (Paris and Pouysségur, 1987). Because vanadate effects were blocked by pretreatment with pertussis toxin, it is suggested that vanadate stimulates PLC activity by

directly activating regulatory G proteins. Like in the study carried out by Jamieson et al. (1988), pretreatment of cells with TPA abolishes the effects of vanadate supporting PKC-dependent inactivation of phospholipase–C. Because PTKs were found to control PI turnover, it is also possible that vanadate indirectly affects PI turnover by its actions on protein-tyrosine phosphorylation which, as mentioned in the previous sections, can be an important regulator phosphoinositol lipid formation and breakdown.

**Mitogenic Effects of Vanadium**

As a result of its early competence actions, vanadium exerts mitogenic activity on quiescent cells, and has synergistic effects with mitogenic growth factors EGF and FGF. Vanadate alone is half as much mitogenic for quiescent cultures of human fibroblasts as EGF, as shown by stimulation of incorporation of [$^3$H]–thymidine into DNA and increase in cell number. The synergistic effects of vanadate and EGF produces 3- to 6-fold increase in stimulation of DNA synthesis as compared to EGF alone (Carpenter, 1981). In quiescent cultures of Swiss mouse 3T3 and 3T6 cells, both vanadyl and vanadate were found to be mitogenic in the concentration range of 5-50 μM. The mitogenic effects of vanadium, which were potentiated by insulin in absence of serum, were additive to the effects of EGF (Smith, 1983). Interestingly, both vanadyl and vanadate were found to stimulate rather than inhibit the Na/K pump, as shown by a stimulation of ouabain-sensitive $^{86}Rb^+$ uptake. The growth stimulatory effect of vanadate has also been observed in a clone of mast cells (IC2 cells) which are strictly dependent on interleukin 3 (IL–3) for growth and survival. Vanadate, at the optimal concentration of 12.5 μM allowed these cells to grow and survive in absence of IL–3, for up to 48 hr by which time these cells normally die when IL–3 is absent (Tojo et al., 1987). The growth characteristics of IC2 cells in presence of vanadate were similar to those grown in presence of IL–3. It is thought that vanadate sustains the growth of IC2 cells by increasing glucose uptake and oxidation or by activating an ATP-generating system which appears to be the main identified action of IL–3 (Tojo et al., 1987).

**Vanadium and Cell Transformation and Differentiation**

The effects of vanadium on cultured cells, measured within minutes or hours are reflected in its observed profound actions on cell transformation and differentiation upon long time treatments (days). In friend erythroleukemia, for example, which can be committed to differentiate into hematopoietic cells, by treatment with dimethylsulfoxide (DMSO), vanadate was found to inhibit DMSO-induced differentiation and inhibit hemoglobin synthesis which is a marker for erythropoietic commitment (English et al., 1983). Vanadate was also found to mimic TPA and FGF angiogenic actions on capillary endothelial cells grown in three dimensional collagen gels. It induces endothelial cells to invade the collagen gels and to form tubular structure (Montesano et al., 1988). Vanadate induced invasive phenotype in these cells by inducing expression of tissue-type and urokinase-type of plasminogen activators. In $BC_3H1$ tumor-derived cells, a nonfusing muscle cell line which undergoes reversible differentiation when arrested in $G_1$ upon serum or growth factor starvation, vanadate was found to fully mimic the competence actions of pituitary-derived FGF (Wice et al., 1987). Vanadate was found to reverse the differentiated phenotype of $BC_3H1$ cells and suppress expression of muscle specific proteins $\alpha$-actin and creatin phosphokinase. In addition it also induces $BC_3H1$ cells to enter the cell cycle as indicated by the induction of c-*fos* expression which marks the transition from $G_0$ to $G_1$ phase of the cell cycle.

Long-term treatment with vanadate also results in profound changes in cellular phenotype reminiscent of those of retroviral-transformation. As mentioned in the previous sections, vanadate induces a transformed phenotype in NRK, NIH–6 Cl 32, and $10T^1/_2$ cells (Klarlund, 1985). Kato et al., (1987) have indicated that in chondrocytes, vanadate, which at low concentrations stimulates matrix proteoglycan synthesis, induces a phenotype characteristic of RSV-transformed chondrocytes at high concentrations (20-60 $\mu$M). Vanadate also mimics RSV-mediated transformation as shown by down-regulation of junctional cell-cell communication in *ts*-RSV-transformed 3T3 cells treated with vanadate (Rose et al., 1986). In BHK cells, vanadate in micromolar concentrations causes F-actin redistribution and formation of podosomes characteristic of RSV-transformed cells (Tarone et al., 1985). In addition to its effects on cytoskeleton remodeling, which were potentiated by simultaneous treatment with TPA, vanadate was also found to increase the cellular content of phosphotyrosine-protein (Marchisio et al., 1988).

## IV). SECRETED PROTEINS AS MARKERS FOR NEOPLASTIC TRANSFORMATION.

**Tumor Promotion-Specific Genes**

The study of acute transforming retroviruses and use of DNA transfection techniques have identified a large number of viral oncogenes as the primary agents responsible for cell transformation *in vitro* and development of tumors in animals *in vivo* (Bishop, 1985) Although the molecular cloning of retroviral oncogenes has allowed extensive genetic analyses to be carried out, their mechanism of action remains largely unknown. In this context, the case of protein tyrosine kinase class of oncogenes is a very illustrative example. However, it is believed that oncogenes initiate and maintain transformation not by introducing new functions into the cells, but by disturbing the pre-existing cellular mechanisms involved in control of cell growth. The existence of chemical and physical agents which obviously do not bring new genetic information into the cells and yet cause cell transformation supports this view and stresses the oncogenic potential of cellular oncogenes (proto-oncogenes) from which viral oncogenes are thought to be derived. These agents, however, do alter gene expression or distort the function of cellular genes in a manner which favors the expression of the malignant phenotype. Altered expression or malfunction of one or more cellular oncogene has indeed been detected in naturally occurring or experimentally induced malignancies. Because viral or activated cellular oncogenes cannot be directly implicated in each of the many unrelated aspects of the transformed phenotype, a great deal of work has concentrated in the identification of genes which are expressed exclusively as a response to the transforming agents mentioned above. Identification of these genes and their products is important as they could be the direct mediators of cell transformation (Weinstein, 1987).

Tumor promoters such as phorbol esters are one class of those agents whose actions are widely

studied (Castagna et al., 1987). The phorbol ester tetradecanoyl phorbol acetate (TPA) induces a myriad of cell responses, of which some lead to mitogenesis or terminal differentiation, depending on the cell type. Although they are not carcinogenic by themselves, they most interestingly induce responses which result in malignant transformation in tumor-initiated cells (Colburn, 1987). The mouse epidermis JB6 cell line responds to TPA treatment by an irreversible transition from nontumorigenic to tumorigenic phenotype in a process called tumor promotion. By successive subdivision (or sib selection), two active genes, pro-1 and pro-2, which determine the responsiveness to TPA have been identified and cloned from promotable ($P^+$) JB6 cDNA library and were found to confer the promotion sensitivity to a nonpromotable ($P^-$) variant of the JB6 cell line (Colburn, 1987). The availability of $P^+$ and $P^-$ variants of this cell line, which are, respectively, defined as such by their ability or inability to grow in soft agar in the presence of TPA, make this cell line particularly useful to select genes which are specifically expressed in $P^+$ cells in response to TPA and which therefore are very likely to cooperate with the pro genes in the process of tumor promotion.

**Identification and Cloning of 2ar as a Tumor Promoter-Inducible Gene**

One of the TPA-inducible messages in $P^+$ JB6 cells is called 2ar, described by Smith and Denhardt (1987). 2ar was cloned from JB6 cells cDNA library that has been enriched, by hybrid selection, for sequences which are relatively more abundant or selectively-induced in TPA-treated $P^+$ compared to TPA-treated $P^-$ JB6 cells. 2ar mRNA is a unique 1.6 kB transcript of a single copy gene. Despite the procedure used to clone 2ar, it was found that it is induced by TPA in $P^-$ cells as well, although at more variable levels than in $P^+$ cells. Kinetic studies indicate that the induction of 2ar expression by TPA is biphasic and depends on cell population density, but not on the duration of exposure to TPA. In subconfluent cells, the induction is rapid and transient, and in confluent cells a second stable reinduction appears after three days and persists up to eight days. In addition to TPA, 2ar is also induced by growth factors such as PDGF, EGF, basic FGF and embryonal carcinoma-derived growth factor (Smith and Denhardt, 1987; Nomura et al., 1988). The facts that 2ar is induced both in $P^+$ and $P^-$ JB6 cells and that it is not expressed in mouse skin epidermis treated with TPA *in vivo*, exclude 2ar from having a role in tumor promotion. However, because of its induction by growth

factors and as will be seen later, it is very likely to play a role in some aspect of cell transformation.

**Characteristics of 2ar/Osteopontin**

Examination of the localization of 2ar protein product indicates that it is secreted in the form of an MSP. Analysis of the cDNA sequence (Craig et al., unpublished) also revealed that it shares 84% homology with rat osteopontin (Oldberg et al, 1986) at the deduced amino acid sequence level (Smith and Denhardt, 1987; Denhardt et al., 1988). Osteopontin was originally purified from mineralized bone matrix after extraction with 0.5 M EDTA and 4 M guanidinium chloride (Fisher et al, 1985; Prince et al., 1987). Osteopontin, also previously known as bone sialoprotein I or (BSP I) or 44k bone phosphoprotein (44k BPP), was also found to be secreted as a phosphoprotein by osteoblast-like osteosarcoma cells (Prince et al, 1987). It is composed of about 300 amino acids and has a molecular weight of about 44 kDa as determined by sedimentation equilibrium analysis. Both secreted and bone-extracted osteopontin and the product of *in vitro* translation of 2ar exhibit abnormal mobility on SDS-PAGE; they show a molecular weight of 75 kDa on 5-15% gradient gels and 45 kDa on 15% gels; the *in vitro* translation of 2ar yields a peptide of a molecular weight of 60 kDa on SDS-PAGE which is considerably larger than the ~30 kDa calculated for the core protein (Craig et al., submitted)[1]. These discrepancies are likely to be due to complex patterns of post-translational modifications and to high content of negatively charged amino acid residues. Osteopontin contains 16.6% carbohydrate of which 1 is N-linked and 5-6 are O-linked. Carbohydrate composition analysis indicates that it is rich in N-acetylneuraminic (sialic) acid residues (10.4 per mol; hence the name BSP I). It also contains 12 phosphoserine and 1 phosphothreonine residues. It is an acidic protein because of high aspartic acid and glutamic acid content and the additional negative charges introduced by post-translational modifications. Osteopontin binds to hydroxyapatite with high affinity (Fisher et al., 1987; Prince et al., 1987).

The cDNA sequence data reported by Oldberg et al. (1986) and that of Craig et al.(submitted)[1] is consistent with this chemical characterization. It predicts a protein 317 amino acids long, with a sequence identical to the chemically determined amino terminal sequence of osteopontin (Prince et

[1]Craig; A. M , J. H. Smith, and D. T. Denhardt. (*submitted*)

al., 1987), starting at amino acid 17 from the methionine encoded by a putative AUG initiation codon. The other 16 amino acids probably represent the signal sequence cleaved during the translocation of the nascent protein across the membrane into the lumen of the endoplasmic reticulum. It has several Ser-Xaa-Glu sequences, likely sites for attachement of O-linked, and one Asp-Xaa-Ser for N-linked carbohydrates.

Osteopontin also contains a functional RGD cell recognition sequence found in ECM proteins, and osteopontin isolated from bone promotes adhesion and spreading of fibroblasts and osteosarcoma cells in a dose dependent manner (Oldberg et al., 1986; Somerman et al., 1987; 1987a). As in the case of fibronectin, the osteopontin mediated cell-substratum attachment is RGD-sensitive in that it is also inhibited by soluble RGD-containing peptides. Unlike fibronectin, however, whose attachment-promoting activity ceases within a few hours, osteopontin continues to enhance cell attachement for up to 24 hours. It is also different from fibronectin (and laminin) in that it does not promote attachement of epithelial cells, does not promote adhesion of fibroblasts to surfaces coated with type-I collagen and is not inhibited by the presence of anti fibronectin antibodies (Somerman et al., 1987).

**Tissue Distribution and Developmental Expression of 2ar/Osteopontin**

Using immunohistochemical techniques with affinity-purified anti-osteopontin antibodies, Mark et al. (1987; 1987a) have examined the distribution of osteopontin in developing bone. Osteopontin was found in the ECM of both endochondral and membranous bones as well as the osteoid. Staining was found in pre-osteoblasts, osteoblasts and osteocytes where it is localized to the Golgi apparatus, indicating that bone matrix osteopontin is synthesized by bone cells. Osteopontin is localized at the bone ECM in early stages of development, before bone mineralization, indicating that this localization is independent of the presence of hydroxyapatite crystals to which osteopontin has a high affinity. Osteopontin was also detected in kidney and nervous tissues of newborn rats (Mark et al., 1987b). The study of Nomura et al. (1988) has also supports that osteopontin expression is not confined to bone cells, as previously thought, but is expressed in the developing non osteogenic tissue as well. They have examined the developmental expression of osteopontin, in mouse, by Northern analysis

and *in situ* hybridization using 2ar-derived DNA probe. Osteopontin expression is detected in the auditory nerve (cochlea) and sensory epithelium (uticle and saccule) of embryonic ear, in the tubules of embryonic and newborn mouse kidney, in the granulated metrial gland cells in placenta and deciduum as well as in the osteoblasts and pre-osteoblasts in the developing bone. 2ar expression could not be detected in fibroblasts, epidermal or mesenchymal cells of adult mice. Therefore, it has been suggested that expression of osteopontin in cultured cells is a response to cell culture, due to continuous exposure of cells to inducing agents or relief from the action of an *in vivo* inhibitory factor (Nomura et al., 1987).

**Major Secreted Phosphoproteins as Markers for Malignant Transformation**

Because cell transformation results in alteration of cellular functions, an alternative approach to understand the process of transformation is to directly investigate and define functions which are altered by transformation. With this view in mind, studies have been sought to find molecular and biochemical markers which distinguish transformed from normal cells. Studies have been centered on transformation-specific or transformation-induced secreted proteins which are of primary importance for the transformed phenotype as they are the means by which cells interact with their extracellular environment. Quantitative and qualitative alterations in a number of secreted proteins have been observed in transformed cells. These include growth factors some of which (TGFs) are preferentially secreted by transformed cells and act as autocrine mitogens (DeLarco and Todaro, 1978); ECM components which determine the stationary, invasive or migratory state of malignant cells; and matrix degrading proteases which play a central role in invasion and metastasis (Denhardt et al., 1986). Realizing their importance in the transformed phenotype, investigators have concentrated their efforts on detailed analysis of secreted proteins using computerized techniques (Celis et al , 1988).

A number of other proteins whose functions are not yet known are secreted exclusively or at higher levels by transformed cells. Of particular relevance to this study are MSPs originally described in transformed cells. Work on major transformation-specific secreted phosphoproteins has been initiated by Senger and his co-workers. In their earlier studies (Senger et al., 1979), they have shown that mammalian cells transformed by various agents secrete immunologically related proteins with molecular weights of about 58 kDa which are not secreted by their nontransformed counterparts. They also secrete unrelated phosphoproteins of heterogeneous molecular weights also in the 58 kDa range. The size of these phosphoproteins, which are not related to any known oncogene product (Senger et al., 1980), is species specific and does not depend on the transforming agent. These phosphoproteins, collectively referred to as pp62, have been found to be secreted in almost undetectable amounts by nontransformed cells, if at all. In a subsequent study (Senger et al., 1980a),

they have shown that all the phosphoproteins secreted by cells of different species origin are immunologically related, as indicated by their cross-reactivity with an anti-serum raised against the protein secreted by the rodent B77-NRK cell line. Comparisons of the levels of secreted pp62 in various cells indicated that it is markedly elevated in neoplastic, epithelial and fibroblastic cells and is therefore, a marker which distinguishes neoplastic from nonneoplastic and normal cells (Senger et al., 1983). A survey of human cell lines has also resulted in detection of MSPs of molecular weight 66-69 kDa which are also cross reactive with the anti-serum raised against the rodent phosphoprotein (Senger et al., 1985). In particular, it has been found that, regardless of the transforming agent, all malignant human cells, in the form of established cell lines or fresh tumor explants secrete this protein, and that nonmalignant counterparts do not. It has been suggested that these transformation-specific or -marker proteins play a role in tumor growth *in vivo*. Recently, Senger et al. (1988) have detected proteins in rat and human plasma and serum which cross-reacted with tha antiboby prepared against rodent pp62. These blood proteins, as well as pp62 secreted by cultured cells, were found to be cleaved during blood coagulation, and by purified thrombin, into a 30 kDa protein as. The protein was purified to homogeneity and an N-terminal 9 amino acid-fragment has been sequenced. Although the authors stated that this sequence is not homologous to any other protein, it is, in fact, identical to that of 2ar/osteopontin determined either by protein (Prince et al., 1987) or cDNA sequencing (Oldberg et al., 1986; Craig et al., Unplublished).

**Major Secreted Phosphoproteins of Normal and Transformed Cultured Cells**

Studies of secreted phosphoproteins have been undertaken in our laboratory to further characterize and more importantly to determine to which of the parameters of the transformed phenotype they most closely relate, information which is likely to provide insight as to their functional role. The rationale behind initiating these studies is manifold. First, the specific pattern of secretion of these phosphoproteins by normal and transformed cells, and in benign and malignant tumor cells indicates that they are not secreted fortuitously and that they have a role to play in the malignant phenotype. Second, not many secreted proteins are phosphorylated; these proteins would be among the few which would act extracellularly. Third, as phosphoproteins, they deserve further

study, since phosphoproteins play key roles in cellular regulation.

To approach this problem, studies in our laboratory have taken advantage of agents such as retinoids, which interfere with the transformed phenotype. These have been used to probe the effect of reversion of the transformed phenotype on the expression and phosphorylation of these secreted proteins. These studies have shown that RSV-transformed rat (RR1022) and vole (SR-1T) cell lines secrete a phosphoprotein having a molecular weight of 62 kDa called pp62 (Chackalaparampil et al., 1985). Treatment of these cells with retinoic acid (RA) causes a loss of some properties of the transformed phenotype, particularly the rounded morphology and anchorage independent growth. The kinase activity of $pp60^{v-src}$ and secretion of TGFs were not affected. Other transformation-specific parameters such as alteration of EGF binding, ornithine decarboxylase activity, and aspects of cell matrix and skeleton were not examined in this study but were previously found to not be affected by retinoic acid treatment (Mukherjee et al., 1982). Concomitant with the loss of anchorage independent growth and restoration of normal morphology following RA treatment was a decrease in secretion of pp62 and a gradual appearance of a slightly larger protein of M. W. 69 kDa (pp69). Examination of nontransformed NRK and Rat-1 cells indicate that they also secrete a protein of the same size, 69 kDa. The 69 kDa phosphoproteins secreted by normal cells and by RA-treated RSV-transformed cells are similar in their V8 protease peptide maps and isoelectric points, and also share fragment homology with pp62. An anti-serum raised against the NRK-secreted pp69 eluted from gel bands precipitates pp69 from NRK and RA-treated RR1022, pp62 from untreated RR1022 cells and both pp62 and pp69 from Ki–MSV-transformed rat (KNRK) cells as well as MSPs from mouse and avian cell lines (Laverdure et al., 1987; Mukherjee et al., unpublished; and RESULTS section). The secretion of either pp69 or pp62 is also temperature-dependent in *ts*–RSV-transformed cells (Mukherjee et al., unpublished). These studies have established a close relationship between the secretion of pp69 or pp62 and anchorage dependent or independent growth in RA-treated and untreated RSV-transformed cells and these proteins are now considered as markers for nontransformed and transformed phenotypes, respectively. During the course of these studies, it has also been found that the secretion and phosphorylation of pp69 in NRK cells is

modulated by growth factors (Laverdure et al., 1987). EGF alone, or EGF and TGF–β which induce colony formation in NRK cells increase the secretion of pp69 whereas TGF–β alone decreases it in a dose-dependent manner. Although there is no direct evidence to demonstrate that the proteins studied in this laboratory are related to the ones reported by Senger's group, the fact that the same RR1022 cell line was found, by both laboratories, to secrete a major phosphoprotein with identical molecular weight of 62 kDa indicates that they are most certainly the same. The cross-reactivity of anti-rat phosphoprotein antisera, prepared in both laboratories, with major secreted proteins of cells of various species of origin also supports this view. In addition, since the recent N-terminal sequencing of transformation-associated pp62 indicates that pp62 is identical to 2ar/osteopontin, the same conclusion could be drawn for a relationship between pp69/pp62 and 2ar/osteopontin.

## V). AIMS OF THIS STUDY.

This study is a contribution to the characterization of major secreted transformation-sensitive proteins described in the previous section. Whereas previous studies have shown that the secretion of pp62 or pp69 is a clear indicator of anchorage-independent or -dependent growth, respectively, a number observations made in this laboratory indicate that this is not an absolute correlation. First, secretion of low, albeit significant levels of pp69 by RR1022 cells is occasionally observed. Second, ras-transformed cells such as KNRK cells grow at high efficiency in soft agar but secrete both pp62 and pp69, in variable amounts, but not pp62 alone.[2] Third, mouse cells, although not as rigourously examined, appear to secrete immunologically-related phosphoproteins of lower molecular weight (58-65 kDa) regardless whether or not they grow in soft agar. Fourth, NRK cells treated with EGF and TGF-β can be plated with high efficiency in soft agar but do not secrete pp62. Finally, with increasing passages and time in culture, NRK cells, which at low passage secrete uniquely pp69, gradually start secreting pp62 in addition to pp69, in nearly equal proportions although their growth in soft agar is not increased proportionately. Therefore, secretion of either pp69 or pp62 appears to correlate tightly with anchorage dependence or independence only in src-transformed cells.[3] However, because secretion of pp62 is observed in non-src-transformed cells (KNRK cells) and NRK cells grown to high passage, at which point irreversible transformation is more likely to spontaneously occur, it appears that secretion of pp62 is associated with irreversible cell transformation and that it is a general phenomenon for all transformed cells.

The question addressed in the experiments described in this study concerns the transformation

[2]KNRK cells have not been examined for their secreted proteins in the presence of retinoic acid treatment since they are resistant (Mukherjee et al., 1983) or respond differently to retinoic acid (Jetten et al., 1986).

