GROWTH FACTOR REGULATION OF A 69kDa PHOSPHOPROTEIN SECRETED BY NRK-49F CELLS

GUY R. J. LAVERDURE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biology
McGill University
Montreal

@ Guy R.J. Laverdure
April 1989
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"being broad-minded is a virtue that can ruin an experimentally inclined biologist"

Racker, E. 1983

- to the memory of my father -
ABSTRACT

Our study shows that the secretion of a major glycosylated, phosphoprotein with a molecular weight of 69kDa (pp69) is a specific marker for non-transformed NRK-49F cells. Antibody raised against pp69 recognizes, in addition to pp69, another major phosphoprotein with a molecular weight of 62kDa (pp62) secreted by RR1022 and spontaneously transformed NRK-49F cells (spt-NRK-49F). Immunoprecipitation of total cell lysates from both NRK-49F and RR1022 cells with anti-pp69 antibody detected only pp69. Treatments with: epidermal growth factor (EGF), transforming growth factor-β (TGF-β), retinoic acid (RA), and TPA modulate the levels of pp69 present in the conditioned media. Furthermore, TPA and EGF induce the synthesis of 3 internal peptides with molecular weights of 58, 54, and 44 kDa which appear to be pre-processed forms of pp69.

Treatment of NRK-49F cells with insulin, EGF, TGF-β, PPA, levamisole and spermine clearly demonstrate alterations in the phosphorylation of pp69, concomitant with changes in extracellular phosphatase activity. EGF-induced increase of secreted pp69 reaches its highest level at approximately 12hr post-EGF treatment. This 12hr peak is accompanied by a pronounced 66% (p<0.02) reduction in secreted ALP activity as well as a reduction in secreted protein phosphatases. Treatment of NRK-49F cells with 5uM spermine inhibits 2-fold the secretion of the phosphorylated pp69 species and also causes a 50% reduction in cell proliferation. Insulin (20ug/ml) which activates protein phosphatases (Londos, et.al., 1986) also causes a 50% reduction in the phosphorylation of pp69. Levamisole (0.01 & 0.1mM), an ALP inhibitor and PPA (0.1mM), a specific protein phosphatase inhibitor enhance cell proliferation by 25% and 50% respectively, similar to levels of enhanced cell proliferation obtained with EGF alone. PP69 secreted by EGF and levamisole treated cells demonstrate increased ALP sensitivity. The data presented here show that there is a relationship between pp69 and cellular proliferation. In essence, the data suggests that EGF induces growth and affects morphology in part by affecting two regulatory events involving pp69, an osteopontin-like protein: 1) secreted protein level alterations with, 2) changes in the phosphorylation of pp69 coupled to altered extracellular phosphatase activity.
RÉSUMÉ

Notre étude démontre que la sécrétion d'une importante phosphoprotéine glycosylée ayant un poids moléculaire de 69kDa (pp69) est un marqueur spécifique des cellules NRK-49F non transformées. Les anticorps élevés contre pp69 reconnaissent, outre pp69, une autre importante phosphoprotéine d'un poids moléculaire de 62kDa (pp62) sécrétée par RR1022 et transforment spontanément les cellules NRK-49F (spt-NRK-49F). L'immunoprécipitation des lysats totaux des cellules NRK-49F et RR1022 avec l'anticorps anti-pp69 n'a permis de détecter que pp69. Le traitement au facteur de croissance épidermique (EGF), au facteur de croissance transformant de type bêta (TGF-β), à l'acide rétinolique (RA) et au TPA module les concentrations de pp69 présentes dans les milieux conditionnés. En outre, le TPA et l'EGF provoquent la synthèse de 3 peptides internes dont les poids moléculaires sont de 58, 54 et 44 kDa, qui semblent être des formes pré-traitées de pp69.