[3]However, secretion of pp62 is independent of the PTK of the src protein since retinoic acid affects its secretion without altering the kinase activity of $pp60^{src}$ (Chackalaparampil et al., 1985).

sensitivity of secretion and phosphorylation of MSPs. In this study, the vanadium-induced cell transformation is used to probe for what effects this transformation would have on MSPs. The initial goal was to determine whether or not secretion of pp62 will be induced in vanadium-treated cells, which would support the generality of the phenomenon of secretion of pp62 in transformed cells. Specifically, it will allow to determine whether secretion of pp62 is favored in conditions of increased tyrosine phosphorylation. This system was chosen because transformation is induced reversibly, therefore making it possible to determine whether secretion of pp62 is restricted to irreversibly transformed cells. In addition, because such a system allows to induce transformation in a dose-dependent manner, it allows a better examination of which transformation parameter secretion of these proteins is most closely related. Furthermore, in contrast to growth factors, which have pleiotropic effects and their mechanisms of action are not precisely known, vanadium acts on relatively fewer cell functions. In the micromolar concentrations used in this study most of these actions are, as pointed in numerous studies, attributable to inhibition of tyrosine dephosphorylation; other actions observed at higher concentrations are, thus excluded. The cell line NRK-49F was chosen to carry out this study for its growth properties; it can be plated with high efficiency therefore can be maintained easily. It is also the cell line of choice for the assessment of transformation parameters, and the responses to vanadium by a related clone, NRK–1, are known. In addition, most of the initial work on secreted phosphoproteins has been carried out using this cell line.

The other possibility which existed is that phosphorylation of MSPs is also transformation-sensitive, a possibility which has a special value in determining the functional role of these proteins and in relating phosphorylation to their function. To address this question, secreted proteins have been analysed by two-dimensional gel electrophoresis which separates phosphorylated from nonphosphorylated proteins as well as differentially phosphorylated isoforms of a given protein.

During the course of this study, it has also been reasoned that like most secreted proteins, in order for MSPs to carry out a functional role, they must, by some means, interact with the cells surface. Therefore their cell surface localization has been examined by cell surface radioiodination and immunoprecipitation.

Because both 2ar and osteopontin have both been found to be secreted phosphoproteins, their immunological relationship to MSPs has been examined. This was important since 2ar/osteopontin have been characterized in more detail and their primary structure, suggestive of an adhesive function, is known; demonstration of a relationship between these proteins is of special importance in both understanding their functional roles and the basis for size heterogeneity in normal and transformed cells and its possible functional implications.

# VI). MATERIALS AND METHODS.

## CELL CULTURE.

### Cell Lines and Growth Conditions.

The cell lines used in this study are: NRK-49F, an normal rat kidney cell line, sub-clone 49F (DeLarco and Todaro. [1978]. *J. Cell. Physiol.*, **94**:335); KNRK, Kirsten murine sarcoma virus-transformed NRK cells (Aaronson and Weaver. [1979]. *J. Gen. Virol.*, **13**:245); RR1022, Schmidt-Rupin Rous sarcoma virus-transformed rat cells (Garbers. [1982]. *Virol.*, **118**:419); KA31, Kirsten murine sarcoma virus-transformed mouse Balb/c 3T3 cells (Aaronson and Weaver. [1979]. *J. Gen. Virol.*, **13**:245); PT-NRK are high passage number NRK cells (≥ 30); agar colony-selected PT-NRK cells were obtained by Dr. G. Brasileiro in this laboratory and are available in frozen stock under the name Col. NRK xx; [xx] denotes the number of times passaged after selection, before freezing.

NRK, KNRK, RR1022 and KA31 cells were obtained from the American Type Culture Collection (ATCC Rockville, MD) and grown in DMEM supplemented with 10% Newborn Calf Serum (NCS, GIBCO) and antibiotics (streptomycin 63 µg/ml, penicillin 12.5 µg/ml, and gentamicin sulfate 1.25 µg/ml.) Cells were fed every second day and subcultured every 3-4 days before reaching confluency. NRK cells were routinely assayed for anchorage independent growth, and only early passages were used for experiments except where otherwise stated. All cell lines are free of mycoplasma contamination as determined by Hoeschst staining (Chen, 1977).

### Cell Treatment.

Vanadyl sulfate and sodium orthovanadate were purchased from Aldrich Chemical Company and

prepared as 1 mM stock solutions in $ddH_2O$, sterilized by filtration and stored in aliquots at -20 °C without any further treatment. Cells were treated by direct dilution of these stock solutions into growth medium to the final concentration for the periods of time indicated for individual experiments.

## ANALYSIS OF CELL GROWTH.

### Monolayer Growth.

For the analysis of monolayer growth, NRK cells were prepared by trypsinisation, counted and seeded at a density of 2.4 X $10^4$ cells/35 mm tissue culture dish in seven sets for each concentration in triplicate dishes. The cells were allowed to adhere for 24 hr and vanadyl sulfate and/or sodium orthovanadate were added to the desired final concentrations. The initial cell number (day zero) was determined by counting the cells in three randomly chosen dishes. The cells were grown at 37 °C, fed every third day and counted every 24 hr, starting 24 hr post-treatment. Cells were harvested in 1.0 ml of trypsin-EDTA (1 mg/ml) at 37 °C for 5 min, then trypsin inhibitor (mg/mg) was added in PBS and the cells were aspirated gently through a narrow gauge pipette to separate the cultures into single cells in a total volume of 20.0 ml of PBS. For each culture the cell number is determined five times using a coulter counter and normalized for the reading error of the machine, where applicable.

For analysis of contact inhibition of growth, cells were first plated in vanadium-free medium, allowed to reach confluency (4 days) and then vanadium was added to the indicated final concentrations. Cells were counted as described above.

### Soft Agar Growth.

Colony formation assay was carried out, based on that of Macpherson and Montagnier (1964), in 0.5% agarose (base layer) and 0.3% agarose (soft suspension layer). The base layer was made by dilution of a 3% agarose solution with DMEM supplemented with NCS and antibiotics to normal final concentrations. Each dish received 5.0 ml of this solution which is then allowed to solidify at room temperature for 1 hr or more. The cells were harvested by trypsinisation, counted and resuspended in 0.3% agarose in DMEM and adjusted to 5. $10^3$ cells/ml. Two milliliters of the cell suspension were

poured on the top of the hardened base layer. Where used, vanadium was included to the indicated final concentrations in both base and suspension layers. Cultures were grown in a humidified incubator at 37 °C in the presence of 5% $CO_2$ and 95% air. Cells were fed every fourth day by adding fresh DMEM in 0.3% agarose. After 10 days, the formed colonies were photographed and counted under a calibrated grid using an inverted microscope; colonies in five randomly chosen area were counted.

## LABELING PROCEDURES.

### Metabolic Radiolabeling.

For metabolic radiolabeling, subconfluent, actively growing cells were labeled 24 to 36 hr after plating in 35 or 60 mm tissue culture dishes (Nunc) at a density of 5 $X10^4$ or $10^5$ cells/dish, respectively. For labeling of phosphoproteins, cells were washed twice with serum-free and phosphate-free DMEM and incubated in this medium during 0.5-1 hr for phosphate starvation. For the treated cells, vanadyl and vanadate were omitted from the incubation medium during this period to avoid accumulation of vanadium to high intracellular concentrations because of the lack of competition from phosphate which is transported by the same mechanism (Ramasarma and Crane, 1981; Klarlund, 1985). Cells were then labeled in 1.0 ml of phosphate-free and serum-free DMEM supplemented with 400 µCi/ml of carrier-free [$^{32}$P]-$PO_4$ (Amersham) for 4 hr at 37 °C in a humidified incubator in the presence of 5% $CO_2$.

For $^{35}$S labeling, cells were labeled as above with 100 µCi/ml of [$^{35}$S]-Methionine (specific activity 1.25-1.425 mCi/mmol, Amersham) in 1.0 ml of serum-free and methionine-free DMEM in the presence of the indicated concentrations of vanadyl after 1 hr of methionine starvation in methionine-free DMEM.

### Cell Surface Radioiodination.

Radioiodination of cell surface proteins was carried out by the lactoperoxidase procedure. Semi-

monolayer cultures were washed twice with Dulbecco's complete PBS (see below) supplemented with 0.9 mM $CaCl_2$ and 0.49 mM $MgSO_4 . 7H_2O$). The iodination reaction was carried out in 2.4 ml of complete PBS containing 300 µCi of Na[$^{125}$I] (specific activity 100 µCi/µl, Amersham), 300 µl of β–D–glucose (5 mg/ml, Sigma), 150 µl of glucose oxidase (0.5 mg/ml, Fraction VII, Sigma) and started by addition of 150 µl of a 1.0 mg/ml solution of lactoperoxidase (Sigma). The reaction was allowed to proceed at room temperature for 30 min, then the reaction mixture was aspirated off and the cell layers were washed twice with phosphate buffered iodide (PBI; [g/L], KCl; 0.2, $Na_2HPO_4 . 7 H_2O$; 2.16, NaI; 22.48, $KH_2PO_4$; 0.2, pH 7.4). Cell lysis and immunoprecipitation were carried out as described below.

## SAMPLE PREPARATION.

### Conditioned Media.

After the labeling period, conditioned media were collected in eppendorf microfuge tubes and adjusted to 5 mM phenylmethylsulfonylfluoride (PMSF). Floating cells were peleted intact by low speed centrifugation at 2,500g for 5 min and cell debris were further removed from the supernatant by centrifugation at 10,000g. The cleared conditioned media were used immediately or stored at -20 °C until use.

For electrophoretic analysis of radiolabeled proteins in the conditioned media, aliquots containing equal trichloroacetic acid (TCA)-precipitable $^{35}$S counts were precipitated by addition of 4-6 volumes of acetone or adjusting to 12% TCA and incubation at 4 °C for 1 hr. The precipitated proteins were then peleted at 10,000g for 10-15 min at 4°C and the supernatant aspirated off in the case of acetone precipitation; in the case of TCA precipitation, the supernatant is first neutralized by 0.75 M Tris hydrochloride, pH7.2, incubated for 3-5 min at room temperature and then discarded. The protein pellets were washed twice with ice-cold acetone (0.45 ml) allowed to dry at room temperature (5 min) and dissolved by heating at 95°C in Laemmli SDS-PAGE sample buffer (0.07 M Tris-HCl pH6.8, 3% SDS [W/V], 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) for one dimensional gel electrophoresis, or dissolved (without heating) in iso-electric focusing lysis buffer of O'Farrel (9.5M urea, 2.0% NP40, 1.6% pH7-5 ampholytes, 0.4% pH3-10 ampholytes, 5.0% β-mercaptoethanol) for

iso-electric focusing.

For Western immunoblotting, unlabeled conditioned media (~25 ml) of sub-confluent NRK cells, growing in roller bottles were harvested and cleared from floating cells and debris by centrifugation as above. The media were adjusted to 5.0 mM PMSF and concentrated by ultrafiltration in a stirred cell chamber (Amicon) fitted with a YM-5 membrane (Diaflo) at 4°C. The concentrated media were cleared by centrifugtion at 20,000g. A typical preparation form a subconfluent culture of cells growing in a roller bottle (~$5.10^5$ cells) yields about 0.75-2.0 mg of protein (as determined using Bio Rad protein assay kit, using BSA as a standard). The proteins were further lyophilized and redissolved in SDS-PAGE sample buffer or IEF lysis buffer.

**Cell Lysates.**

After labeling, the cells were washed three times with 5.0 ml of Dulbecco's phosphate buffered saline (PBS; [g/L], KCl; 0.2 g, $KH_2PO_4$; 0.2 g, NaCl; 8.0 g, $Na_2HPO_4$ . $7H_2O$; 2.16 g, pH 7.4) and incubated on ice in 0.7 ml of radioimmunoprecipitarion assay (RIPA) buffer (50 mM Tris-HCl pH 7.2, 0.15 M NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 Kallikrein inactivator units of aprotinin/ml, 5mM PMSF, 5.0 µg/ml trypsin inhibitor) for 15 min. The residual insoluble material is scrapped using a rubber policeman and the lysates were vortexed briefly to assist dissolution and centrifuged at 10,000g at 4 °C for 25 min. The supernatant is used immediately or stored at -20 °C until use.

## IMMUNOLOGICAL TECHNIQUES.

The antisera used in this study are: Anti-p69 prepared in this laboratory by Dr. I. Chackalaparampil. It was raised by injecting rabbits with gel-purified p69 obtained from the culture supernatants of NRK cells grown in serum-free medium. Anti- fibronectin antibody was purchased from Collaborative Research and was raised against purified human plasma fibronectin. Anti-2ar antiserum was obtained from Dr. D. Denhardt (Cancer Research laboratory, University of Western Ontario). It was obtained as a mixture of two antisera raised in rabbits against (N-terminus and C-

terminus)2ar-Cro-βgal fusion proteins produced in bacteria. Anti-P–Tyr monoclonal antibody, 1G2, coupled to CNBr-activated Sepharose was obtained from Dr. J. Bell (Department of Biochemistry, McGill University).

**Immunoprecipitation.**

Aliquots of conditioned media containing 5.104 cpm of $^{32}P$ or 105 cpm of $^{35}S$ were diluted with one volume of RIPA buffer. Cell lysates were used without further dilution. Fifty microliters of anti-p69 antiserum were added and the mixture is incubated for 2 hr or overnight at 4°C in siliconized microfuge tubes with continuous end-to-end agitation. Non-immune serum was also used as a control. Thirty to fifty microliters of 100 mg/ml suspension of *Staphylococcus aureus* protein A-Sepharose (3-5 mg/reaction) were added and incubated for one additional hr. The immune complexes bound to protein A-Sepharose were peleted by centrifugation and washed, by pelleting/resuspension, four times in 0.7 ml of RIPA buffer and once with PBS. The beads were finally transferred to clean tubes in 1.0 ml of PBS, peleted, washed in 250 µl of $ddH_2O$ and dried. Proteins were eluted by heating for 3 min in SDS-PAGE sample buffer and analysed or with the appropriate (enzyme assay) buffer for further analysis (see below).

**Affinity Purification of Phosphotyrosyl Proteins.**

For affinity purification of P–Tyr-containing proteins, sub-confluent cultures were first labeled with 1.0 mCi of [$^{32}P$]–$PO_4$ for 3 hr. After labeling, the culture plates were immediately transferred onto ice and the labeling medium removed. Then, each culture received 0.4 ml of extraction buffer (1% Triton X-100, 10 mM Tris/HCl, pH 7.6 [at 4 °C], 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 µM $Na_3VO_4$, and 0.1% BSA) extemporaneously adjusted to 1 mM PMSF. The monolayers were scrapped twice in 0.4 ml of extraction buffer into microfuge tubes. Extraction was carried out for 20 min on ice with frequent vortexing to assist extraction. The extracts were cleared by centrifugation for 15 min at 10,000g at 4 °C. Aliquots of the cell extracts were the added to tubes already containing 20 µl of 50% anti-phosphotyrosine–Sepharose beads in extraction buffer. The mixtures were incubated for 2.5 hr at 4 °C with continuous end-to-end agitation. The

beads were then washed as above by peleting/resuspension 3 times in 1.0 ml of extraction buffer and twice in modified extraction buffer (without BSA). Bound phosphotyrosyl proteins were specifically eluted by incubation for 10 min at 4 °C in elution buffer (extraction buffer without BSA; with 0.01% ovalbumin; with 1.0 mM phenyl phosphate as P–Tyr competitor) with occasional vortexing. The beads were then peleted by centrifugation for 10 sec at 10,000g and the supernatants (~ 40 µl) containing P–Tyr-proteins were collected using a Hamilton microsyringe. Samples were diluted with one volume of 2X SDS-PAGE sample buffer, heated at 95 °C and resolved by SDS-gel electrophoresis.

**Immunoblotting.**

Protein samples were loaded along the top of a 4% polyacrylamide stacking gel at 150-200 µg of protein per centimeter of gel and fractionated on a 10% polyacrylamide resolving gel. At the end of the run, the gel is rinsed in water and equilibrated for 1 hr in 0.02 M Tris-base , 0.192 M glycine (pH 8.3), 0.1% SDS and 20% methanol (equilibration buffer) on a rotary shaker. Protein transfer to nitrocellulose filter is carried out electrophoretically for 16 hr at 250 mA at room temperature in the buffer above without SDS (transfer buffer). The efficiency of the transfer is monitored by coomassie blue staining of gel strips prior and after the run and by the migration of prestained molecular weight marker ( Rainbow Markers, Amersham). The blotted filter is washed in 10% ethanol in PBS to remove residual SDS, rinsed three times in PBS and incubated in 100 µg/ml of bovine serum albumin in PBS for 16 hr at 4°C. The filter is then rinsed and dried at room temperature and a line is drawn across the bottom of the filter to help in manipulation in the subsequent steps. Individual 0.75 cm strips were cut from the filter and incubated separately with specific antisera. Anti-fibronectin and anti-p69 were used at 1:500 and 1:250 fold dilutions in TBST (50 mM Tris-HCl pH 7.9, 150 mM NaCl and 0.05% Tween20), respectively. Non-immune serum is used at 1:250 dilution. The filters were incubated at room temperature for 1.5 hr with continuous shaking, followed by three washes in 10.0 ml of TBST. Detection of bound primary antibodies was carried out by incubation with biotinylated goat anti rabbit IgG and subsequently by staining with avidin-biotinylated horseradish peroxidase (A-BHRP), following the supplier's instructions (Clontech Laboratories Inc., Palo Alto, CA). Incubation with goat anti rabbit

anti-rabbit IgG (1.5 mg/ml) was carried out in 10.0 ml of TBST (1:500 dilution) at room temperature for 30 min with constant shaking. The filters were subsequently washed in three changes of TBST and incubated with the A-BHRP complex. The A-BHRP complex is prepared 30 min before use by mixing 40.0 µl of avidin solution and 40.0 µl of biotin horseradish peroxidase conjugate (concentrations not specified by supplier) in 10.0 ml of TBST. After incubation at room temperature for 30 min as above, the filters were washed thoroughly in TBS and incubated for 30 min in the substrate solution (0 5 mg/ml 4-chloro-1-naphtol, 8.3 M imidazole, 0.012% $H_2O_2$) in 83% TBS (TBST without Tween20) made up from stock solutions immediately before use. After the staining developed, the filters were washed thourruoghlg in dd$H_2O$, dried and phoiographed.

## ENZYME TREATMENTS.

### V8 Protease Digestion for Peptide Mapping.

*In situ* peptide mapping by limited proteolysis using S. *aureus* V8 protease (Cleveland et al., 1977) was carried out as described by Gooderham (1984). Relevant protein bands were located on 1-d and 2-d gels by radiography, excised and incubated in 5.0 ml of equilibration buffer (125 mM Tris-HCl pH 6.8, 0.1% SDS, 1 mM $Na_2$-EDTA) with gentle shaking for one hr. Gel slices were then placed in wells of a 5.0 cm long, 4% SDS-PAGE stacking gel. The slices were overlaid with 30 µl of overlay buffer (125 mM Tris-HCl pH6.8, 0.1% SDS, 1.0 mM EDTA, 20% glycerol and 0.001% bromophenol blue). 0.05 µg of V8 protease (Sigma) were added per gel slice in a total volume of 10.0µl in protease buffer (125 mM Tris-HCl pH6.8, 0.01% SDS, 1.0 mM $Na_2$-EDTA, 20% glycerol and 0.001% bromophenol blue) prepared by dilution of a stock solution of 1.0 mg/ml of protease (in water) stored at -20°C in frozen aliquots. Electrophoretic migration through the stacking gel was performed at 20 mA constant current. Peptides were resolved in a 15.0 cm long 15-20 % linear gradient gel at 40 mA and visualized by exposing to a Fuji XAR film for 10 days at -70 °C.

### Bacterial Collagenase Digestion.

Collagenase treatment was carried out by the modification described by (Broek et al., 1985).

Briefly, $^{35}S$-labeled conditioned media (2.0 ml) from NRK and vanadyl-treated NRK cells were desalted by two rounds of dilution in collagenase assay buffer (5 mM Tris/HCl, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$ and 10 mM NEM) and concentration by ultra-filtration through centricon (Ym5, Amicon) membrane, and finally brought to approximately 10% of the starting volume. Aliquots were diluted with 2 volumes of collagenase buffer and ten units of bacterial collagenase (Advanced Biofactures) were added and incubated for 18 hr at 25 °C . Control samples without the enzyme were also included. Proteins recovered by TCA precipitation were dissolved in reducing SDS-PAGE sample buffer, and analysed by electrophoresis.

**Alkaline Phosphatase Treatment.**

After immunoprecipitation and washing of $^{35}S$- and $^{32}P$-labeled pp69, the immune complexes coupled to protein A-Sepharose were suspended in phosphatase assay buffer (50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 2 mM PMSF, 1.0 mg/ml trypsin inhibitor and 100 kallikrein inactivator units of aprotinin/ml) and 2 Units of bacterial alkaline phosphatase (Sigma) were added. The mixtures were incubated at 25 °C for 16 hr with gentle agitation in a total volume of 250 µl. The beads were then collected by centrifugation and the proteins were eluted and dissolved in lysis buffer for two-dimensional gel electrophoresis or in sample buffer for SDS-PAGE.

**Neuraminidase Digestion.**

Neuraminidase treatment was carried out as described (Le Cam et al., 1985; Cunningham et al., 1984). $^{32}P$-labeled NRK conditioned media were immunoprecipitated as above. After the final rinse in distilled water, the immune complexes were resuspended in 40 µl of 0.5% SDS, heated to 95 °C for 3 min and 160 µl of $H_2O$ were added to bring the concentration of SDS down to 0.1%. The samples were heated again at 95 °C for 3 min, then centrifuged to pellet the beads. The supernatant containing the eluted proteins was separated into two halves to which one volume of double strength (2X) neuraminidase assay buffer (0.2 M sodium citrate, 4 mM $CaCl_2$, 0.4 mM EDTA, pH 5.5, adjusted extemporaneously to 1 mM PMSF). To one half-sample 5 µl of *Clostridium perfrigens* neuraminidase

(Sigma) solution (10 U/µl; in neuraminidase buffer, stored in aliquots at -20 °C) were added. The samples were incubated at 37 °C for 18 hr. Proteins were concentrated by TCA precipitation, dissolved in SDS-PAGE sample buffer and analysed.

**Endo H Digestion.**

For endo-β-*N*-acetylglucosaminidase H (Endo H) treatment (Cunningham et al., 1984, Julius et al., 1984), samples were prepared as above except that they were diluted in 2X Endo H buffer (0.3 M sodium citrate, pH 5.5) and extemporaneously adjusted to 5 mM PMSF. Endo H (Sigma) was added to 25 mU/ml and the digestion was carried out for 37 °C for 18 hr. After digestion, samples were processed for SDS-PAGE as described for neuraminidase treatment.

## ANALYTICAL PROCEDURES

**Slab Gel Electrophoresis.**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous gel system of Laemmli (1970). Proteins were separated on a 10% polyacrylamide gel made from 13.3 ml of a stock solution of 30% acrylamide:*N, N*-methylene *bis*-acrylamide (29.2:0.8), 10.0 ml of a resolving gel buffer (1,5 M Tris HCl pH 8.8, 0.4% SDS) and $ddH_2O$ to 40.0 ml final volume. Polymerization was initiated by addition of ammonium persulfate (APS) and *N,N,N',N'*-tetramethylethylendiamine (TEMED) to 0.05 and 3.3 mM final concentrations, respectively The gels were casted 10.0 cm long and 1.5 mm thick, overlayed with 0.5 ml of $ddH_2O$ or 50% isopropanol and allowed to polymerize at room temperature for 1 hr or more. The stacking gel is made 4% polyacrylamide from the same acrylamide stock solution above (1.8 ml) and 3.0 ml of stacking gel buffer (0.5 M Tris-HCl pH 6.8, 0.4% SDS) and $ddH_2O$ to 12.0 ml final volume. APS and TEMED were added at the same final concentrations, and polymerization was achieved in 30-60 min. Electrophoresis was carried out at 55 V constant voltage or 12 mA constant current for 14-17 hr in a freshly made running buffer (0.25 M Tris-base, 0.92 M glycine and 1% SDS, pH 8.8). The gels were calibrated with $^{14}C$-methylated or prestained molecular weight markers (myosin 200.0 kDa,

phosphorylase b 92.5 kDa, bovine serum albumin 69.0 kDa, carbonic anhydrase 30.0 kDa, trypsin inhibitor 21.5 kDa and lysozyme 14.3 kDa, Amersham). For $^{35}S$-labeled proteins, the gels were rinsed briefly in $ddH_2O$ and incubated in 16.0% sodium salicylate for 30 min, at room temperature with gentle agitation. The gels were then dried on Whatmann 3MM paper and fluorographed. Gels containing $^{32}P$-labeled proteins were dried immediately after a brief rinse in water and autoradiographed. The gels were exposed to Kodak XR-5 or Fuji RX films in a cassette with double intensifying screens for 3 days to 2 weeks (Fluorography) or 24-72 hr (autoradiography; Laskey and Mills, 1977) at -70°C.

**Two Dimensional Gel Electrophoresis.**

Two dimensional iso-electric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis (2-d IEF/SDS-PAGE) was carried out as described by Pollard (1984) based on the original procedure of O'Farrell (1975). For the first dimension, IEF gel solution (10.0 ml) was made by dissolving 5.5 g urea in 1.33 ml of 30% acrylamide stock solution (28.38% [w/v] acrylamide, 1,62% [w/v] bisacrylamide, 2.0 ml of 10% Nonidet P-40, 0.6 ml of ampholines pH 5-7, 0.115 ml of ampholines pH 3-10 and 1.7 ml of $ddH_2O$. The solution is degassed under vacuum for 5 min and 7.0 µl of TEMED and 10.0 µl of 10% APS were added. Gels were casted 10.0 cm long in 1.5 mm internal diameter and 12.0 cm long glas tubes. They were overlayed with 20.0 µl of $ddH_2O$ and allowed to polymerize for 1 hr or more. After polymerization the gel edges were washed in $ddH_2O$, then the tubes were placed in electrophoresis tank and and the gels were overlaid with 20 µl of IEF lysis buffer (9.5 M urea, 2% v/v NP-40,1.6% ampholines pH 5-7, 0.4% ampholines pH 3-10, 5.0% [V/V] β-mercaptoethanol, stored in aliquots at -20°C ) and 20 µl of sample overlay buffer (8.0 M urea, 0.8% ampholines pH 5-7, 0.2% ampholines pH 3-10, stored in 0.5 ml aliquots at -20 °C) and filled with cathode electrode solution (see below). The pH gradient is established by pre-electrophoresis at 200 V for 15 min, 300 V for 30 min, and 400 V for 1 hr or more in 20 mM degassed NaOH (cathode electrode solution, prepared from 1.0 M NaOH stock solution) and 10 mM phosphoric acid (anode electrode solution, prepared from 1.0 M stock solution of $H_3PO_4$). At the end of the pre-run, the gel tops were washed three times with $ddH_2O$ and the samples loaded in 30-70 µl volumes in lysis buffer, overlayed with sample overlay solution and the tubes filled with cathode solution. Focusing is performed at 400V for 17 hr and 800V for 1 hr in the

same anode and cathode electrode solutions. Prestained IEF markers (Bio Rad) were also run to monitor the focusing, and to allow comparisons to be made between different runs. At the end of the focusing, gels were removed from the casting tubes and placed individually in 5.0 ml of SDS-PAGE equilibration buffer (0.06 M Tris-HCl pH6.8, 2% w/v SDS, 5% v/v β-mercaptoethanol, 10% v/v glycerol), and stored -20°C if not to be used immediately. Equilibration was achieved by incubation of the gels in three changes of 5.0 ml equilibration buffer, 30 min each. For approximate determination of iso-electric points, four "blank" gels focused with the samples, and were aligned against each other, cut into 0.5 cm pieces and incubated in 7.0 ml of degassed deionized dd$H_2O$ in capped vials overnight on a rotary shaker. The pH range in the IEF tubes was determined by pH reading in individual vials.

For the second dimension, slab gels were made 10% resolving and 4% stacking polyacrylamide gels as above. The equilibrated IEF gels were layered atop the stacking gel, sealed in place with 1% agarose in SDS-PAGE sample buffer. All the subsequent steps were carried out as described above

## OTHER METHODS.

### Alkali Treatment of $^{32}P$ Gels.

Alkali treatment was carried out as described by Feuerstein and Cooper (1983). Gels to be treated were first fixed in 50% methanol and 10% acetic acid for 30 min, dried and autoradiographed The dried gels were rehydrated by soaking in 1.0 M NaOH for 30 min at room temperature with gentle shaking. Filter paper and debris were removed, and the gels were extracted in 1.0 M NaOH at 37 °C for 1 hr, followed by three washes in 1 M NaOH and re-extraction as above. The gels were finally rinsed in distilled water and incubated for 2 hr in 7.0% acetic acid, 7.0% methanol, dried and re-exposed.

### Determination of Acid-Insoluble Radioactivity.

For determination of radioactivity incorporated into proteins, 10.0 µl aliquots (in duplicates) were

taken form various samples and added to 0.5 ml of 50 µg/ml of a solution of bovine serum albumin. To the mixture are added 2.5 ml of 20% ice-cold TCA and incubated on ice for 10-30 min. The precipitates were collected on 0.45 µm pore diameter filters (Millipore) or on Whatmann glass fiber discs. The filters were washed four times with 5.0 ml of cold 5% TCA, dried and counted by scintillation counting in 20.0 ml of 0.4% omnifluor in scintillation-grade toluene.

**Preparation of Membrane Vesicles.**

Membrane vesicles were prepared as described (Banerjee et al., 1986). Briefly, $^{32}$P-labeled cultures were rinsed 3 times with ice-cold PBS or serum-free DMEM, than scrapped into 15 ml Falcon tubes in 2 ml of cold DMEM and incubated on ice for 2 hr (cold shock) and for 2 hr at room temperature. The tube contents were then layered atop of a discontinuous 5%-10% Ficoll (Pharmacia) gradient and centrifuged for 12 min at 5,000-7,000g in a clinical centrifuge. Membrane vesicles near the 5-10% Ficoll interface were collected and peleted by centrifugation at 12,000 rpm in a TI ss34 rotor (Sorvall). After removing the supernatants, the vesicles were dissolved in SDS-PAGE sample buffer. Volumes (10-70 µl) containing equal acid-insoluble cpms were loaded on 10% SDS-PAGE gels and analysed.

## VII). RESULTS.

### Characteristics of NRK Cells Grown in the Presence of Vanadium.

As an initial step in this study, the concentration of vanadyl sulfate which allows optimum growth with minimal toxicity has been determined. NRK cells were grown for several days in DMEM in the presence of various concentrations of vanadyl sulfate ranging from 0 to 50 μM. These initial experiments indicated that NRK cells can be maintained for long periods of time in presence vanadyl sulfate in concentrations up to 40 μM provided normal culturing conditions are maintained. Concentrations above 40μM are toxic particularly for sparse cultures.

### Morphological Transformation of Vanadyl Sulfate-Treated Cells.

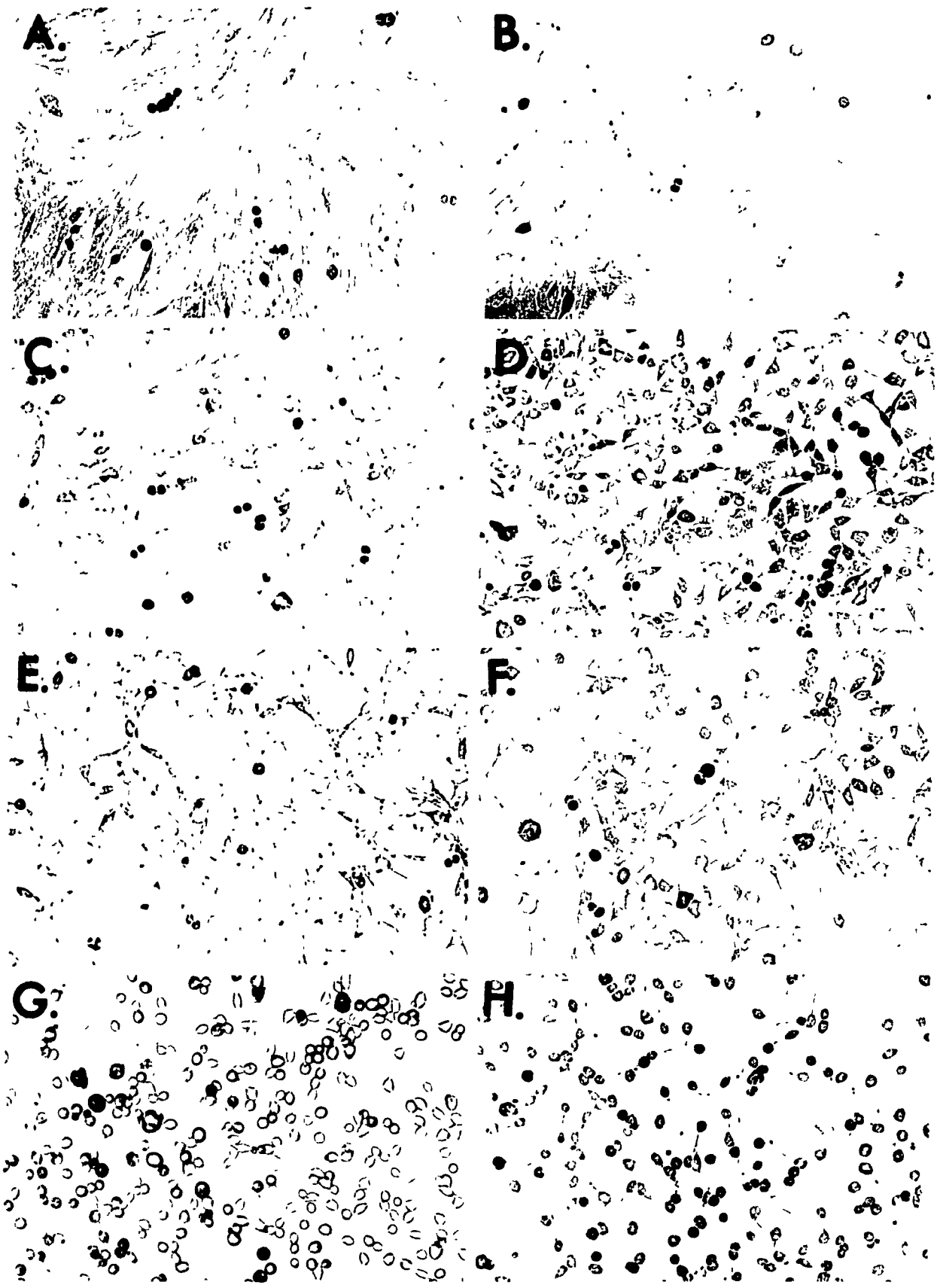
Figure 1 shows the morphological appearance of untreated and treated NRK cells grown for three days in presence of various concentrations of vanadyl sulfate Untreated NRK cells have a normal, flat morphological appearance of normal fibroblasts (Fig. 1, *A*). After reaching confluency, they align against each other in regular arrays forming a uniform monolayer at which stage further growth is inhibited. In contrast to NRK cells, treated cells, depending on vanadyl concentration (Fig 1, *B-H*), show a full spectrum of morphological changes ranging from polygonal to extremely rounded shape with long cytoplasmic processes characteristic of transformed cells. At confluence, treated cells do not form contact-inhibited monolayers but grow on top of each other in multilayers and attain high cell densities.

The extent of morphological transformation is dose-dependent Cells grown in medium containing more than 40 μM vanadyl are extremely rounded, adhere poorly to the culture substratum and cell detachment often occurs. However, the detached cells are still viable as indicated by their ability to re-adhere and grow once transferred to vanadyl-free medium or media containing lower vanadyl concentrations The time of onset of transformation also depends on vanadyl concentration, but generally 48-72 hours of treatment allow treated cells to display maximum degrees of transformation at their respective doses Finally, removal of vanadyl from the culture medium causes the cells to regain their normal morphology. The results show that NRK cells can be cultured in

presence of up to 40 μM vanadyl without any toxic effects. They also show that morphological transformation is completely reversible and dose-dependent.

**Figure 1.** *Morphological transformation of NRK cells by vanadyl sulfate treatment.*

Bright field photomicrographs of untreated and vanadyl-treated NRK cells. Cells were grown in absence (*A*) or presence of 5, 10, 20, 25, 30, 35, and 50 μM vanadyl sulfate (*B-H*). Cells were photographed using an inverted microscope.


A.
B.
C.
D.
E.
F.
G.
H.

**Monolayer Growth of NRK Cells in the Presence of Vanadyl Sulfate.**

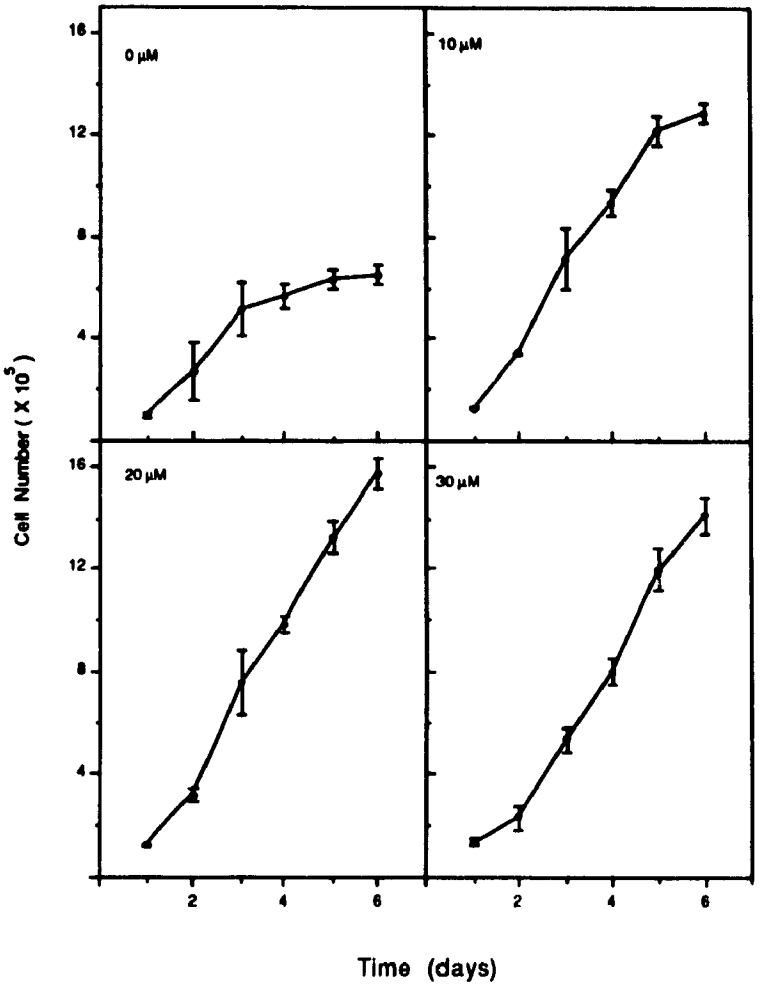
Neoplastic cells have deregulated growth control and as a result, they generally have shorter population doubling times than normal cells, they are not sensitive to serum starvation and their growth is not contact-inhibited (Holley, 1975). Vanadium ions are mitogenic for a number of cell lines and mimic the actions of growth stimulatory factors (Carpenter, 1981; Smith, 1983). After establishing that cells can be grown for several days in presence of up to 40 μM vanadyl sulfate with minimum toxicity, the growth properties of NRK cells in presence of various concentrations of vanadyl sulfate were examined. This was important since it can indicate whether or not, and to which extent, vanadyl treatment mimics oncogenic transformation-induced effects on cell growth. In order to examine the effects of vanadyl on NRK cell growth, NRK cells were plated in various concentrations of vanadyl sulfate and counted every 24 hours starting 24 hours after treatment. Figure 2*A* shows that the overall growth is stimulated in V–NRK cells as early as 24 hours post-treatment. At the active phase of growth, stimulation is much higher at low vanadium concentrations than at higher ones, but as the cell number increases growth stimulation becomes evident even at high concentrations (35 μM) The maximum growth stimulatory effect of vanadyl is at 10-20 μM and depends on cell number and vanadyl concentration.

After day 4, having reached confluency, untreated NRK cells stop growing due to contact inhibition of growth. V–NRK cells, on the other hand, continue to grow and reach high densities indicating that they have lost contact inhibition of growth. The loss of contact inhibition in V–NRK cells is dose-dependent and is illustrated in Figure 2*B* where cells were treated with vanadyl sulfate only after the cultures reached monolayer. These results demonstrate that vanadyl confers to NRK cells other properties of transformed cells, namely high mitotic activity and loss of contact inhibition of growth.

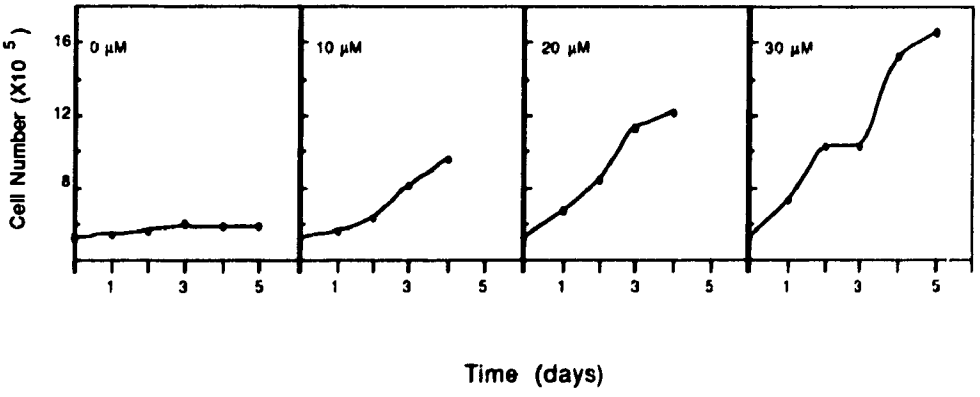
**Figure 2.** *Monolayer growth of NRK cells in presence of vanadyl sulfate.*

***A.*** Cells were treated with the indicated concentrations of vanadyl sulfate 24 hours after plating 2.4 X $10^4$ cells/35 mm tissue culture dish. The cell number at each time point in each growth curve represents average cell number of triplicate cultures (± S.D.). ***B.*** Cells were grown in duplicate 35 mm culture dishes for 5 days to reach confluency, then treated with the indicated concentrations of vanadyl sulfate. Cell counting started 24 hours after treatment. The results show average cell number/duplicate cultures of one representative experiment.

A.


0 µM
10 µM
20 µM
30 µM
Cell Number ( X 10 5 )
16
12
8
4
2
4
6
Time (days)

B.


0 µM
10 µM
20 µM
30 µM
Cell Number (X10 5 )
16
12
8
1
3
5
Time (days)

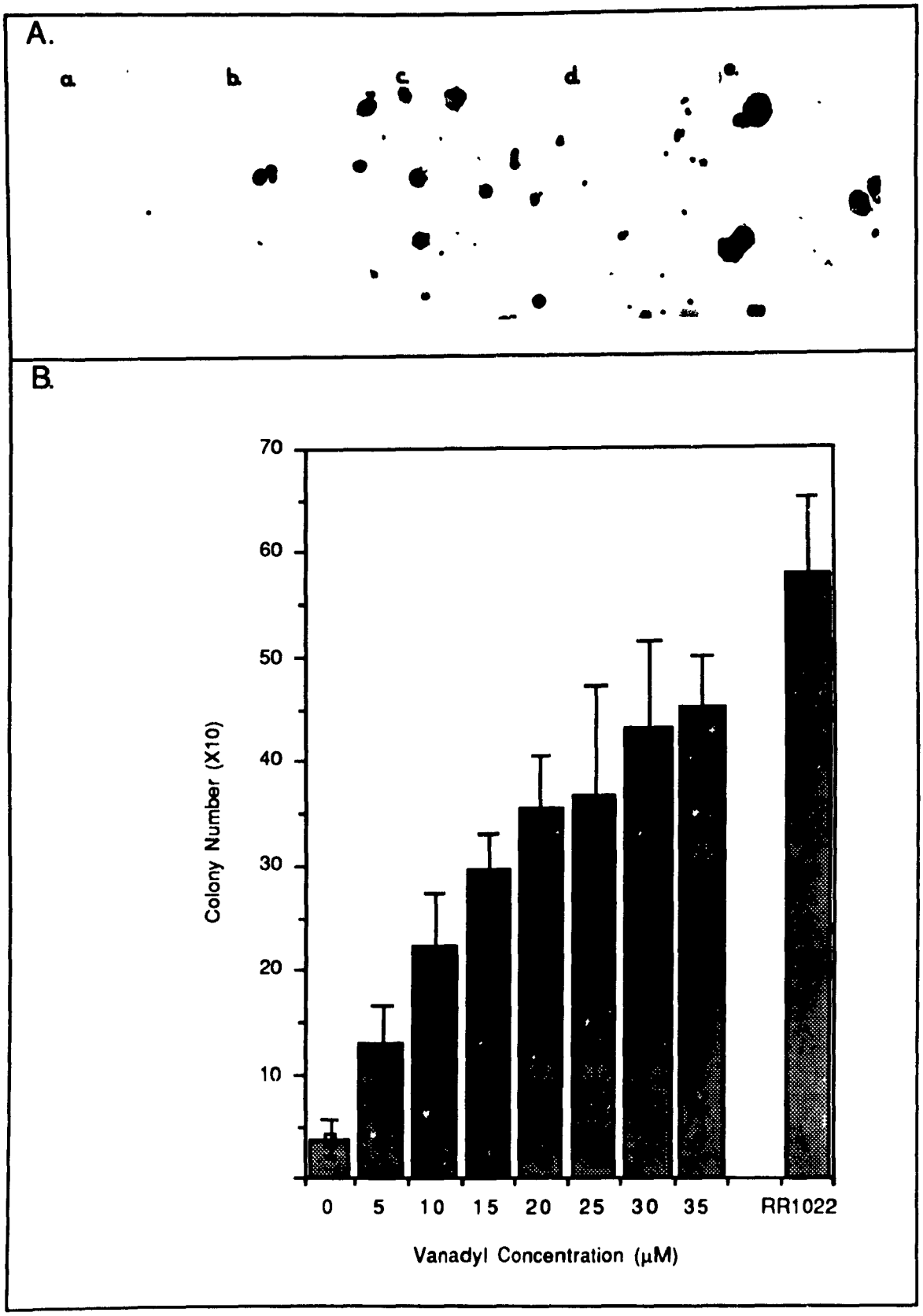
**Anchorage Independent Growth of V-NRK Cells**

In order to investigate any effect of vanadium-induced transformation on major secreted transformation-sensitive phosphoproteins (MSPs), it was important to determine first at which extent phenotypic transformation is induced. Because anchorage independent growth is the most consistent *in vitro* correlate of transformation, the growth of NRK cells in soft agar in presence of vanadyl was examined. Untreated and vanadyl-treated NRK cells were plated in soft agar to assay for their ability for anchorage-independent growth KNRK, RR1022 and KA31 cells were also grown in the same conditions as positive controls. Figure 3*A* shows the colonies formed by NRK, V-NRK and KA31 cells 10 days after plating. RR1022, KNRK (not shown) and KA31 (Fig. 3*A*, e) cells formed numerous well-defined colonies whereas NRK cells did not (Fig. 3*A*, a). Vanadyl-treated cells formed colonies varying in size and number depending on vanadyl concentration (Fig. 3*A*, b-d). At high concentrations (20-35 μM) a large number of colonies were formed although of reduced size. At lower concentrations (5-15 μM) smaller number of colonies with larger size were formed.(Fig.3*B*). Further culturing for 10 more days did not increase the size or the number of the colonies formed by V-NRK cells, whereas colonies formed by RR1022, KNRK and KA31 cells continued to grow to larger sizes. This indicates that the growth arrest of V-NRK colonies could be due to toxicity of vanadyl (see DISCUSSION) However, the cells from the growth-arrested colonies spread and grew again when plated on a solid substratum indicating that they were still viable. When these cells were plated again in vanadyl-free soft agar medium they failed to form colonies indicating that vanadyl conferred anchorage-independent growth to NRK cells in a reversible manner. These results indicate that the phenotypic transformation of NRK cells is extended to the ability for anchorage-independent growth. This ability is acquired in a dose-dependent and reversible manner, but is limited by comaprison to virus-transformed cells.