Le traitement des cellules NRK-49F à l'insuline, à l'EGF, au TGF-β, au TPA, au Levamisole et à la spermine démontre clairement des modifications dans la phosphorylation de pp69, parallèlement à des changements dans l'activité des phosphatases extracellulaires. L'augmentation des pp69 sécrétées induite par EGF
atteint son plus haut niveau environ 12 heures après le traitement à l'EGF. Toutefois, on n'a détecté aucun changement important dans la cinétique de sécrétion de pp69 après traitement à l'EGF. Cette pointe à 12 heures s'accompagne d'une baisse marquée de 66 % (p< 0,02) dans l'activité de l'ALP sécrétée ainsi que d'une réduction des phosphatases protéiques sécrétées. Le traitement des cellules NRK-49F à la spermine 5uM inhibe 2 fois la sécrétion de l'espèce pp69 phosphorylée et entraîne également une baisse de 50 % de la prolifération des cellules. L'insuline (20ug/ml) qui active les phosphatases protéiques (Londos et coll. 1986) provoque également une diminution de 50 % de la phosphorylation de pp69. Le Levamisole (0,01 et 0,1mM), inhibiteur de l'ALP, et le PPA (0,1mM), un inhibiteur spécifique de la phosphatase protéique, augmente la prolifération cellulaire respectivement de 25 % et de 50 %, ce qui est semblable aux niveaux de prolifération cellulaire augmentés obtenus avec l'EGF seulement. Le pp69 sécrété par l'EGF et les cellules traitées au Levamisole affichent une plus grande sensibilité à l'ALP. Les données présentées ici donnent à penser qu'il existe un rapport entre pp69 et la prolifération cellulaire. Dans le fond, ces données semblent indiquer que l'EGF provoque la croissance et affecte la morphologie en prenant part à deux événements de régulation mettant en cause pp69, une protéine semblable à l'ostéopontine: 1) modification des niveaux de protéines sécrétées et, 2) changement de la phosphorylation de pp69 associé à une modification de l'activité de la phosphatase extracellulaire.
CONTRIBUTION OF AUTHORS

This thesis is assembled in accordance with the regulations of the Faculty of Graduate Studies and Research. It consists of an Abstract in French and English, a general introduction, literature review and two chapters of results and a general conclusion. Portions of chapter 1 - work involving crude TGF - have previously been presented in abstract form (Laverdure et al., 25th Congress of the American Society for Cell Biology, 1985) As well, portions of chapter 1 (in particular, Figures 1 4.1, 1 4.2, 1 5, 1 6 A and 1 6 B) have been previously published, Laverdure GR Banerjee D. Chackalaparampil I & Mukherjee BB (1987). Epidermal and Transforming Growth Factors Modulate Secretion of a 69kDa Phosphoprotein in Normal Rat Kidney Fibroblasts, FEBS 222, 261-265 Figure 1 4 1 C, lanes 6 & 7, were provided by Dr Chackalaparampil whose work I have successfully confirmed I am thankful for Dr Banerjee’s expertise and collaboration in supervising the running of the isoelectric focussing for the 2-D electrophoretic analysis Chapter 2 is presented in manuscript form suitable for publication in a learned journal which has been previously presented in abstract form (Laverdure & Mukherjee, 4th International Congress of Cell Biology, 1988) and will soon be submitted. Materials and Methods is present as a single section to avoid unnecessary duplication. A related research project I performed will be submitted (Immunofluorescent Localization of p53 Comparison of Primary mouse embryo cells and tissue slices with cTGF treated cells) but is not included in this thesis. All of the Results presented in this thesis, save those mentioned above, are the work of the author.
AKNOWLEDGEMENTS