**Figure 3.** ***Anchorage independent growth of NRK cells in presence of vanadyl sulfate.***

***A.*** Cells were pretreated for 3 days with the indicated concentrations of vanadyl sulfate to induce transformation, then plated in soft agar at a density of $10^4$ cells/60 mm tissue culture dish as described in MATERIALS AND METHODS. The assay was carried out for 10 days then photographed using an inverted microscope. Randomly chosen areas in one experiment are shown. a: untreated NRK cells, b, c and d: NRK cells treated with 5,15, 1nd 35 μM vanadyl sulfate, respectively, e: KA31 cells.

***B.*** Quantitative analysis of colony formation by vanadyl-treated NRK cells. Cells were plated at a density of $10^4$ cells/soft agar assay in triplicate cultures and grown for 10 days as above, in presence of indicated vanadyl concentrations. The colonies formed in five randomly chosen areas/assay were counted visually under a calibrated grid using an inverted microscope. *Columns* indicate the total number of colonies formed/assay (± S.D ). Inset *bars* represent the number of colonies with a diameter ≤20μm.


A.
a.
b.
c.
d.
B.
Colony Number (X10)
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
RR1022
Vanadyl Concentration (μM)

**Phosphorylation of Transformation-Marker Plasma Membrane Proteins.**

Previous studies from this laboratory have established that phosphorylation of a group of low molecular weight (14-30 kDa) proteins, associated with shed plasma membrane vesicles, is tightly correlated with the ability of cells to grow in an anchorage independent manner. Such proteins are phosphorylated in transformed cells, but remain nonphosphorylated in nontransformed and in certain transformed cells after treatment with retinoic acid (RA). In addition, it has been suggested that RA inhibits anchorage independent growth by interfering with the phosphorylation of these proteins which can be considered as a marker for transformation (Banerjee et al, 1986). Therefore, we determined whether or not this marker is expressed in vanadium-treated NRK cells. Shedding of plasma membrane vesicles by $^{32}P$-labeled cells was cold-induced and vesicles were purified as described in the METHODS section. Figure 4*A* shows that membrane vesicle preparations from RR1022 cells contain 5-7 major phosphorylated proteins in the 14-30 kDa range (*lane* 4) whereas similar preparations from NRK cells do not (*lane* 1). Treatment of NRK cells with either vanadyl or vanadate, at a concentration and length of time which induce transformation, did not induce the phosphorylation of these proteins (*lanes* 2 and 3, respectively). Examination of membrane vesicles prepared from high passage NRK cells, which show significant growth in soft agar and a proportion of which are transformed, indicates that they contain these phosphoproteins (Fig. 4*B*, *lane* 1) Treatment with vanadyl or vanadate does not alter their phosphorylation (*lanes* 2 and 3, respectively). These experiments indicate that vanadium does not induce transformation by inducing phosphorylation of this group of low molecular weight plasma membrane proteins. This also indicates that phosphorylation of these proteins is not under direct control of PTKs and PTPases, which is consistent with the findings that they are not phosphorylated in NRK cells in response to EGF treatment (Pentney and Mukherjee, Unpublished).

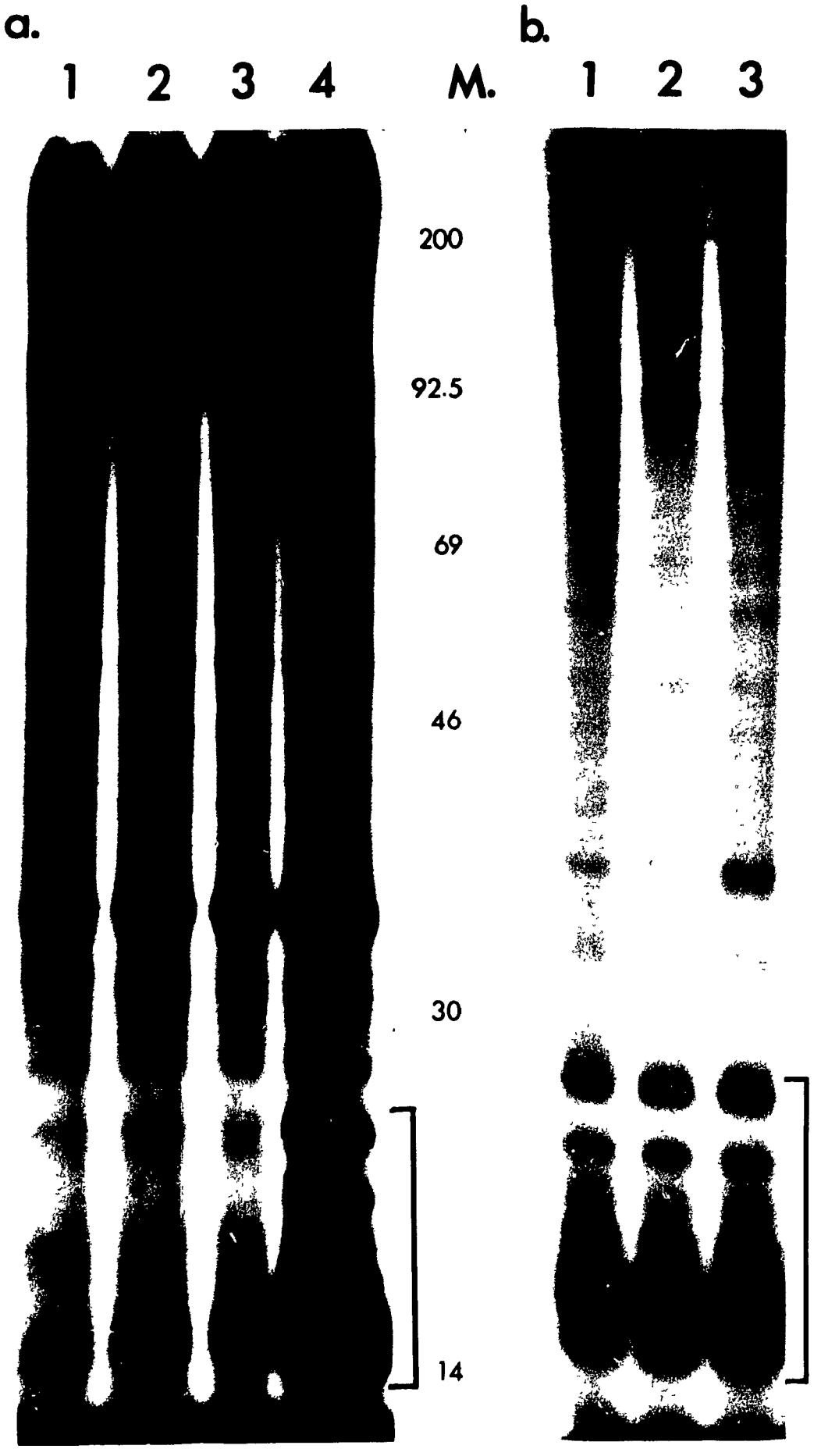
**Figure 4.** *Phosphorylation of low molecular weight proteins associated with shed plasma membrane vesicles.*

Shedding of plasma membrane vesicles was induced by a cold shock then the vesicles were purified by centrifugation through a Ficoll gradient. Proteins were solubilized by sample buffer and aliquots containing equal (~50,000) acid-insoluble $^{32}P$ cpm were analysed by SDS-PAGE.

***A.*** Shows phosphoproteins associated with membrane vesicles of NRK cells (*lane* 1) and vanadyl- and vanadate-treated NRK cells (*lanes* 2 and 3, respectively) and RR1022 cells (*lane* 4).

***B.*** Phosphoproteins of untreated (*lane* 1) , vanadyl- and vanadate-treated (*lanes* 2 and 3, respectively) PT-NRK cells.

The phosphoproteins of interest are indicated by *brackets*. Exposure time is 24 hours.


a.
1
2
3
4
M.
200
92.5
69
46
30
14
b.
1
2
3

**Tyrosyl-Protein Phosphorylation.**

Similar effects on cell morphology and growth characteristics have also been obtained by treating NRK cells with vanadate, which are in agreement with the results reported by Klarlund (1985). Although a number of studies have indicated that vanadate in this concentration range acts by inhibiting phosphotyrosyl-protein phosphatases, this has not been demonstrated for vanadyl *in vivo* or *in vitro*. In an attempt to determine whether vanadyl also acts by increasing P–Tyr-proteins, total cell phosphoaminoacid analysis was carried out by the method of Cooper and Hunter (1983), but without conclusive results; P–Tyr residues were not consistently detected either in untreated, vanadate-treated, vanadyl-treated NRK cells, or in RSV-transformed RR1022 cells (not shown). Therefore, immunoaffinity purification, a more sensitive and a much simpler method, using anti-P–Tyr monoclonal antibody 1G2 was carried out. Untreated NRK and RR1022 cells as controls, and NRK cells treated for 4 days with 25μM vanadyl and vanadate were analysed. Because the sensitivity of RR1022 cells to vanadium treatment is not known, they were treated only overnight with the same concentrations of vanadyl and vanadate and examined for comparison. Cells were then metabolically labeled with [$^{32}$P]$PO_4$, extracted and P–Tyr-proteins purified as described in MATERIALS AND METHODS. Figure 5 shows that NRK cells contain a basal level of P–Tyr-phosphorylated proteins (*lane* 1) which is increased by both vanadyl (*lane* 2) and vanadate (*lane* 3) treatment, as indicated by the increase in the intensity of the bands. In RR1022 cells (*lane* 5), the basal level of P–Tyr-proteins is higher than in NRK cells and is also increased by both vanadyl (*lane* 6) and vanadate (*lane* 7) even after only 18-24 hr of treatment. In both cases, however, vanadate is (2-4 times) more effective in increasing the the levels of P–Tyr-proteins than vanadyl.

It is likely that not all the radioactive $^{32}$P[$PO_4$] detectable on the autoradiograms is incorporated in the form of P–Tyr; these proteins could be phosphorylated on serine and threonine as well, as is often the case for most known PTK substrates Nevertheless, all the purified proteins should contain phosphotyrosine since both adsorption and elution of these proteins are specific to phosphotyrosine; the antibody recognizes P–Tyr, but not P–Ser or P–Thr, and the eluting agent,

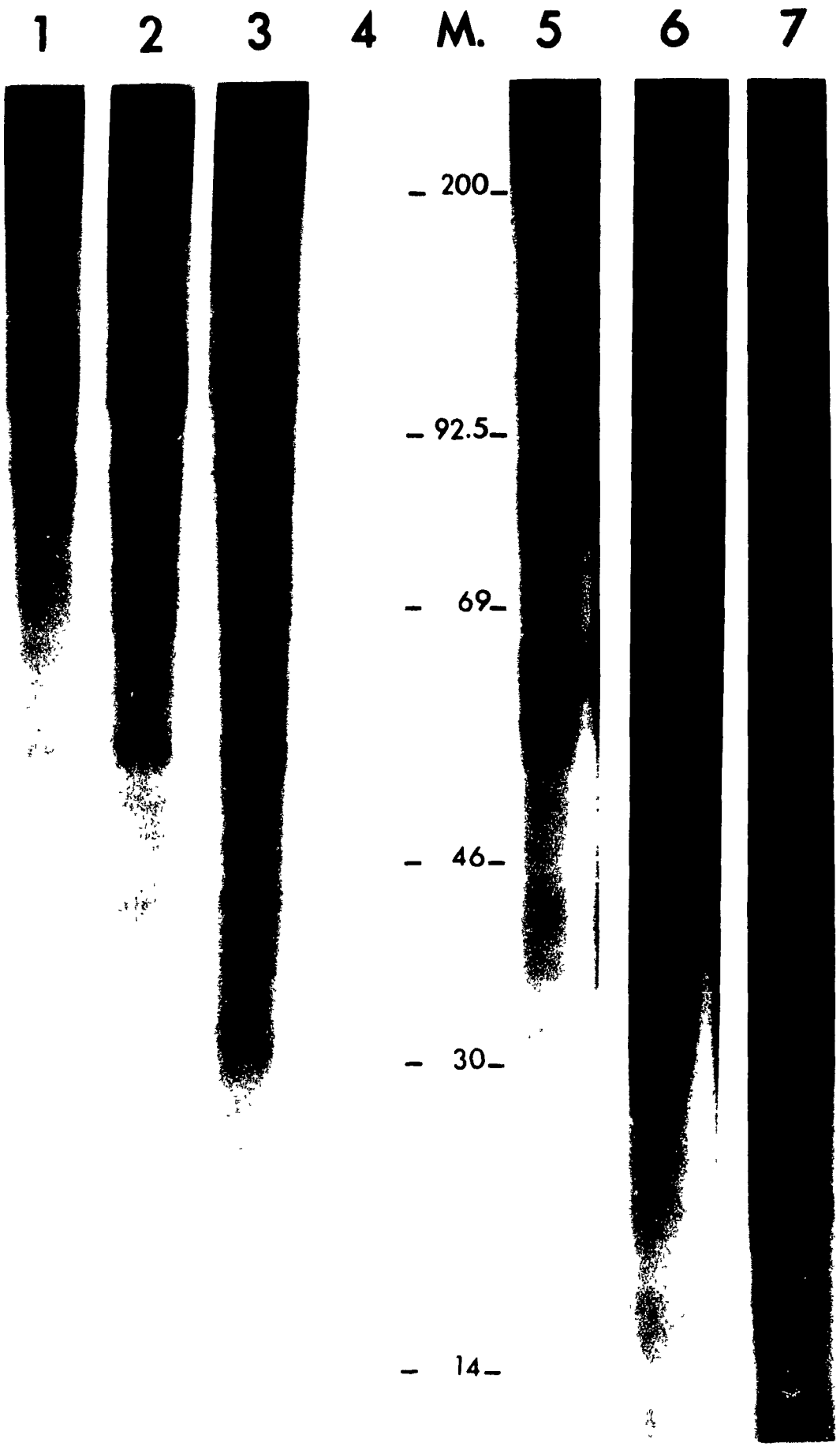
cell extracts with antibody is carried out in the presence of a competitor (2 mM phenyl phosphate), no proteins could be detected (Fig. 5, *lane* 4) also indicating that these proteins contain phosphotyrosine and are purified on the basis of their P–Tyr content. Although for exact determination of the proportions of P–Ser, P–Thr and P–Tyr in these proteins, phosphoaminoacid analysis needs to be carried out, this has not been done as it was not the primary objective of this study.

The data presented here indicate that vanadyl treatment induces transformation by increasing the basal level of cellular phosphotyrosine content. However, this does not demonstrate directly that vanadyl acts as a phosphotyrosine phosphatase inhibitor It is possible that the observed increase in P–Tyr-proteins is due to the actions of vanadate resulting from oxidation of vanadyl which could occur in the culture medium. Because both vanadyl and vanadate have similar effects on cell growth and morphology, and because the intracellular vanadium is in the vanadyl form, the subsequent studies have been carried out using vanadyl to induce cell transformation.

**Figure 5.** ***Affinity purification and SDS-PAGE analysis of phosphotyrosine-containing phosphoproteins.***

After metabolic labeling with $[^{32}P]PO_4$, cells were lysed and extracted and P–Tyr-containing proteins were batch-purified by adsorption to monoclonal antibody 1G2-Sepharose and elution by phenyl phosphate, then analysed by SDS-PAGE and autoradiography.

*Lanes*: 1, untreated NRK cells; 2 and 3, NRK cells treated with 25 μM vanadyl sulfate and sodium orthovanadate, respectively; 4, RR1022 cell lysates adsorbed to affinity beads in presence of 2 mM phenyl phosphate; 5, RR1022 cells; 6 and 7, RR1022 cells treated overnight with 25 μM vanadyl sulfate and sodium orthovanadate, respectively. *M* indicates the position of molecular weight markers. Exposure time: 12 hours.


1
2
3
4
M.
5
6
7
200
92.5
69
46
30
14

**Analysis of Secreted Phosphoproteins.**

To analyse the effects of vanadyl treatment on secreted phosphoproteins, NRK cells were grown for 4 days in presence of 20 μM vanadyl sulfate, a concentration that is both nontoxic and induce cell transformation, then labeled with [$^{32}$P]-$PO_4$ and the secreted proteins analysed. To compare secreted protein profiles, secreted proteins from untreated NRK and transformed RR1022 cells as well as high passage NRK (PT-NRK) cells were analysed. Figure 6*A* shows equal aliquots of the $^{32}$P-labeled total phosphoproteins secreted into the conditioned media of untreated NRK, vanadyl-treated NRK (V-NRK), RR1022 and PT-NRK cells, analyzed by SDS-PAGE and autoradiography. As previously shown, NRK (*lane* 1) and RR1022 (*lane* 4) cells secrete only 69-kDa (pp69) and 62-kDa (pp62) phosphoproteins, respectively. PT-NRK cells (*lane* 3), on the ohther hand, secrete both pp69 and pp62, reflecting their partial transformed state. NRK cells treated with 20 μM vanadyl sulfate, a concentration which induces transformed morphology, anchorage-independent growth and causes loss of contact inhibition of growth, secrete only pp69 but at a significantly reduced level (*lane* 2), as compared to untreated NRK cells (*lane* 1). However, secretion of pp62 is not induced by vanadyl treatment. The reduction in the level of $^{32}$P-labeled pp69 with vanadyl treatment is also confirmed by analyzing phosphoproteins immunoprecipitated from the conditioned media of NRK, V–NRK and RR1022 cells (Fig. 6*B lanes* 3, 1 and 2, respectively) using anti-pp69 antiserum. The lack of additional low molecular weight phosphoproteins in the conditioned medium of V–NRK cells, relative to untreated NRK cells, also indicates that the reduction in the level of pp69 is not due to its degradation.

The finding that vanadyl treatment causes a decrease in in the levels of pp69 was unexpected in view of the fact that vanadium is a phosphatase inhibitor and a kinase activator. Because it acts on other phosphate transfer reactions as well, this raised the possibility that its effect on secreted pp69 is nonspecific. To address this question, its effect on pp69 secreted by PT-NRK cells was examined. These cells offer the advantage that, in addition to pp69, they also secrete pp62 which could be considered as an internal marker. When PT-NRK cells are treated with micromolar concentrations of vanadyl sulfate, they show a transformed phenotype similar to that of the vanadyl-treated NRK cells

vanadyl sulfate, they show a transformed phenotype similar to that of the vanadyl-treated NRK cells (not shown). Analysis of total secreted or immunoprecipitated $^{32}$P-labeled proteins (Fig. 6*C* and *D*, respectively) shows significant reduction in the level of pp69 in the conditioned media of vanadyl-treated PT-NRK cells (*lanes* 2) compared to their untreated counterparts (*lanes* 1). However, the level of pp62 in the conditioned medium remains unaltered upon vanadyl treatment of these cells. These results indicate that vanadyl specifically affects the levels of secreted pp69 protein because pp62 which is immunologically related to pp69 is not affected. The effect of vanadyl on pp69 is also not due to non-specific interference with protein phosphorylation reactions, since other secreted proteins and cell and plasma membrane-associated phosphoproteins (Figs. 4 and 5) are phosphorylated efficiently in its presence.

Because only vanadate has been shown to inhibit PTPases *in vitro* and to increase the levels of P–Tyr-proteins *in vivo*, and as shown in Figure 6*A*, is more potent in increasing P–Tyr-protein levels, it was important to examine the vanadate effects on secreted phosphoproteins. It was possibe that pp62 secretion is induced only in conditions of high P–Tyr-protein levels. The immunoprecipitation data presented in Figure 7*A* show that treatment of NRK cells with vanadate (*lane* 3), like vanadyl (*lane* 2), also causes a decrease in the level of $^{32}$P-labeled pp69 as compared to that of untreated NRK cells (*lane* 1), but without inducing secretion of pp62. No major band in the 62-kDa range could be detected on the autoradiogram, even after prolonged exposures (not shown), indicating that secretion of pp62, a characteristic of oncogenically-transformed cells, is not induced in vanadyl-and vanadate-transformed NRK cells. We also examined the whether the decrease in pp69 levels is due phosphorylation on tyrosine in vandium-treated cells as this may alter kinase-substrate affinity Previous studies have shown that pp69 is phosphorylated only on serine residues (Chackalaparampil et al., 1985). Therefore to examine the above possibility, the gel autoradiographed in Figure 7*B*, which shows $^{32}$P-labeled secreted proteins of NRK and V–NRK cells (*lanes* 1, 2, respectively), was subjected to alkali treatment, which hydrolyses P–Ser; any residual radioactivity will be indicative of P–Tyr phosphorylation. Figure 7*B* shows that all the radioactivity associated with pp69 disappeared after such treatment (*lanes* 3, 4). This indicates that pp69 is not phosphorylated on tyrosine in V–NRK cells, therefore, further phosphoaminoacid analysis was not carried out

**Figure 6.** *SDS-PAGE analysis of secreted phosphoproteins.*

Nearly confluent culture were metabolically labeled with [$^{32}$P] $PO_4$, then aliquots of total or immunoprecipitated phosphoproteins were analysed.

***A.*** Total secreted proteins from NRK cells (*lane* 1); 20 μM vanadyl sulfate-treated NRK cells (*lane* 2), high passage PT-NRK cells (*lane* 3); and RR1022 cells (*lane* 4).

***B.*** Immunoprecipitation using anti-p69 antiserum of phosphoproteins from NRK cells (*lane* 3); RR1022 cells (*lane* 2) and 20 μM vanadyl sulfate-treated NRK calls (*lane* 1).

***C.*** Total and ***D.*** immunoprecipitated phosphoproteins from untreated (*lanes* 1) and and 20 μM vanadyl sulfate-treated PT-NRK cells (*lanes* 2).

The positions of pp69 and pp62 and prestained molecular weight markers are indicated

Exposure times: ***A*** and ***C***, 24 hours; ***B*** and ***D***, 48 hours.

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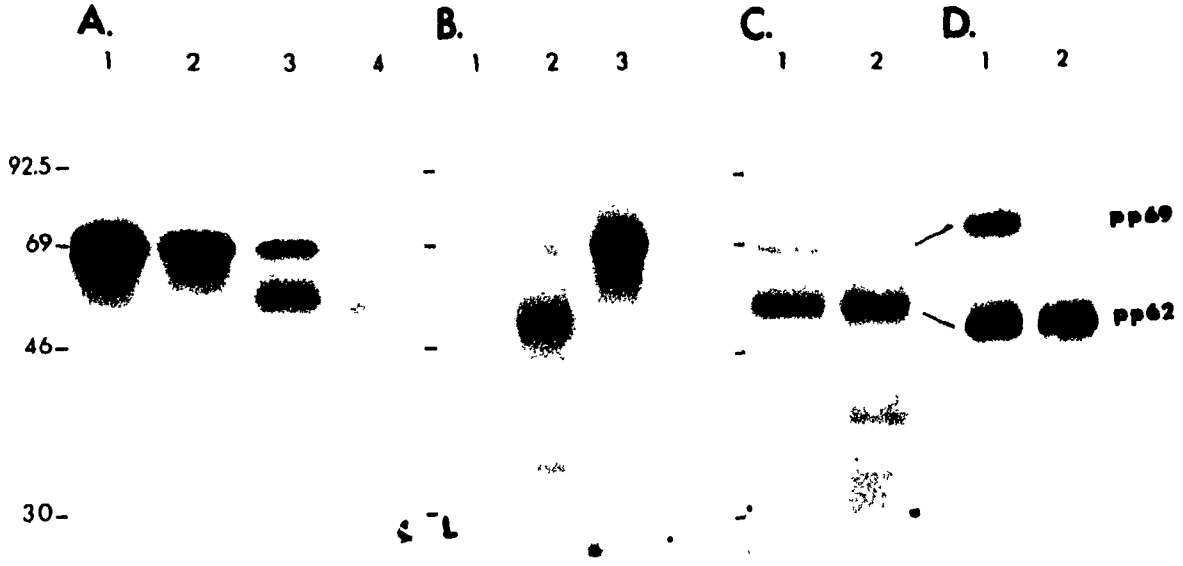
**Figure 7.** *Phosphorylation of pp69 in vanadium-treated cells.*

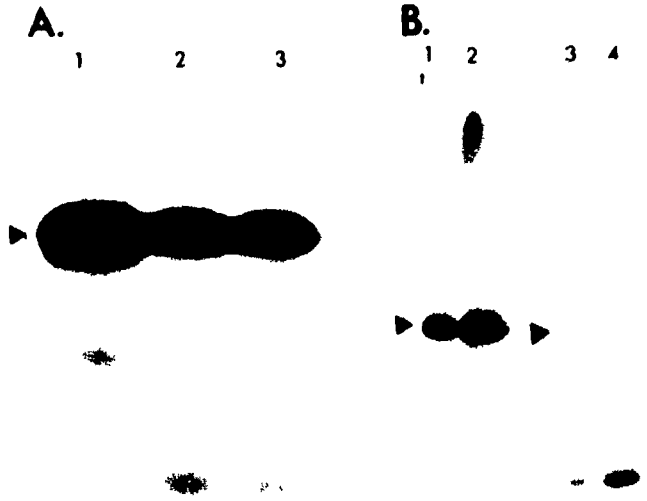
***A.*** Both vanadyl and vanadate decrease the secretion of pp69.

Aliquots of conditioned media containing equal acid-soluble cpm were immunoprecipitated from the conditioned medium of NRK (*lane* 1); vanadyl and vanadate-treated NRK cells (*lanes* 2 and 3, respectively) and analysed. Exposure time is 24 hours

***B.*** pp69 is phosphorylated through alkali-labile phosphodiester linkages in both NRK and vanadyl-treated NRK cells

Phosphoproteins from untreated (*lanes* 1, 3) vanadyl sulfate-treated (*lanes* 2, 4) NRK cells were fractionated by SDS-PAGE and autoradiographed before (*lane* 1, 2) and after (*lanes* 3, 4) alkali treatment. Five-fold excess of cpms were loaded on *lane* 2. Exposure time is 24 hours (*lanes* 1, 2) and 72 hours (*lanes* 3, 4).


A.
B.
C.
D.
1 2 3 4
1 2 3
1 2
1 2
92.5–
69–
46–
30–
pp69
pp62


A.
B.
1 2 3
1 2 3 4

**Analysis of Total Secreted Proteins.**

Another possibility for the decrease of pp69 levels in V-NRK cells was that vanadyl inhibits its secretion, but not phosphorylation. To examine this possibility, cells were metabolically labeled with [$^{35}$S]-methionine and total secreted proteins were analysed by SDS-PAGE and fluorography. Figure 8*A* shows that the protein banding at 69 kDa is increased in the conditioned medium of vanadyl-treated (*lane* 2) relative to untreated NRK cells (*lane* 1). In RR1022 cell conditioned medium (*lane* 3) a 69 kDa protein (p69) is also present at comparable levels to that of NRK cells, although undetectable when labeled with $^{32}$P, which suggests that the 69 kDa protein, secreted in elevated levels in V–NRK cells, is in a nonphosphorylated form. The increase in p69 secretion is also shown by immunoprecipitation (Fig. 8*C*, *lane* 4; compare with Fig. 12*A*, *lane* 1); in both NRK and V–NRK cells, p69 resolved into a doublet in which the faster migrating band is increased and the slower migrating band is decreased upon vanadyl treatment.

As mentioned previously, we suspected that high passage NRK cells consist of a mixed population of nontransformed and spontaneously transformed cells as indicated by phosphorylation of plasma membrane vesicles-associated proteins, secretion of pp62 and growth in suspension. Therefore, the transformed cells were selected by two rounds of growth in soft agar and their secreted proteins analysed. Figure 8*B* shows that after selection, PT-NRK cells secrete reduced levels of $^{32}$P-labeled pp69 and pp62 (*lane* 3), but labeling with [$^{35}$S]-methionine indicates that, like V–NRK cells, they secrete a 69 kDa protein which resolves into a doublet in which the faster migrating component is increased approximately 10-fold as compared to that of NRK cells (Fig. 8*C*, *lanes* 3 and 5).

**Figure 8.** *Analysis of total secreted proteins.*

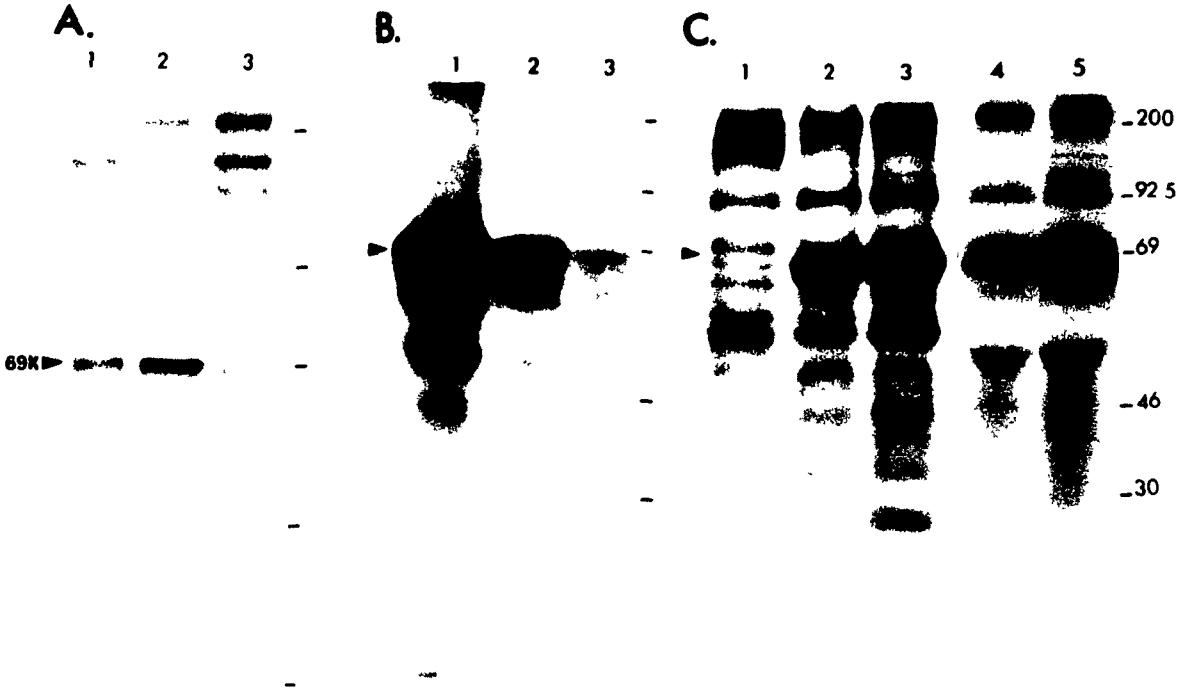
Twenty-four hours after plating, subconfluent cultures were metabolically labeled with [$^{35}$S]-methionine or [$^{32}$P]$PO_4$ for 4 hours.The conditioned media were collected and aliquots containing equal TCA-precipitable $^{35}$S cpm were precipitated with either TCA or by anti-p69 antiserum and analysed by SDS-PAGE.

***A.*** Total secreted proteins of NRK (*lane* 1); vanadyl-treated NRK (*lane* 2); and RR1022 cells (*lane* 3).

***B.*** Analysis of total secreted $^{32}$P-labeled proteins of NRK (*lane* 1); vanadyl-treated NRK (*lane* 2) and agar-selected PT-NRK cells (*lane* 3).

***C.*** Analysis of $^{35}$S-labeled total secreted (*lanes* 1-3) and immunoprecipitated (*lanes* 4,5) proteins from the conditioned media of NRK (*lane* 1); vanadyl-treated NRK (*lanes* 2, 4); and agar-selected PT-NRK cells (*lanes* 3, 5). The immunoprecipitated sample of NRK cells is shown in Fig. 12***A***, *lane* 1.

*Arrowheads* point to the position of p69. The positions of molecular weight markers are indicated on the right.


A.
1
2
3
69K
B.
1
2
3
C.
1
2
3
4
5
200
92.5
69
46
30

**Relationship Between the 69 kDa proteins.**

The data on analysis of secreted proteins and phosphoproteins, summarized in Figure 8, indicated that both V–NRK and agar-selected PT-NRK cells secrete the 69 kDa protein in elevated amounts, but in a form which is not detectable when the cells are labeled with $^{32}P$. Although both components of the 69 kDa doublet are specifically immunoprecipitated by anti-p69 antiserum, this was not enough evidence to indicate whether or not they are the same protein. The anti-p69 serum used in this study was originally raised against a 69 kDa protein eluted from preparative polyacrylamide gel bands after identification with coomassie blue staining. Therefore, it is possible that the antiserum was raised against two or more distinct proteins. Particularly, fibronectin is consistently detected in immunoprecipitates using this antiserum, and 3 proteins of M.W. 140-180, identified as collagens (see below), were decreased concomitant with an increase in the levels of the faste-migrating 69 kDa band. Therefore, it was possible that this protein is is a degradation product of fibronectin or one of the collagens. On the other hand, by analogy with other reported proteins which have anomalous mobility on SDS-PAGE, depending on their phosphorylation state, we hypothesized that the fast migrating band is a nonphosphorylated form and the slower one is a phosphorylated form of p69 (np69 and pp69, respectively).

For a more detailed analysis, $^{35}S$-labeled and $^{32}P$-labeled proteins secreted by NRK cells were subjected to two-dimensional IEF/SDS-PAGE. Figure 9*A* shows NRK cells secrete two proteins in the 69 kDa range. The approximate pI values were determined by reading the pH on four separate IEF gels ( see Fig. 11*D*). The most acidic protein has a pI value of approximately 3.8 The second one, less acidic, resolves into several discrete spots with heterogeneous pI and M W., indicative of differential glycosylation commonly observed in secreted proteins, has a pI value of ~4 5 The position of phosphorylated 69 kDa protein (pp69) was inferred from the position of major $^{32}P$-labeled protein secreted by NRK cells (Fig. 9*B*) which also corresponds to pI 3 8. To determine whether the charge difference in the two 69 kDa proteins is due to phosphate groups, $^{32}P$- and $^{35}S$-labeled 69 kDa proteins were isolated by immunoprecipitation from NRK conditioned media and treated, on immunoaffinity support, with alkaline phosphatase (ALP). Figure 9*C*, *lane* 1 shows that ALP

efficiently dephosphorylates $^{32}$P-labeled pp69. Figure 9*D* indicates that after such treatment, only one spot can be detected, indicating the co-migration of the two proteins. This result indicates that the two proteins are phosphorylated and nonphosphorylated forms of the same 69 kDa protein, designated as pp69 and np69, respectively. For further confirmation $^{32}$P- and $^{35}$S-labeled pp69 and $^{35}$S-labeled np69 were isolated from 2-d gel bands and subjected to peptide mapping using S. *aureus* V8 protease. Figure 9*E* shows that both $^{32}$P- (*lane* 3) and 35S- (*lane* 1) labeled pp69 exhibit similar peptide pattern and share fragment homology with np69 (*Lane* 2).

Similar analysis of V–NRK cells (Fig. 10*A*) indicates that they secrete elevated amounts of np69, but low, undetectable levels of pp69, thus confirming that vanadyl treatment increases the secretion of nonphosphorylated (np) p69. Figure 10*B* shows that RR1022 cells secrete normal levels of p69 relative to NRK cells, but also in an np form. To locate the position of pp62, PT–NRK and vanadyl-treated PT–NRK cells were labeled with [$^{32}$P]$PO_4$ and the secreted phosphoproteins were analysed by 2–d gel electrophoresis. Figure 11*A* shows that in PT–NRK cells pp69 migrates in several spots with pI values ranging from 3.8 to close to 4.5 and that pp62 has a pI of 3.8, the same than that of pp69. The identity of these proteins as pp69 and pp62 is confirmed by analyzing $^{32}$P-labeled proteins secreted by PT–NRK cells after vanadyl treatment (Fig. 11*B*), which reduces the levels of pp69, but does not affect pp62 (Fig. 6*C* and *D*). The migration patternof pp69 in PT–NRK cells is indicative of different levels of pgosphorylation. However, it was not possible to determine unambiguously the position of $^{35}$S-labeled pp62 on 2-d IEF/SDS-PAGE (e.g. in RR1022 cells; Fig. 10*B*).

**Figure 9.** ***Relationship between the two p69 secreted proteins.***

Two-dimensional IEF/SDS-PAGE analysis of total secreted $^{35}$S-labeled (***A.***) and $^{32}$P-labeled (***B.***) secreted proteins of NRK cells.

***C.*** SDS-PAGE of immunoprecipitated, $^{32}$P-labeled phosphoproteins incubated with (+) or without (-) alkaline phosphatase (ALP.)

***D.*** 2-D analysis of immunoprecipitated $^{35}$S-labeled secreted proteins after treatment with alkaline phosphatase.

***E.*** *In situ* peptide mapping of $^{35}$S-labeled (*lane* 1) and $^{32}$P-labeled (*lane* 3) pp69 and $^{35}$S-labeled np69 (*lane* 2) after proteolytic digestion using V8 protease.

*Filled arrows* denote the position of pp69; *open arrows* indicate the position of np69; *asterisks* point to the position of reference proteins. The positions of molecular weight markers are indicated. Peptides resulted from the protease digestion were separated on a 15-20% cross-liked gradient polyacrylamide gel. Exposure times were: 3 days (***A.***); 48 hours (***B.***); 24 hours (***C.***); five days (***D.***); and 10 days (***E.***)

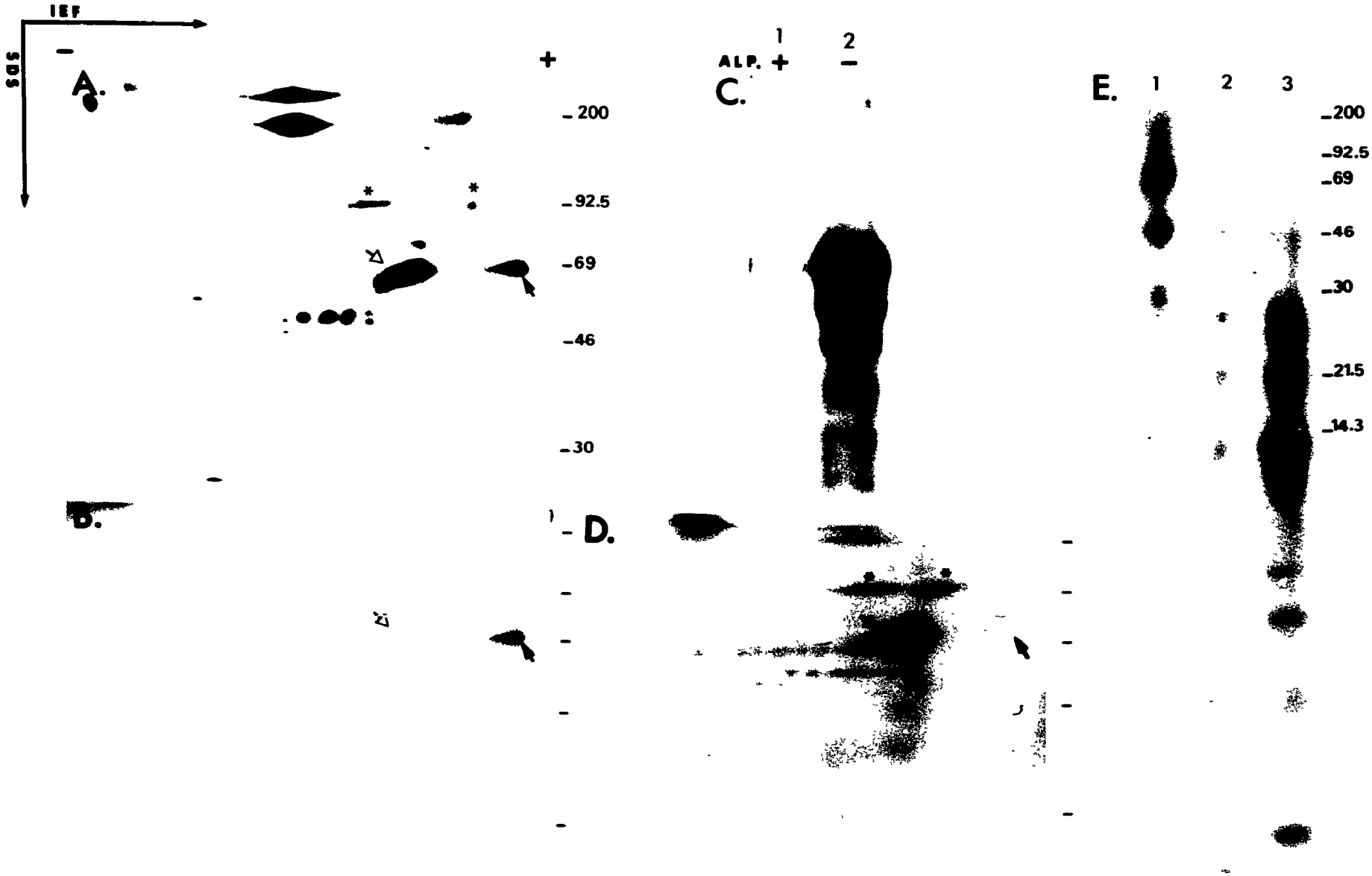

IEF
SDS
A.
B.
C.
D.
E.
ALP.
1
2
3
200
92.5
69
46
30
21.5
14.3

**Figure 10.** *Two-dimensional IEF/SDS-PAGE analysis of secreted proteins in transformed cells.*

The figure shows fluorograms of $^{35}$S-labeled secreted proteins from the conditioned media of vanadyl-treated NRK cells (***A.***) and RR1022 cells (***B.***).
Legend as in Fig. 9.