I first wish to thank Dr. B.B Mukherjee, my thesis Director for his critical analysis throughout this work. I am also indebted to many individuals without whom completion of this project would not have been possible. In particular, Robert Pentney for his expertise in membrane phosphorylation analysis of NRK-49F cells and for his invaluable friendship, and Michael DeVouge for his ability in helping our laboratory run more efficiently. I thank Mabel Rodrigues, Dr. Isaac Chackalaparambil and Dr. Deborata Banerjee, Mohammed Nemir and Franz Omman for their constant support, direction and expertise. I wish to thank others of the Biology Department, Dr. Yutaka Nishioka for his support of my abilities through the course of this work, Robert Lamarche and Guy L’Heureux for photographic and computer assistance, Karen Ruthman, Claude Lalonde, and Bruce Goodchild for helping to instill constant enthusiasm and motivation during my teaching assistantship, as well, all the members of the softball and hockey teams. I thank Dr. Anita Roberts for providing TGF-β. I also thank my family for their constant encouragement and support during the final stages of this work. This work was supported by a grant (MT-2169) from the Medical Research Council of Canada.
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<tbody>
<tr>
<td>AcP</td>
<td>acid phosphatase</td>
</tr>
<tr>
<td>AIG</td>
<td>anchorage-independent growth</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ASV</td>
<td>avian sarcoma virus</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degree centigrade</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine mono phosphate</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>cTGF (SGF)</td>
<td>crude transforming growth factor</td>
</tr>
<tr>
<td>2d</td>
<td>two dimensions</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethyl amino ethyl</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxy ribonucleic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modification of Eagle's medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetate</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>elf-2</td>
<td>initiation factor-2</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>g</td>
<td>gram(s) or gravity</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>h (hr)</td>
<td>hours</td>
</tr>
<tr>
<td>KiMSV</td>
<td>Kirsten murine sarcoma virus</td>
</tr>
<tr>
<td>Kd (kDa)</td>
<td>kilodalton</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------------------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>Levamisole</td>
<td>L-(−)-2,3,5,6-tetrahydro-6-phenyl imidazo [2,1-6] thiazole</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>M</td>
<td>Molar concentration</td>
</tr>
<tr>
<td>m</td>
<td>milli (10^-3)</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule associated protein</td>
</tr>
<tr>
<td>mEGF</td>
<td>mouse epidermal growth factor</td>
</tr>
<tr>
<td>min.</td>
<td>minutes</td>
</tr>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MuSV</td>
<td>murine sarcoma virus</td>
</tr>
<tr>
<td>n</td>
<td>nano (10^-9)</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>prot/ser-P</td>
<td>protein, serine-specific phosphatase</td>
</tr>
<tr>
<td>pp69</td>
<td>phosphorylated 69 kilodalton protein</td>
</tr>
<tr>
<td>PPA</td>
<td>phenyl phosphonic acid</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>Rf</td>
<td>relative mobility</td>
</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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</tbody>
</table>
SDS  sodium dodecyl sulfate
TCA  trichloroacetic acid
TGF-a (α)  transforming growth factor type alpha
TGF-b(β)  transforming growth factor type beta
TLC  thin layer chromatography
TPA  12-O-tetradecanoyl-phorbol-13-acetate
TPCK  L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone
u  micro (10^-6)
um  micro mole
v (V)  volts

**Cell lines**

A31  Balb/c 3T3 clone
A431  epidermal carcinoma cells
AKR-2B  mouse embryo fibroblasts
B77-NRK  avian sarcoma virus transformed NRK cells
3B11-1C  moloney murine sarcoma virus transformed mouse 3T3 cells
Balb/c-3T3  mouse fibroblasts
BSC-1  African green monkey kidney cells
FaO  rat liver hepatoma
KA31  KiMSV transformed A31 cells
LA-23  avian sarcoma virus transformed NRK cells
NIH-3T3  mouse fibroblasts
NRK-2  normal rat kidney fibroblasts clone 2
NRK-49F  normal rat kidney fibroblasts
<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
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<tbody>
<tr>
<td>KNRK</td>
<td>KiMSV transformed NRK cells</td>
</tr>
<tr>
<td>Rat-1</td>
<td>embryonic rat fibroblasts</td>
</tr>
<tr>
<td>ROS 17/2</td>
<td>rat osteosarcoma cells</td>
</tr>
<tr>
<td>RR1022</td>
<td>Schmidt-Rupin strain RSV transformed rat fibroblasts</td>
</tr>
<tr>
<td>spt-NRK</td>
<td>spontaneously transformed NRK cells</td>
</tr>
<tr>
<td>SR-iT</td>
<td>RSV transformed vole cells</td>
</tr>
<tr>
<td>d10MEp8</td>
<td>day 10 mouse embryo cells passage #8</td>
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LITERATURE REVIEW

INTRODUCTION

A normal cell's capacity to maintain regulatory control over cellular proliferation and the molecular mechanisms by which activation of signal transduction systems can effect the malignant phenotype (see Lockwood, et.al., 1987) are important links in understanding the pathogenesis of neoplasia (see Shirrmacher, 1985). Although growth control in normal cells remains poorly understood, it has been well established that a number of intracellular properties, such as transport of nutrients, cyclic nucleotide concentration, RNA and protein synthesis, phosphorylation of the ribosomal protein S6 (Chambard & Pouyssegur, 1986), and an increase in intracellular pH (Burns & Rozengurt, 1983) to activate the Na+/H+ exchanger (Moolenar, et.al., 1984), change when quiescent cells are stimulated to grow by the addition of serum or polypeptide growth factors (Holley, 1975). Growth factors such as PDGF, TGF-α, TGF-β, EGF, FGF, and hormones such as insulin, are secreted by a variety of embryonic and adult cells in culture (Goustin, et.al., 1986), and act in turn in an autocrine fashion upon cellular stimulation (Lippman, et.al., 1987, Sporn & Roberts, 1985). These growth factors are required for continued cell proliferation and act at different points of the cell cycle (Goustin, et.al., 1986).