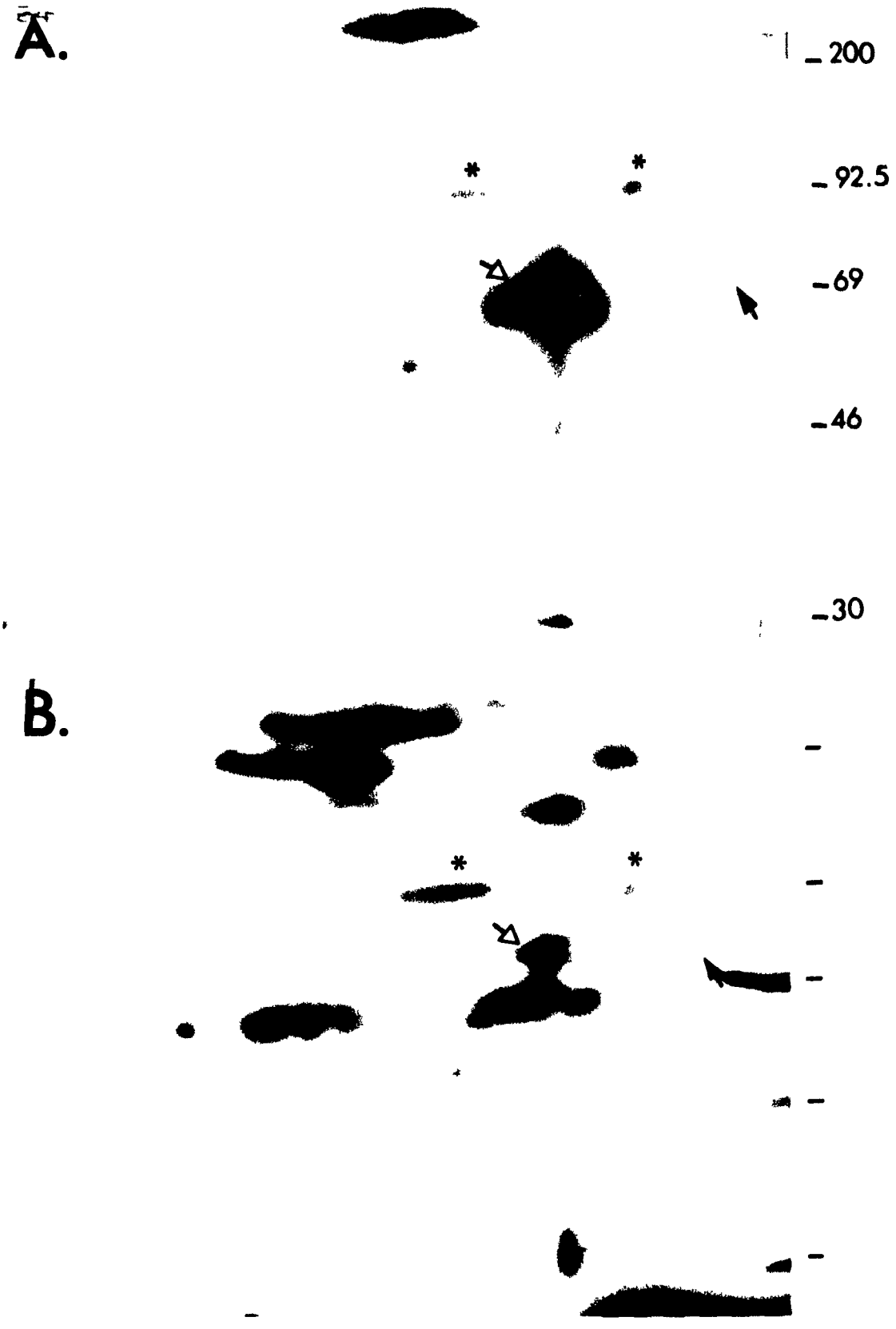

A.
200
92.5
69
46
30
B.

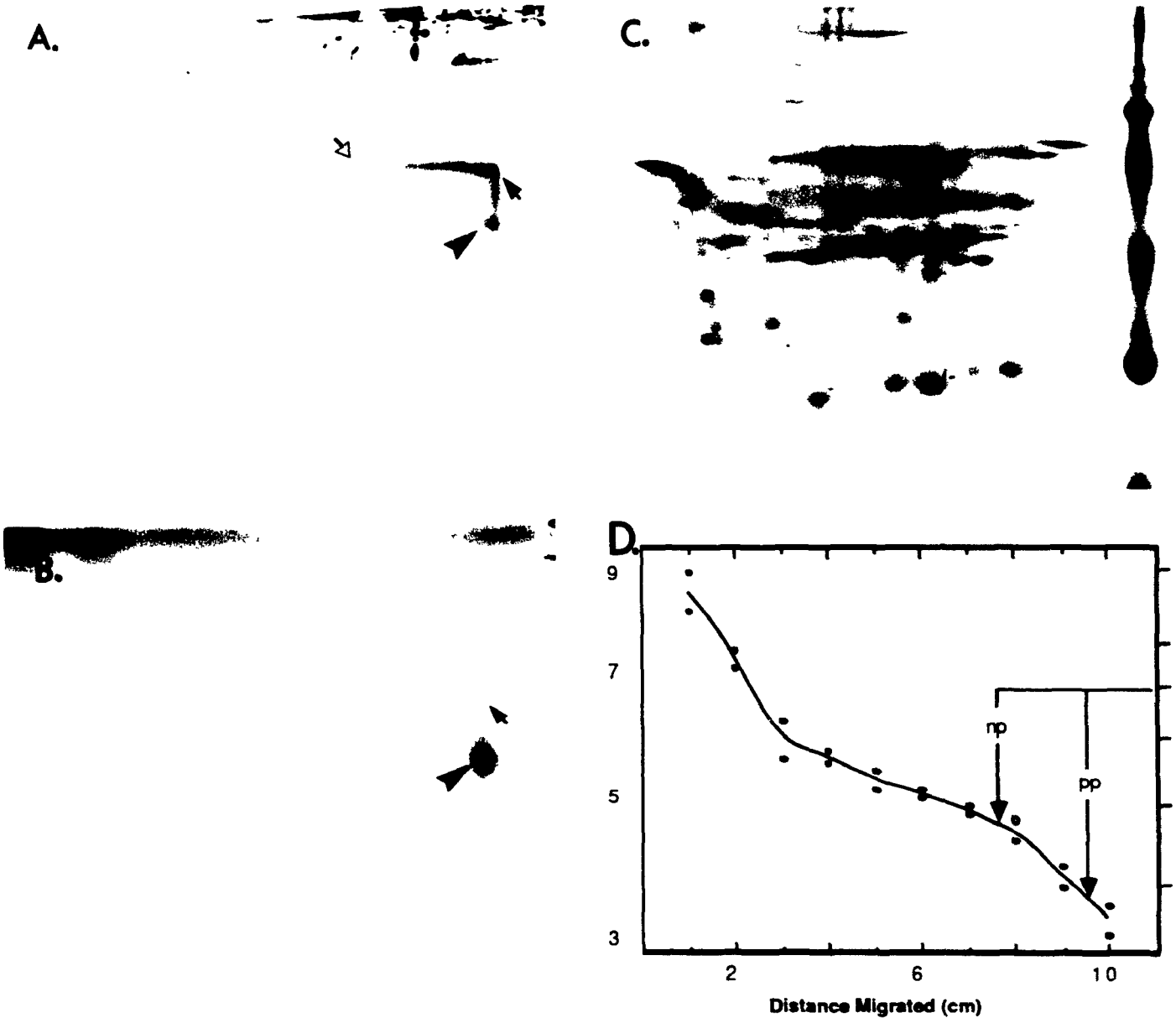
**Figure 11.** *2-D IEF/SDS-PAGE of secreted proteins of PT-NRK and NRK cells.*

Figure shows $^{32}$P-labeled conditioned medium of untreated (***A.***) and vanadyl-treated (***B.***) PT-NRK and unlabeled conditioned medium of untreated NRK cells (***C.***). ***A.*** and ***B.*** are autoradiograms; ***C.*** is a coomassie blue-stained gel.

In ***C.*** the lane on the right indicates the the migration molecular weight standards lane; standard proteins run slightly faster than sample proteins because they were loaded directly in the stacking gel. *Arrowhead* indicates the position of pp62. Other legend as in Fig. 9.

In ***D.*** the pH values read in four IEF gels (two by two) are plotted against distance from the top. The approximate positions of pp69 and np69 are indicated.*

* Note that the pH values change abruptly near the cathode and the anode which may be due to the fact that the gels were soaking in the cathode and anode solutions. For this reason, the pI values assigned here to pp69 and np69 may not be accurate.


A.
C.
B.
D.
9
7
5
3
2
6
10
np
pp
Distance Migrated (cm)

**A Possible Molecular Interaction between np69 and Fibronectin.**

As mentioned previously, the presence of fibronectin (Fn) in anti-p69 immunoprecipitates was consistantly observed. Fibronectin is an adhesive protein, therefore its presence in immunoprecipitates is not surprising. However, we observed its co-precipitation with p69 regardless of the stringency of the immunoprecipitation conditions. Figure 12*A* shows that anti-p69 antiserum precipitates in addition to p69 and p62, an unidentified 92.5 kDa protein and fibronectin (*lane* 1). Interestingly, anti-Fn antiserum also precipitates np69 in addition to fibronectin (*lane* 3). When the conditioned media were heated to 95 °C in presence of 0.2% SDS prior to immunoprecipitation, anti-p69 precipitates only p69 and anti-Fn precipitates only fibronectin (*lanes* 2 and 4, respectively) Therefore, denaturation of conditioned media abolishes co-precipitation of np69 and fibronectin. This is confirmed by western blotting of serum-free NRK conditioned medium shown in Figure 12*B*; anti-Fn antiserum detects only fibronectin and anti-p69 detects only the 69 kDa band and to a lesser extent, the 62 kDa band (*lanes* 2 and 1, respectively). These results indicate that anti-p69 does not recognize directly Fn and suggest a possible association between Fn and np69. This putative association, if any, does not involve disulfide linkages as the migration of either np or pp69 is not altered in reducing or non reducing conditions. Similar results were obtained by diagonal reducing/non-reducing 2--d SDS-PAGE (not shown). A possibility which cannot be excluded is that native np69 and fibronectin possess some homology in antigenic determinants, which are masked by phosphate groups in pp69, and which are abolished following denaturation.

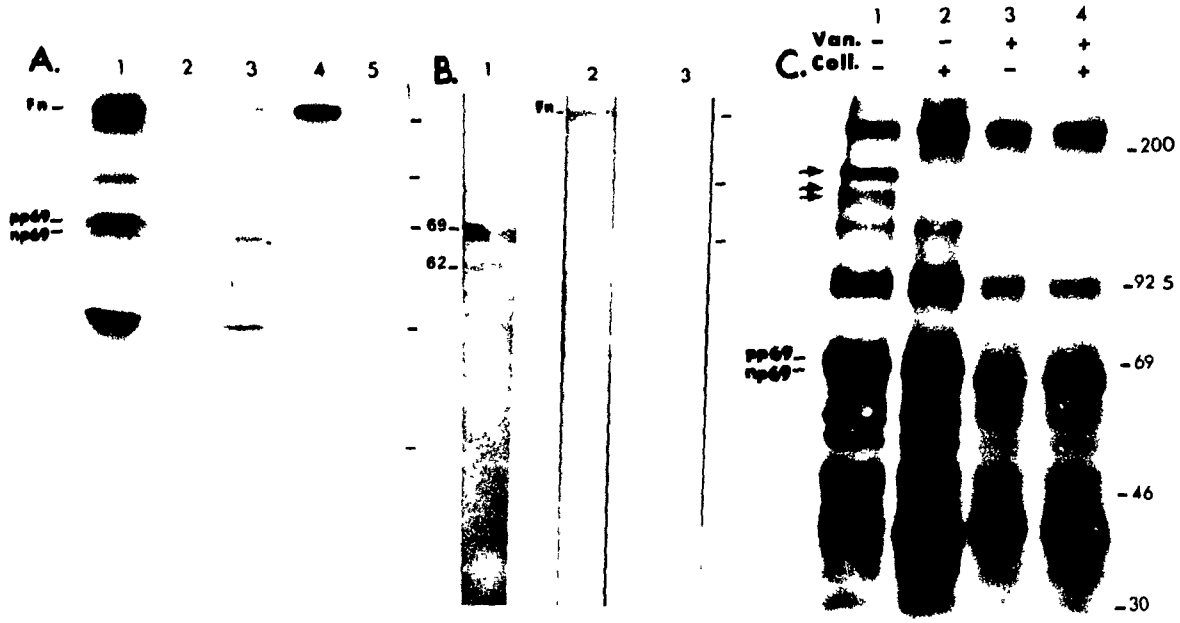
**Figure 12.** *Co-precipitation of np69 and fibronectin and collagenase digestion of vanadium-decreased proteins.*

***A.*** $^{35}$S-labelec' conditioned medium of NRK cells was precipitated using either anti-p69 antiserum (*lanes* 1, 2) or anti-fibronectin antiserum (*lanes* 3,4) with (*lanes* 2, 4) or without (*lanes* 1, 3) prior heat treatment (95 °C, 5 min) in presence of 0.2 % SDS. *Lane* 5 is NRK conditioned medium precipitated with a non-immune rabbit serum.

***B.*** Western blotting. Unlabeled serum-free conditioned medium of NRK cells was prepared, and the proteins fractionated on a 10% polyacrylamide gel, transferred to a nitrocellulose filter and individual strips of the filter were probed using either anti-p69 (*lane* 1) or anti-fibronectin (*lane* 2) antiserum. *Lane* 3 was probed using non-immune rabbit serum. The sample used in this experiment is shown in Fig. 11***C.***

***C.*** Collagenase assay. $^{35}$S-labeled proteins from the culture supernatants of vanadyl (*Van.*)-treated (+) or untreated (-) NRK cells were incubated in absence (-) or presence (+) of highly purified bacterial collagenase (*Coll.*) as described in MATERIALS AND METHODS and analysed by SDS-PAGE and fluorography.

The positions of np and pp69 are indicated. *Arrows* indicate the proteins selectively digested by the collagenase. Molecular weight markers are indicated on the right.


A.
1
2
3
4
5
Fn
pp69
np69
B.
1
2
3
Fn
69
62
C.
1
2
3
4
Van.
Coll.
200
92.5
69
46
30

**Dose-Dependent Effects of Vanadyl on p69.**

Examination of effects of vanadyl on cell growth and morphology indicated that whereas morphological transformation is induced in a dose-dependent manner, its effects on cell growth (anchorage-dependent and -independent) are not. Growth stimulatory effects of vanadyl are more pronounced at lower concentrations (10-20 μM) in low density cultures whose growth appears to be inhibited at higher concentrations (>30 μM). Therefore through analysis of effects of different concentrations of vanadyl on p69 secretion and phosphorylation, we have exploited this discriminative effect of vanadyl on cell growth and morphology to gain insight as to which parameter p69 is related. Figure 13*A* shows the $^{32}$P-labeled phosphoproteins of NRK cells treated with 0, 10, 20, 30, and 35 μM vanadyl sulfate and panel *B* shows total secreted proteins of NRK cells treated at the corresponding concentrations. Panel *A* shows that the decrease in the levels of pp69 is dose-dependent. Panel *B* shows that concomitant with the dose-dependent decrease in pp69, is a dose-dependent increase in np69 secretion. However, densitometric tracing of the fluorograms indicate that the levels of pp69 and np69 are not additive for every vanadyl concentration, but there is an overall increase in p69 production. This dose-dependent effect of vanadyl suggests that p69 may not be related to cell growth since there is no correlation between growth characteristics of V-NRK cells and p69 secretion or phosphorylation; these latter two parameters correlate better with morphological changes which are also induced in a dose-dependent manner, suggesting p69 is involved in maintenance of some aspect of cell morphology.

**Action of Vanadyl on Extracellular Matrix Collagens.**

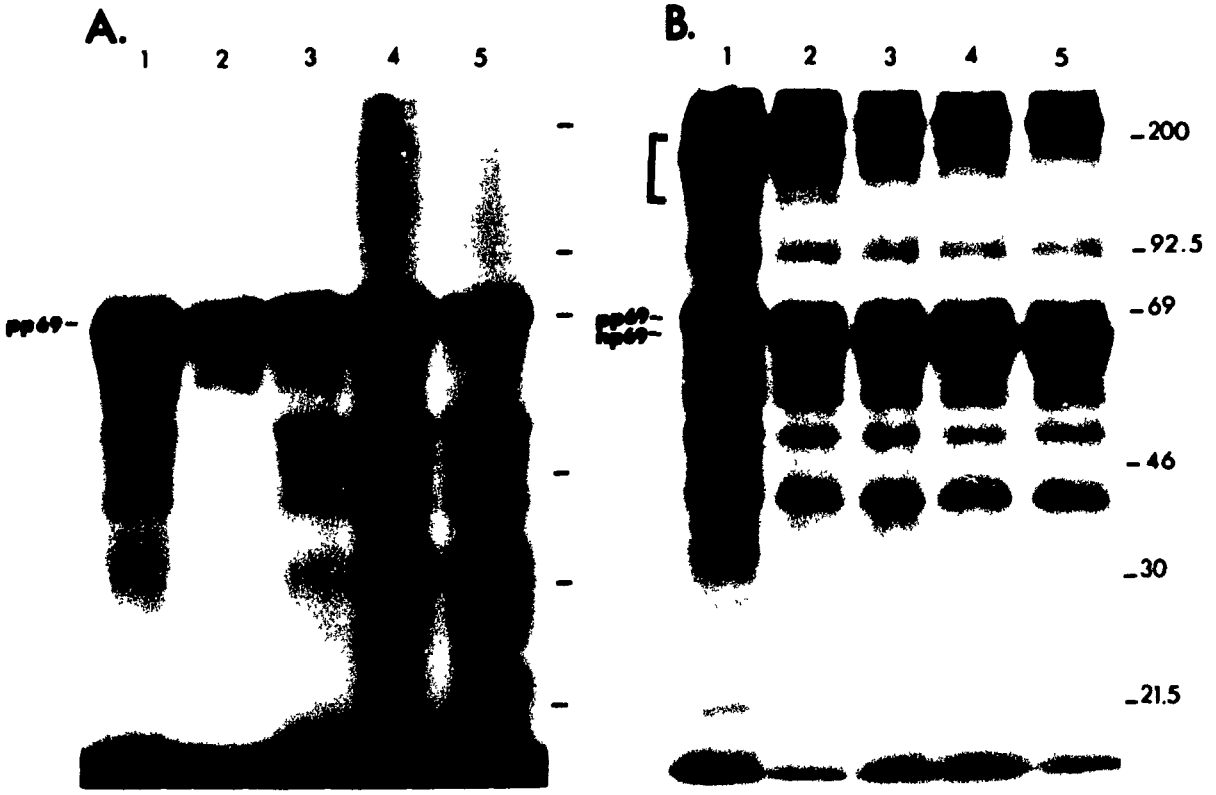
In addition to its effects on p69, vanadyl treatment also causes a decrease in the levels of three high molecular weight proteins (140-170 kDa) in a dose-dependent manner (Fig. 8, *lane* 2; Fig. 13*B*, *lanes* 2-5). It was suspected that these proteins are collagen-like molecules by comparison with other published studies (Ignotz and Massagué, 1986; Broek et al., 1985). To examine this possibility, $^{35}$S-labeled secreted proteins were digested with highly purified bacterial collagenase in conditions which

typical migration patterns in reducing and non-reducing SDS-PAGE (not shown). The levels of soluble fibronectin are not affected by vanadyl treatment.

**Figure 13.** *Dose-dependent effect of vanadyl sulfate on p69 secretion and phosphorylation.*

Aliquots of $^{35}S$- or $^{32}P$-labeled conditioned media containing eq' al $^{35}S$ counts were precipitated by 10% TCA and analysed. ***A.*** $^{32}P$-labeled and ***B.*** $^{35}S$-labeled total secreted proteins of untreated NRK cells (*lanes* 1) or treated with 10, 20 30 and 35 μM vanadyl sulfate (*lanes* 2-5, respectively).

The positions of np69, pp69, fibronectin (Fn.) and molecular weight standards are indicated. *Bracket* denotes the collagenase-sensitive proteins.


A.
1
2
3
4
5
pp69
B.
1
2
3
4
5
200
92.5
69
46
30
21.5

**Cell Surface Localization of pp69.**

Some observations made during the course of this study prompted examination of the cell surface localization of p69. First, the co-precipitation of np69 with fibronectin suggested that it may result from an association between np69 and Fn after or prior to incorporation of Fn into the cell matrix. Second, the complete correlation between p69 secretion and phosphorylation and morphological changes observed in V-NRK cells suggested that p69 may be involved in the maintenance of cell morphology through interaction with ECM components. Finally, like most secreted proteins, p69 should interact with the cell surface in order to carry out some functional role. This possibility was examined by radioiodination of cell surface proteins followed by immunoprecipitation. This was the method of choice since purified p69 was not available to directly demonstrate its interaction with the cell surface or its incorporation into the cell matrix. Affinity labeling of cell surface p69 or immunofluorescence studies were also not performed. Therefore NRK cells were cell surface labeled with $^{125}$I in mild conditions to avoid cell lysis or disruption. After labeling, cells were examined under microscope for their structural integrity, and intact cultures were lysed and immunoprecipitated using anti-p69 antiserum. Figure 14*A* shows the total $^{125}$I-labeled cell surface protein profile (*lane* 1) and the result of the immunoprecipitation (*lane* 2). Comparison with the migration of $^{35}$S-labeled secreted proteins (*lane* 4) indicates the immunoprecipitated $^{125}$I-labeled p69 co-migrates with pp69, but not np69. To further examine this point, V–NRK cells, which at this concentration secrete predominantly np69, have been analysed as above. Figure 14*B* shows that the p69 localizing to the cell surface of V–NRK cells (*lane* 2) also co-migrates with pp69 of untreated cells (*lane* 1). In addition, the amount of p69 detectable on the cell surface appears to be increased by vanadyl treatment (see Figure legend). This indicates that p69 detectable on the cell surface is qualitatively and quantitatively different from the soluble p69 present in the conditioned medium and suggest that p69 phosphorylation is critical for its cell surface localization. To further confirm this point, PT-NRK cells were made quiescent by growth to confluence and serum starvation. Figure 14*C*, *lane* 6, shows that when PT-NRK cells are serum-starved they secrete low levels of $^{32}$P-labeled pp69 compared to normal growth conditions (*lane* 5). However, the levels of $^{125}$I-labeled p69 present on the cell surface

is increased in quiescent (*lane* 3) compared to growing cells (*lane* 1). In addition, in KNRK cells, which secrete elevated amounts of pp69 and pp62 in comparable amounts (see below; Fig. 15*B*), only little pp69 can be detected on their cell surface (*lane* 2).

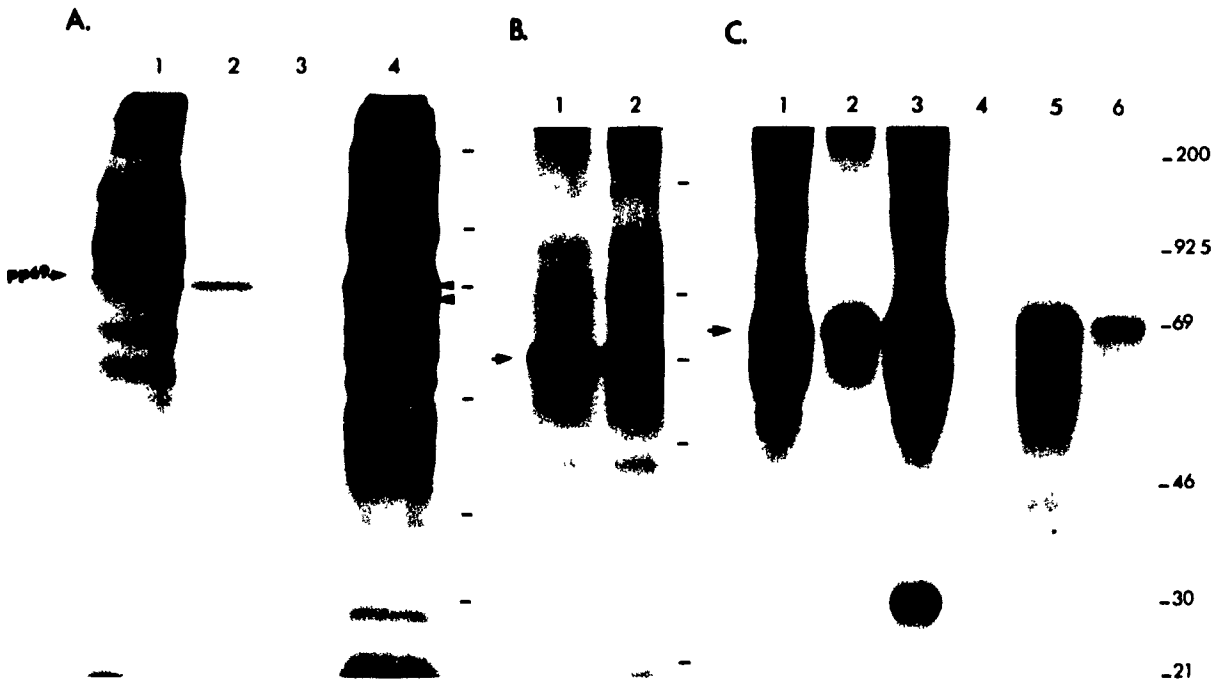
**Figure 14.** *Cell surface localization of pp69.*

Nearly confluent cultures were $^{125}$I–cell surface-labeled with [$^{125}$I]NaI using lactoperoxidase, examined under microscope and intact cultures were lysed and immunoprecipitated.

***A.*** Detection of pp69 on the cell surface of NRK cells. *Lane* 1 total cell lysate; *lane* 2, cell lysate precipitated with anti-p69 antiserum; *lane* 3, cell lysate precipitated with non-immune rabbit serum; *lane* 4, total secreted $^{35}$S-labeled proteins; for comparison, the position of pp69 and np69 are indicated (*arrowheads*).

***B.*** Effect of vanadium on cell surface pp69. cell surface $^{125}$I-labeled pp69 immunoprecipitated from aliquots containing equal precipitable counts of NRK (*lane* 1) and vanadyl-treated NRK (*lane* 2) cell lysates. The labeling of vanadyl-treated NRK cells was 2-3 times more efficient than untreated NRK cells, therefore the size of the sample used for immunoprecipitation was adjusted accordingly. Therefore, it is likely that the increase of pp69 detectable on the cell surface of treated cells is 2-3–fold higher than the actual increase observed on the autoradiogram.

***C.*** Detection of pp69 on the surface of transformed and partially transformed cells.Cell surface labeling and immunoprecipitation: PT-NRK cells (*lane* 1), KNRK (*lane* 2), quiescent NRK cells (*lane* 3), $^{125}$I-labeled PT-NRK cell-lysate precipitated with a non-immune serum (*lane* 4), $^{32}$P-labeled secreted proteins of PT-NRK (*lane* 5), and quiescent PT-NRK cells (*lane* 6). For comparison, a sample of $^{32}$P-labeled proteins secreted by KNRK cells is shown in Fig. 15***B,*** *lanes* 3 and 4.


A.
B.
C.
1
2
3
4
1
2
1
2
3
4
5
6
-200
-92.5
-69
-46
-30
-21

**Immunological Relationship Between 2ar/osteopontin and pp69/pp62.**

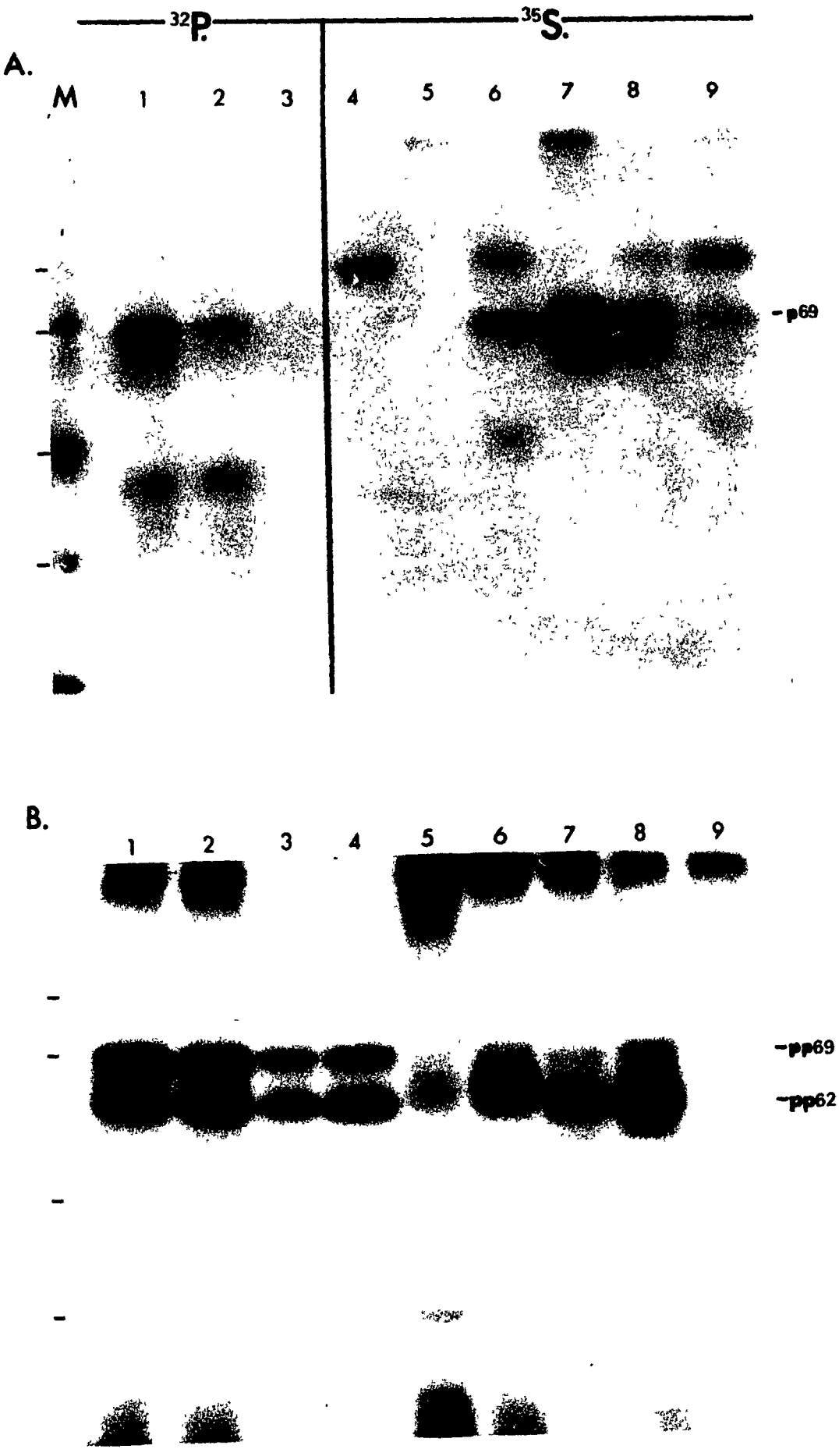
Osteopontin has been originally isolated from calcified rat bone extracts (Prince et al., 1987), and 2ar was cloned as an mRNA whose expression is inducible by TPA in promotable mouse epidermis JB6 cells (Smith and Denhardt, 1987). cDNA sequencing revealed that they are the rat (Odberg et al., 1986) and mouse (Craig et al., submitted)[1] counterparts, and both were identified as secreted proteins representing MSPs in culture supernatants of rat and mouse cells, respectively. Therefore we examined whether or not they are related to the MSPs described in the present study. To address this question, $^{32}P$- and $^{35}S$-labeled secreted proteins were immunoprecipitated from NRK conditioned media using anti-p69 and anti-2ar antisera. Figure 16*A* indicate both antisera immunoprecipitate $^{32}P$-labeled pp69 (*lanes* 1 and 2). When the conditioned medium is subjected to double immunoprecipitation, using anti-2ar first, no further $^{32}P$-labeled protein could be labeled using anti-p69 (*lane* 3) indicating that both antisera recognize the same protein. Figure 15*B* also indicate that both antisera precipitate $^{35}S$-labeled p69 (*lanes* 6 and 7) whose levels are decreased upon reciprocal double immunoprecipitations (*lanes* 8 and 9). The 92.5 kda protein precipitated by anti-p69 is present in non-immune rabbit serum, but is not precipitated by anti-2ar or anti β–galactosidase control serum. The precipitation of pp69 and pp62 (Fig. 15*B*) by anti-p69 (*lanes* 1, 3, 5, 7) and anti-2ar (*lanes* 2, 4, 6, 8) antisera is also demonstrated by immunoprecipitation of $^{32}P$-labeled PT-NRK (*lanes* 1, 2), KNRK (*lanes* 3, 4), KA31 (5, 6) and vanadyl-treated PT-NRK (*lanes* 7, 8) conditioned media These results indicate that pp69 and pp62 are immunologically related to 2ar/osteopontin. Based on this and the facts that 2ar/osteopontin are secreted proteins encoded by a single copy gene in mice and rats, and that pp62 described by Senger and co-workers (Senger et al., 1988) has N-terminal sequence identical to that of 2ar/osteopontin, and that all these proteins are unique major secreted phosphoproteins allow to conclude that they all are the same protein.

**Figure 15.** *Immunological relationship between pp69/pp62 and 2ar/osteopontin.*

***A.*** Both anti-p69 and anti-2ar immunoprecipitate the same protein secreted by NRK cells. Conditioned medium of NRK cells labeled with $^{32}P$ (*lanes* 1-3)or $^{35}S$ (*lanes* 4-9) was precipitated using either anti-p69 (*lanes* 1, 6) or anti-cro–βgal–(N/C)2ar fusion proteins (*lanes* 2, 7). *Lanes* 3, 8 and 9 are double immunoprecipitations. *Lane* was precipitated with anti-2ar, then the supernatant was precipitated with anti-p69. *Lane* 8, same than *lane* 3. *Lane* 9 opposite than *lane* 3. *Lanes* 4 and 5 were immunoprecipitated using a non-immune rabbit serum and anti-cro–βgal control serum, respectively.

***B.*** Immunoprecipitation of pp69 and pp62 secreted by different cell lines. Immunoprecipitation of $^{32}P$-labeled pp69 and pp62 from the conditioned media of PT-NRK (*lanes* 1, 2, 9), KNRK (*lanes* 3, 4), KA31 (*lanes* 5, 6), and RR1022 cells (*lanes* 7, 8), using anti-p69 (*lanes* 1, 3, 5,7), anti-cro–βgal–2ar (*lanes* 2, 4, 6, 8) antisera, or a non-immune rabbit serum (*lane* 9). Immunoprecipitations were carried out as described before, except that 25 μl of anti-p69 and 2 μl of anti-2ar antisera were used.

The positions of p69, pp69 and pp62 are indicated. The migration of $^{14}C$-methylated marker proteins is indicated; from *Top*: 92.5, 69, 46 and 30 kDa, respectively.


32P
35S
A.
M
1
2
3
4
5
6
7
8
9
p69
B.
1
2
3
4
5
6
7
8
9
pp69
pp62

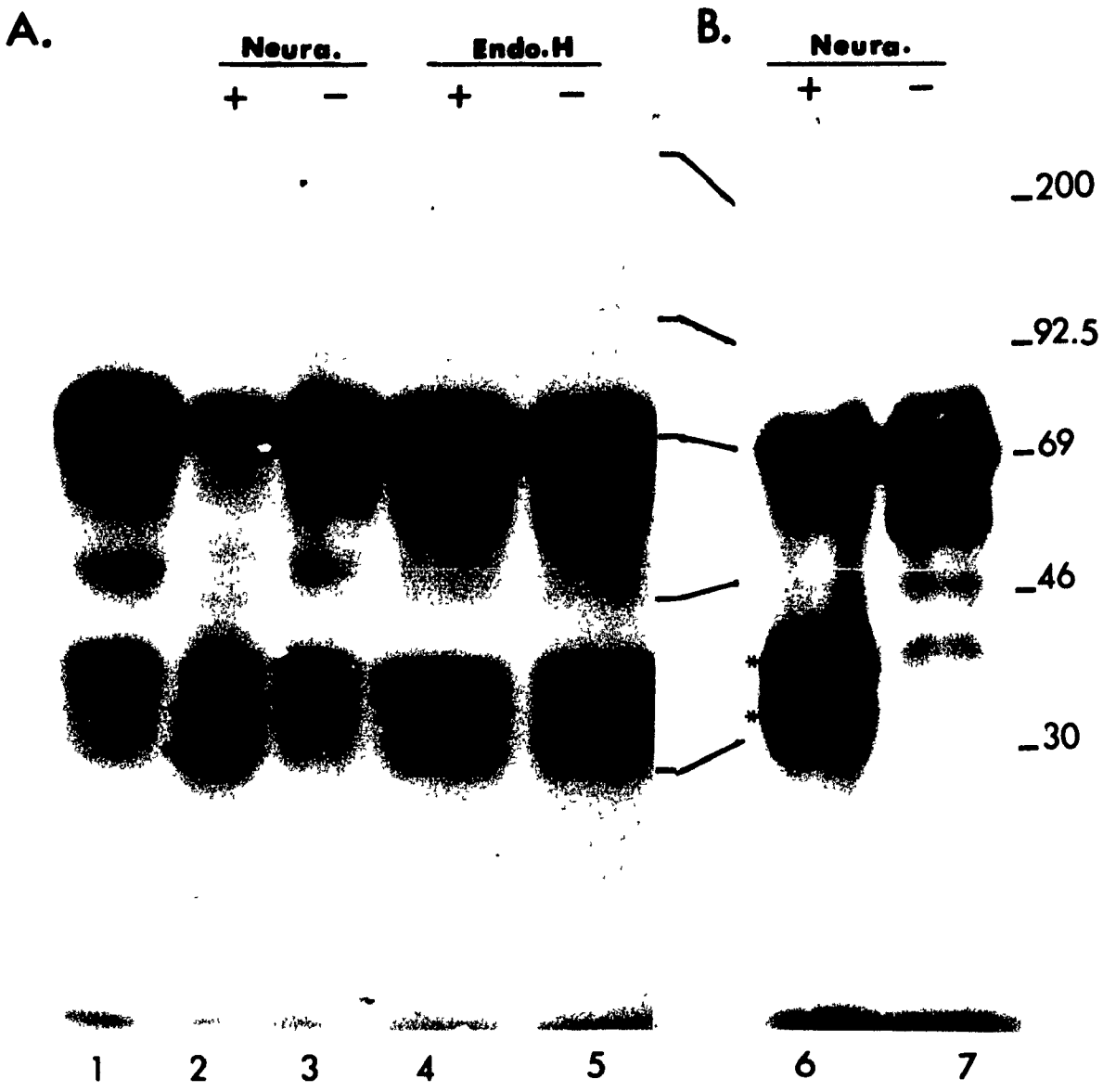
**Glycosylation of pp69.**

For further characterizations of relationship between pp69/pp62 and 2ar/osteopontin we have examined whether pp69/pp62 exhibit some properties of osteopontin. One of these properties is osteopontin's adhesive function. I have previously examined this property for pp69 by cell blotting using the procedure of Hayman et al. (1985), but without success. Cells attached only to fibronectin and to a lesser extent to the proteins identified in this study as pro-collagens, but did not adhere to p69 in various experimental conditions. This could be due to lack of renaturation of p69 on nitrocellulose filter or to absence of an unidentified factor necessary for cell adhesion to osteopontin.

Chemical characterization of osteopontin indicates that it is a glycoprotein rich in sialic acid (Prince et al., 1987; Fisher et al., 1987). Therefore, to determine whether or not pp69 also contains sialic acid, $^{32}$P-labeled pp69 was immunoprecipitated and digested with neuraminidase which removes sialic acid residues and endoglycosidase H which cleaves N-linked carbohydrates. Figure 16 shows that desialiation of pp69 results in two protein bands having a molecular weight of ~36-42 kDa (*lane* 2), absent in non-digested pp69 preparations (*lane* 3). However, the size of the resulting proteins is lower than the predicted size of the core protein of osteopontin. The size difference between neuraminidase-digested and-non-digested pp69 cannot be attributed solely to the removal of sialic acid from carbohydrate chains, but may be explained by the abnormal mobilities of this protein in SDS-PAGE. It is also ruled out that this could be due to protease action since the control sample of immunoprecipitates, incubated exactly in the same conditions, but in absence of neuraminidase (*lane* 3), is unaltered and its profile is identical to an immunoprecipitated, but unincubated sample (*lane* 1). Treatment with endoglycosidase H did not alter the molecular weight of pp69 indicating that it does not contain N-linked carbohydrate chains (*lanes* 4 and 5) Addition of neuraminidase to $^{32}$P-labeled conditioned medium containing pp69 and pp62 results in digestion of both proteins into lower molecular weight proteins (*lane* 6) indicating that pp62 also contains sialic acid and that it does not differ in size from pp69 on the basis of sialic acid content.

**Figure 16.** *Glucosidase digestion of pp69.*

Conditioned medium of NRK (*lanes* 1-5) and PT-NRK cells (*lanes* 6, 7) were incubated in presence (+) or absence (-) of neuraminidase (*Neura.*) or endoglycosidase H (*Endo. H*) as described and fractionated by SDS-PAGE. *Lane* 1 is the result of immunoprecipitation without incubation. In ***B.***, neuraminidase was added directly to the conditioned medium after desalting by ultrafiltration and adjusting the sample with the assay buffer [0.2 mM sodium citrate, pH 5.5], without prior immunoprecipitation.


A.
Neura.
+
−
Endo.H
+
−
B.
Neura.
+
−
200
92.5
69
46
30
1
2
3
4
5
6
7

## VIII). DISCUSSION.

In this study, a novel system whereby reversible cell transformation is induced in nontransformed cells by chemical treatment is used to investigate the transformation sensitivity of MSPs. It is shown here that treatment of NRK-49F cells with vanadyl is as potent in inducing phenotypic transformation as vanadate. Cell transformation is assessed by examining morphological changes in treated cells and their growth properties on solid and semi-solid substratum. Whereas the degree of morphological transformation was dose-dependent, maximum stimulatory effects of vanadium on cell growth were at the concentration range of 10-20 μM; higher concentrations (> 35 μM) were not as growth stimulating and for sparse cultures had growth inhibitory effects. Similarly, in soft agar cultures, at high (25-35 μM) concentrations, vanadium induced a much higher number of cells to grow into colonies, but their growth ceases rapidly, thus only small-size colonies are formed. At lower concentrations, less percentage of cells are induced to form colonies, but attain a larger size. Although the size and number of colonies formed by vanadium-treated NRK cells are not as high as those formed by virus-transformed cells, indicating that vanadium does not fully mimic viral transformation, vanadium-induced anchorage independent growth is, nevertheless, significant when compared to the basal level of colony formation of untreated NRK cells.

This concentration-dependent biphasic effect of vanadium on cell growth is the result of its combined action of its mitogenic effects (Carpenter, 1981; Tojo et al., 1987) and growth factor-synergistic effects (Smith, 1983) at low concentrations, and its toxic effects when it reaches high intracellular concentrations. As mentioned previously, vanadium mimics growth factor actions from post-ligand binding receptor phosphorylation and internalization (Tamura et al., 1984; Torossian et al., 1988) to the last events in the competence pathway that occur upon growth factor stimulation of resting cells (Macara, 1985; Wice at al., 1987; Jamieson et al., 1988). Although the toxicity of vanadium on live animals is known (Ramasarma and Crane, 1981; Nechay et al., 1986), little studies have been aimed to determine its toxicity on cells cultured *in vitro*. Nevertheless, Braken and Sharma (1985) examined some biochemical functions in Martin Darby bovine kidney cells cultured in

presence of 20-500 μM vanadate; toxicity-dependent alterations in DNA and protein synthesis and $K^+$-dependent phosphatase, acid phosphatase and succinate dehydrogenase activities have been observed at concentrations exceeding 100 μM. Although vanadium concentrations used in the present study did not exceed 35 μM, growth inhibition, particularly in soft agar, may be due to vanadium toxicity as its intracellular concentration can reach, with time, 10-fold higher levels than its extracellular concentration (Ramasarma an Crane, 1981).

The present study also shows that in correlation with its dose-dependent effect on cell morphology, vanadium treatment also causes a reduction in the levels of collagens which are major ECM constituents in most cell types. Therefore, morphological transformation can be attributed at least partially to this effect of vanadium on collagens. Although this study does not discriminate whether vanadium acts by reducing collagens synthesis or by enhancing their degradation, it has been shown that vanadate induces expression of collagenase, tissue-type and urokinase-type plasminogen activator (Montesano et al., 1988). Concentration-dependent biphasic effects of vanadium on rabbit costal chondrocytes proteoglycans, which are major ECM constituents in this specialized cell type have also been observed; at low concentrations vanadate causes an increase, and at higher concentrations it causes a decrease in proteoglycan synthesis and a normal or transformed chondrocyte phenotype is expressed accordingly (Kato et al., 1987).

Although vanadium acts on a number of cellular functions, as discussed in other studies, it appears to induce cellular transformation by its PTPase inhibitory actions. Other actions of vanadium, such as inhibition of ATPases or glycolytic enzymes, are excluded since they occur only *in vitro*, or occur *in vivo*, but at much higher (millimolar) concentrations. Klarlund (1985) has shown that the dose-dependent phenotypic transformation of NRK-1 cells is correlated with a dose-dependent increase in cellular P–Tyr-protein content and that concomitant with reversal of transformed phenotype, upon removal of vanadate from the growth medium, a parallel decrease in P–Tyr-proteins is observed. It is shown in the present study that the level of P–Tyr-containing proteins is increased by either vanadyl or vanadate treatment of normal and transformed cells. Increase in P–Tyr content may account for both growth stimulatory effect and morphological transformation of treated cells. First, P–Tyr phosphorylation is thought to have a major regulatory role in phosphatidyl inositol

signalling pathway which is an integral part of the cascade of events that lead to mitogenesis (Macara, 1985) or to constitute a separate signal transducing pathway (Charbonneau et al., 1988). Second, as discussed in the previous sections, phosphorylation of tyrosine in cytoskeletal proteins, most notably integrins, may have a deleterious effect on cytoskeleton organization and cell morphology (Hirst et al., 1986). Marchisio et al. (1988) have shown that vanadate-treated BHK cells assume a morphology similar to their RSV-transformed counterparts concomitant with an increase in P–Tyr-proteins most of which are localized to adhesion pads. Increased P–Tyr content due to PTK activity, at least of the *src* family, may not account for decreased collagen levels as *src*-transformed RR1022 cells show normal collagen levels (Fig. 8).

This study also shows that the observed increase in P–Tyr content in vanadium treated cells is more pronounced in *src*-transformed RR1022 cells than in NRK-49F cells. Therefore, the level of increase in P–Tyr-proteins appears to vary according to the cell lines. Variable levels of increase in P–Tyr-protein content, upon vanadium treatment, have been observed in different cell lines; 3.5-fold increase in nontransformed and 16-fold increase in middle T antigen-transformed rat F1-11 cells (Yonemoto et al., 1987); 40-fold increase in NRK-1 cells treated with 37 μM vanadate and less in mouse cell lines (Klarlund, 1985). Variable levels of P–Tyr-proteins were also observed in BHK cells (Marchisio et al., 1988) and rabbit costal chondrocytes (Kato et al., 1987). As discussed earlier (Yonemoto et al., 1987), this variability may be due different basal levels of P–Tyr phosphorylation in different cell lines. This is consistent with the observation made in this study indicating that RR1022 cells, which are expected to have higher basal levels of P–Tyr-protein content, show more increase in P–Tyr-proteins, upon vanadium treatment, than NRK cells. In addition, this variability could be due, at least in the case of *src* proteins, to differential effect of vanadate on viral and cellular oncogene-encoded PTK activities; whereas v–*src* kinase is stimulated by vanadate (Brown and Gordon, 1984; Collet et al., 1984), the cellular *src* kinase activity is inhibited (Ryder and Gordon, 1987).

The second action of vanadium which may account for the transformed phenotype and which also occurs *in vitro* at micromolar concentrations, is stimulation of $Na^+/H^+$ pump (Paris and Pouysségur, 1987). Stimulation of proton pump leads to cytoplasmic alkalinization, a feature common

to transformed and mitogen-stimulated cells. Recently, evidence has been obtained indicating that activation of $Na^+/H^+$ pump leads to cellular transformation. Expression of the yeast pump in mammalian cells induces a transformed phenotype characterized by abnormal morphology, anchorage independent growth, growth to high saturating densities, and tumorigenicity in *nude* mice (Perona and Serrano, 1988).

By means of 2-d gel electrophoresis, this study showed that NRK cells secrete two 69 kDa proteins, one, phosphorylated (pp), with pI ~3.8, and the second, non-phosphorylated (np) and less acidic, with pI ~4.5, both of which are immunoprecipitated by anti-p69 antiserum. Based on the similarity of their V8 peptide maps and co-migration on 2-d IEF/SDS-PAGE following dephosphorylation by phosphatase treatment, it was concluded that they are the phosphorylated and non-phosphorylated (pp and np) forms of p69. In support for this is the migration pattern of $^{32}P$-labeled p69 secreted by PT–NRK cells which has a pI ranging from 3.8 to close to 4.5 of np69. Iso-electric point difference in the two proteins is due to additional negative charges of phosphates present in pp69 and absent in np69. This has been observed in a number of proteins including neurofilament subunits (Georges et al., 1986) and a growth factor receptor protein substrate, p42 (Cooper and Hunter, 1985). Phosphate groups, presumably by altering protein structure/accessibility to SDS, also result in anomalous (slow) mobility of phosphorylated proteins in 1-d SDS-PAGE, therefore resolving into pp and np "doublets" (Georges et al., 1986; Aletta et al., 1988), as is often the case for p69.

Analysis of RR1022 secreted proteins indicates that they secrete p69 in the np form only. In PT–NRK cells, pp69 displays charge heterogeneity having a pI ranging from 3.8 for pp69 to close to 4.5 for np69. We interpret that charge heterogeneity in pp69 is due to differential phosphorylation, and suggest that phosphorylation of p69 is regulated differently in normal, transformed and partially transformed cells. In support for this is the finding that pp69 is phosphorylated to various degrees in untreated and growth factor (EGF, TGF–β)-treated NRK cells (Laverdure and Mukherjee, in preparation). There is also evidence indicating that pp62 is more phosphorylated than pp69 (on a molar basis). This is based on the the fact that $^{35}S$-labeled pp62 is difficult to detect on either 1-d or 2-d gels. Therefore, the abundance of $^{32}P$-labeled pp62 in the conditioned media of transformed cells

may not reflect its abundance, but its phosphorylation to a higher stoichiometry on additional sites. This is true if the size difference between pp69 and pp62 is due to differential glycosylation: In the case of lack or reduced (competing) O-linked glycosylation in pp62, additional serine residues will be available, which can potentially be phosphorylated.

The data presented in this study shows that p69 is antigenically related to 2ar/osteopontin; both anti-p69 and anti-2ar antisera immunoprecipitate the same protein, p69, from the conditioned media of NRK cells as well as other cell lines (Craig et al., 1988; see Fig. 15). The precipitation of pp69 and pp62 by both antisera provides further evidence that these two proteins are related and strengthens the previously suggested possibility of precursor/product relationship between these two proteins (Chackalaparampil et al., 1985). By Northern and Southern analysis, it was found that in both mouse and rat cells, the 2ar probe hybridized only to one 1.6 kB mRNA species (Craig et al., 1988) and that 2ar is encoded by a single copy gene (Craig et al.,submitted)[1]. Therefore it appears that rat p69 and mouse2ar are the same protein (see below). Interspecies size heterogeneity may be due to post-translational modifications. In support for this, anti-p69 antiserum, which appears to react with post-translational modifications –as judged by its inability to react with 2ar mRNA *in vitro* translation product– (Denhardt, personal Communication), reacts poorly with the mouse MSPs (Fig. 15).

Other published studies characterizing osteopontin extracted from mineralized bone matrix (Prince et al., 1987; Fisher et al., 1987) did not reveal the existence of phosphorylated and nonphosphorylated forms of this protein. This may be due to one of the following biological, functional or technical reasons: i) all the osteopontin produced by bone cells in early developmental stages is phosphorylated and secreted in a pp form, ii) an np form of osteopontin is also produced and secreted but not incorporated into bone matrix, which will be consistent with the finding here that only the pp form of p69 is detectable on the cell surface or, iii) np form of osteopontin is produced, secreted and incorporated into bone matrix, but lost during one of the several purification steps used to isolate osteopontin. Nevertheless, the study of Kubota et al. (submitted)[4] presents evidence for the presence of two forms of osteopontin, one being less phosphorylated than the other.

[4]Kubota; T., J. L. Wrana, R Ber, Q Zhang, J. E Aubin, W. T. Butler, and J Sodek. (submitted)

The application of vanadium-induced transformation system discussed above to study its effects on p69 indicates that in vanadium treatment of NRK cells causes an increase in levels of np69 and a decrease in the levels of pp69 in a dose-dependent manner. The results also show that vanadium does not induce pp62 secretion. These observations allowed to draw two main conclusions with potentially important significance. First, by demonstrating that vanadium increases p69 secretion in predominantly an np form, concomitant with a dose-dependent phenotypic transformation confirms the transformation sensitivity of p69 and indicates that p69 function could be regulated by phosphorylation. Second, the finding that pp62 is not induced by vanadium indicates that pp62 secretion is limited to irreversibly transformed cells. The vanadium effect on p69 phosphorylation can be due to inhibition of a specific kinase or activation of a phosphatase. That np69 is present intracellularly is not known; immunoprecipitation of p69 from $^{35}S$-labeled cell lysates resulted in a large number of proteins making np69 difficult to be unambiguously identified. Priliminary results indicate that dephosphorylation of pp69 into np69, in the conditioned medium, subsequent to its secretion may be ruled out since incubation of $^{32}P$-labeled pp69, on immunoaffinity support, with vanadium-treated NRK cell extracts or conditioned media did not result in loss of radioactivity associated with pp69 (not shown). On the other hand, studies in this laboratory indicate that the differential levels of p69 phosphorylation under various growth factor and drug treatments –as judged by sensitivity of pp69 to phosphatase actions – is correlated with variable levels of protein phosphatase activities (Laverdure and Mukherjee, In preparation).

Analysis of secreted proteins of vanadium-treated PT-NRK cells indicates that vanadium, as in NRK cells, reduces the levels of pp69, but does not affect pp62, indicating that pp69 and pp62 are regulated differently. However, by cell surface radioiodination, we show that the levels of pp69 detectable on the cell surface is increased by vanadium treatment. Therefore, it is possible that the vanadium-induced low levels of pp69 in the culture supernatants is due to its increased association with the cell surface, but not to its reduced secretion (see below). The same explanation may be true to the observed decrease in pp69 levels in NRK cells treated with TGF–β (Laverdure et al., 1987)

which is known to enhance the incorporation of nectins into the ECM (Ignotz and Massagué, 1986).

Although the exact mechanism behind size difference between pp69 and pp62 is not known, indirect evidence for and against protease action is available. Immunoprecipitation of normal and transformed $^{32}$P-labeled cell lysates using anti-p69 antiserum, which cross-reacts with both pp69 and pp62, results in precipitation of pp69 only, indicating that pp62 is not present intracellularly and that only pp69 is initially synthesized (Laverdure et al., 1987; data not shown). In addition, mixing experiments, involving incubation of $^{32}$P-labeled pp69 with conditioned media of RR1022 or KNRK cells, did not result in cleavage of pp69, indicating that pp62 does not result from a protease action present in the conditioned medium (Mukherjee, Personal communication). On the other hand, cleavage of pp69 into pp62 by a specific protease present on the cell surface may occur. Proteases such as thrombin, elastase and urokinase are known to interact with cultured fibroblasts *via* specific sites present on the cell surface and particularly, thrombin is thought to exert its mitogenic effects through its extracellular proteolytic actions (Cunningham et al., 1986). Of these serine proteases, thrombin, which selectively cleaves argenine-glycine peptide bonds and which has high substrate specificity, is of particular interest to this study because it has been shown to cleave pp62 into a 30 kDa protein (Senger et al., 1988). It is interesting to note that 2ar/osteopontin contain a unique potential thrombin Arg-Gly cleavage site which is located within its cell recognition RGD sequence; the functional implications of thrombin-mediated osteopontin cleavage are obvious. In the present study, a ~62 kDa protein is detected on the surface of PT–NRK, quiescent PT–NRK, but not NRK cells, which may be the cleavage product of pp69. Furthermore, a 30 kDa protein is also immunoprecipitated from these cells, but not from NRK cells. Although the identity of this latter was not examined, it may be related to the pp62-thrombin-generated 30 kDa fragment resulting from cleavage of pp69 or pp62 by thrombin or a thrombin-like protease.

Based on the observations made so far, regarding pp69 and pp62, the best working hypothesis is that in both normal and transformed cells, p69 is initially produced and secreted in pp and np forms in varying proportions. Then, phosphorylated p69 interacts with the cell surface *via* its putative cell surface receptor. In transformed cells, where proteolytic activity is markedly increased, pp69 is

cleaved. As a result of protease action, the resulting pp62 will have diminished affinity to its binding site, therefore is released into the culture supernatants and free binding sites are made available. The newly secreted pp69 interacts with the free sites, then cleaved into pp62, which is released into the medium and the cycle is repeated. As a result, the conditioned media of transformed cells will contain predominantly pp62 and little or no pp69. In nontransformed cells, where protease action is reduced and extracellular proteolysis is less pronounced, little or no degradation of pp69 takes place. Therefore, cell surface pp69 is more stable, most pp69 interaction sites are occupied, and all the newly secreted pp69 remain in the medium. As a result, in the conditioned medium of normal cells, only pp69 is detected. In vanadium-treated cells, cell surface localization of pp69 is enhanced, but its rate of degradation is unaffected, therefore, resulting in low levels of pp69 and unaltered levels of pp62 detectable in the conditioned media. Although this model is rather simplistic, it is, nevertheless, compatible with all the presently available data on secretion of pp69 and pp62 by normal, transformed, growth factor- and vanadium-treated and partially-transformed cells. Like any other model, it will allow experiments to be devised and it can easily be subjected to experimental verification.

By cell surface radioiodination and immunoprecipitation, it has been shown that pp69 is detectable on the surface of normal, transformed and partially-transformed cells, regardless of whether they are quiescent or actively growing. In addition, preliminary results obtained by cell matrix extraction using the method of Ignotz and Massagué (1986) and cell surface trypsinisation followed by immunoprecipitation, resulted in anti-p69-precipitable peptides ranging in molecular weight from 69 to ~25 kDa (not shown). Quantitative and qualitative differences have also been observed in different cells indicating that localization of pp69 to the cell surface is not due to nonspecific (e.g. electrostatic) interactions. For example, in vanadium-treated NRK cells, which predominantly secrete p69 in an np form, only pp69 is detected on the cell surface. In addition, quiescent PT-NRK cells, which secrete low amounts of pp69 as indicated by $[^{32}P]$-$PO_4$ metabolic labeling, show elevated levels of cell surface pp69 as compared to actively growing PT-NRK cells. The same argument can be stated regarding KNRK cells. Since p69 is or related to osteopontin, which mediates cell adhesion in

an RGD-dependent manner, it is tempting to speculate that the cell surface localization of pp69 is receptor-mediated. The qualitative and quantitative differences of cell surface p69 mentioned above support this view. The extent of ECM formation does not depend only on the levels of soluble ECM components, but also on the rate of integrin synthesis and its expression on the cell surface (Ignotz and Massagué, 1987; Ruoslahti and Pierschbacher, 1987). It has also been suggested that some transformed cell lines fail to incorporate nectins into their ECMs due to diminished synthesis of integrins or to altered affinity for their ligands following post-translational modifications such as phosphorylation (Ignotz and Massagué, 1986; 1987; Hirst et al., 1986). This may explain why in KNRK cells only low amounts of pp69 is detectable on their cell surface, although they secrete elevated amounts of both pp69 and pp62,.

Although the relationship of p69 and 2ar/osteopontin and complete correlation between the effects of vanadium on cell morphology and p69 are suggestive of involvement of p69 in the maintenance of normal cell morphology, the data on cell surface localization of pp69 indicate that this may not be the case. Elevated levels of pp69 were observed in both vanadium-treated NRK and PT-NRK, yet they exhibited dissimilar morphologies. Whereas vanadium-treated cells display a rounded morphology and tend to grow on top of each other, quiescent NRK cells are flat and form a closely packed monolayer. Conversely, KNRK cells have a similar morphology to vanadium-treated NRK cells, but have a much reduced level of pp69 on their cell surface. Although osteopontin is found to mediate cell adhesion and spreading when used to coat cell culture substrata (Oldberg et al., 1986; Somerman et al., 1987; 1987a), this does not necessarily imply that it has an adhesive function. For example, in contrast to Fn, which promotes both cell adhesion and spreading, tenascin promotes cell adhesion, but prevents spreading even in the presence of Fn; cells plated in presence of tenascin attach, but remain rounded (Ehrismann et al., 1987). It is possible that in V-NRK cells, which show a dose-dependent decrease in cell spreading, concomitant with an abundant secretion of p69 and cell surface pp69, soluble p69 competes with Fn for the same receptor. Binding of pp69/osteopontin to Fn receptor is not unlikely; although each nectin appears to have its own receptor, integrins bind most RGD-containing proteins at varied affinities (Ruoslahti and Pierschbacher, 1986; Hynes, 1987;

Ruoslahti, 1988). Therefore, soluble p69, abundantly secreted by V–NRK and agar-selected PT-NRK cells (also exhibiting a rounded morphology), by competing with Fn for the same receptor, could act like soluble Fn, its cell binding domain or RGD-containing peptides used to demonstrate the dualistic properties of Fn adhesive function (Yamada and Kennedy, 1984). With this view in mind, and with the finding that pp62, which might have a role in tumorigenesis (Senger et al., 1985), is present in serum of patients with disseminated metastases, but not in patients with localized tumors (Senger et al.,1988), an *in vivo* situation can easily be imagined. For example, by binding to tumor cells in the primary tumor, MSPs may participate in the determination of their motile and/or invasive state, as contrasted to their stationary state when attached to Fn or other ECM components. This is also compatible with the finding that 2ar is expressed and its product is localized to cellular matrices during early developmental stages where extensive cell migration and tissue remodeling occur (Nomura et al., 1988; Mark et al., 1987; 1987b).

Using immunoprecipitation on denatured and non-denatured samples, this study shows that fibronectin and np69 co-immunoprecipitate using either anti-p69 or anti-Fn antiserum. When denatured conditioned medium is used, these antisera immunoprecipitate only their cognate proteins. Co-precipitation of proteins in non-denaturing conditions and loss of co-precipitation under denaturing conditions is indicative of molecular interaction between proteins. This was based on a study demonstrating complex formation between v–*fos* and c–*fos* proteins and an associated 39 kDa protein, p39 (Curran et al., 1985). Taking a similar approach, a more recent study has confirmed this complexation of p39, identified as the transcription factor c-Jun/Ap-1, and Fos (Chiu et al., 1988). Similarly, this approach has also been used to demonstrate complex formation between SV40 large T antigen and the retinoblastoma susceptibility gene product (DeCapiro et al., 1988). Therefore it is likely that co-immunoprecipitation of Fn with np69 is due to a molecular interaction or complex formation between the two proteins; a complex which is not preserved under denaturing conditions. By means of its different functional domains, Fn interacts with a number of proteins and ligands including collagen, fibrin, heparin and integrin (Yamada et al., 1985). Fn also transiently interacts with

factor XIII; this interaction has been recently shown to result in factor $XIII_a$ (thrombin-activated factor XIII or plasma transglutaminase)-mediated fibronectin ε–(γ-glutamyl)lysyl cross-linking into larger deoxycholate-insoluble multimeric aggregates (Barry and Mosher, 1988) Most recently, it has been shown that tenascin, also an RGD-containing ECM protein with spatially and temporally restricted tissue distribution, interacts with fibronectin and inhibits its cell adhesion and spreading functions (Ehrismann et al., 1988). An attractive possibility is that the putative np69 interaction with Fn will have some consequences on np69 or Fn function. This may have an effect on the transformed phenotype since transformed cells, like RR1022, vanadium-treated NRK and spontaneously transformed NRK cells predominantly secrete p69 in the np69 form. I have attempted to reconstitute this interaction *in vitro* using fibronectin overlay assay, a procedure which was used to show Fn binding to a 36 kDa protein (Salonen and Vaheri, 1987), but without positive results. Although the possibility of antigenic similarities in non-denatured Fn and np69 cannot be excluded, the possibility of complex formation between np69 and Fn and its presumptive functional consequences is interesting and deserves further study.

The number of reported MSPs, which share some biochemical or biological properties with each other, has increased recently and it is now becoming necessary to consider their relatedness as this may contribute to more understanding of their structure/function. To list a few, early studies have reported a number of MSPs ranging in size from 58 to 67 kDa which are all antigenically related (Senger et al., 1979; 1985). Subsequently, studies in this laboratory have identified two MSPs, pp69 and pp62, which are also antigenically related (Chackalaparampil et al., 1985; Laverdure et al, 1987). Mouse cells secrete related proteins with molecular weights of 58-67 kDa. A recent study by Sodek's group (Kubota et al., submitted)[4] describes two forms of osteopontin secreted by ROS osteosarcoma 17/2·8 rat cells and rat calvarial cells in the form of MSPs one with a M. W. of 44 kDa and the other with M. W. 67 kDa. In addition, Le Cam et al. (1985) described an acidic phosphoprotein secreted by rat hepatocytes with properties expected for osteopontin: It is a neuraminidase-sensitive MSP, with low intracellular levels, synthesized as a ~43 kDa precursor which matures into 53 and 56 intermediates, then into a 67 kDa protein secreted in pp and np forms. Still, another study has reported a 58 kDa

MSP secreted by NRK cells whose secretion is increased by EGF and inhibited by TGF–β (Binas and Grosse, 1987), in a manner similar to pp69 (Laverdure et al., 1987). With the demonstration here of an immunological relationship between p69/p62 and 2ar/osteopontin and with the finding that the N-terminal sequence of the proteins described by Senger et al. (1988) is identical to that of 2ar/osteopontin (Prince et al., 1987), it is likely that all the described MSPs are related. In addition, because 2ar/osteopontin is encoded by a single copy gene, it can be argued that all MSPs are products of the same, but not distinct genes. Some of the heterogeneity in molecular weights (most determined by SDS-PAGE) in the MSPs described by different laboratories can be attributed to technical differences in addition to the reported anomalous mobility of osteopontin in SDS-PAGE (Prince et al., 1987). For example, in our collaborative work with Dr Denhardt's laboratory, using the same cell lines, the size of anti-2ar and anti-p69-precipitable proteins differed in the two laboratories (Craig et al., 1988; and see Fig. 15). However differential RNA processing, translation, post-translational modifications or protease actions in different cell lines and different growth conditions cannot be ruled out. In respect to the two formers, it was found that *in vitro* translation of 2ar mRNA yields one major and two minor proteins of M.W. ~60 kDa (Craig et al., submitted)[1]. Therefore, the other question that remains to be answered concerns the molecular basis behind the size heterogeneity in MSPs. That the size of this protein shows interspecies size heterogeneity is not surprising and can be explained in evolutionary terms. But its variation within the same cell line subsequent to viral or spontaneous neoplastic transformation could have a significant functional consequence or, if not, elucidating the mechanism behind it may reveal an equally important specific biochemical function derepressed or induced by transformation.

As mentioned previously, secretion of phosphoproteins is a rare event and the protein described in this study is one of the few. By establishing that it is secreted in both np and pp forms and that each form has different characteristics, in respect to cell surface localization and co-precipitation with Fn, it is tempting to speculate that its function is regulated by phosphorylation. Phosphorylation-induced conformational/functional changes with subsequent alterations in cognate ligand binding is known for a number of proteins including growth factor receptors, synapsin, and

most likely integrins (Sibley et al., 1988; Petrucci and Morrow, 1987; Hirst et al., 1986). If the function of p69 is to interact with Fn, then phosphorylation serves to mask the site of interaction on p69. On the other hand, the fact that only p69 is detectable on the cell surface, by the method used in this study, indicates that phosphorylation may be crucial for such localization. Phosphorylation in this case may serve to provide the protein with a suitable conformation, making its cell recognition sequence more exposed for interaction with its putative cell surface receptor. This is true for nectins and integrins which interact with low affinity as compared to other ligand-receptor systems; any structural changes may have deleterious effects on such interactions (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Obara et al., 1988). Regulation of ligand-receptor binding by phosphorylation usually occurs at the receptor level, but not at the ligand level; with the exception of lysosomal enzymes, the only known phosphorylated ligand is fibronectin (Ali and Hunter, 1982), but Fn phosphorylation does not appear to affect its receptor binding activity. However, such comparison cannot be made between p69 and Fn because the latter is a much larger protein and is phosphorylated only in some cell types to a much lower stoichiometry. Therefore, phosphorylation or dephosphorylation events are expected to have more pronounced consequences on p69 structure and function than on Fn.

It is often difficult to relate some phenomena occurring in monolayer culture under certain growth conditions to anchorage independent growth. Although previous studies have demonstrated a complete correlation between secretion of pp62 and pp69 in RSV-transformed cells and their anchorage independent growth ability and inability, respectively, definite proof for their role in the process of colony formation or significance of such correlation has not been established. In addition, this correlation is not observed in reversibly transformed cells. However, in both vanadium- and growth factor-transformed cells secretion and phosphorylation of p69 is affected. By establishing that p69 is 2ar/osteopontin and by analogy with ECM fibronectin, which supplies an anchoring matrix, it is plausible to consider that MSPs may play a role in growth in soft agar of nontransformed cells. This study also shows that vanadium at concentrations that induce anchorage independent growth

increases the overall secretion of p69 and the proportion of pp69 present in the cell surface, and the same could be true for effects of TGF–β on pp69. A similar situation is known with respect to the action of TGF–β on ECM components, fibronectin and collagens; by enhancing synthesis of fibronectin and its incorporation into the ECM, TGF–β induces anchorage independent growth in non-transformed cells (Ignotz and Massagué, 1986; 1987). Therefore the vanadium-induced increase p69 secretion and pp69 localization to the cell surface may account for some aspect of vanadium-induced anchorage independent growth of NRK cells. The establishment of the cell surface localization of pp69 and its relationship with osteopontin, will allow to devise further experiments to directly test the possible involvement of pp69 and pp62 in anchorage independent growth.

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