With an increase in the understanding of events involved in cell proliferation, embryogenesis, as well as wound healing and growth factor stimulation, several cell cycle growth control models have been proposed (Pardee, et.al., 1985; Scott & Fiorine, 1982) One of these models examines the role of negative control in cells, in other words, under conditions of growth factor or
serum deprivation, contact/density inhibition, and intracellular acidification (Chambard & Pouyssegur, 1986). Under these conditions inhibition of both protein synthesis and progression through G0/G1 phase of the cell cycle takes place and cells become quiescent (Holley, 1975). Additionally, the loss of, or inability to control the response to a growth inhibitory or a growth stimulatory substance could lead to uncontrolled growth. An example to illustrate the latter possibility can be found in retinoblastoma cells which do not respond to TGF-β, whose inhibitory role in many cell lines has been identified (Hunter, 1986), because these cells have lost their receptors for this protein (Marx, 1988).

The search for "mitotic factors" which govern the transitional state of the cell has led to the description of a diverse array of molecules apparently involved in the proliferation of normal and cancer cells (Croy & Pardee, 1983, Lipkin & Knecht, 1974, Pardee, 1987), many of which appear to be phosphoproteins (Adlakha, et al., 1985). For example, Chakrabarty, et al., (1985) identified intracellular pp66 whose phosphorylation pattern in vivo/in vitro is altered by various transforming agents such as, hormones, and growth factors, including TGF-β.

Protein phosphorylation/dephosphorylation is a prominent and well understood mechanism by which cellular events can be regulated. Although other post-translational modifications of proteins such as methylation, acetylation, and glycosylation exist, it appears, according to E Racker, (1983) that "a disturbance in the balance of phosphorylation/dephosphorylation of proteins is the basic defect in growth regulation".

The primary goal of this work is to gain an understanding of the role of these growth stimulatory and inhibitory "factors", such as EGF and TGF-β (Massague, 1985), with respect to a secreted phosphoprotein (pp69). These studies
of the changes in the extracellular environment at the cellular and biochemical levels are crucial towards a more cohesive picture of the proliferative response.

**SARCOMA GROWTH FACTOR**

Work by Delarco & Todaro, (1978) led to the identification of a factor initially termed sarcoma growth factor (SGF) isolated from the conditioned media of Moloney murine sarcoma virus (MuSV) transformed normal rat kidney cells (NRK), as well as from acid/ethanol extracts of AKR-2B (Tucker, et al., 1983) and MuSV transformed NIH-3T3 cells (Roberts, et al., 1980). SDS-PAGE revealed that SGF's consisted of three heat-stable, trypsin-sensitive peptides with molecular weights of 25, 12, and 7 kDa all with a pI range of 6.8-7.0, which were found to compete with the EGF receptor in a reversible fashion (Delarco & Todaro, 1980). However, apparent loss of the epidermal growth factor receptor and release of transforming growth factors does not always correlate with sarcoma-virus induced transformation (Brown, et al., 1985).

Some fundamental epigenetic changes take place when fibroblast cells are treated with SGF (Knowles, et al., 1985). These include, loss of actin fibers, and altered cell morphology (Racker, 1983) which are reversible with the removal of SGF from the cell growth medium. Retinoids (RA) which can also reverse AIG of transformed mouse fibroblasts also inhibit these effects seen with SGF (Todaro & Delarco, 1980). In attempting to understand the biochemistry of SGF action Binas, et al. (1985), analyzed the effects of crude TGF (SGF) on secreted protein profiles in human epithelial and fibroblast cells. They found that four low molecular weight proteins were affected but no further characterization of these proteins has been
carried out. As well, crude TGF treatment of AKR-2B cells altered the cellular phosphoprotein profiles in a similar fashion to those cells treated with a chemical carcinogen (Chakrabarty & Brattain, 1985). The finding that transformed cells could produce a factor which acts in a manner analogous to a tumor promoter suggests that endogenous factors may be involved in tumor progression, and further, that cell proliferation could be due to an elevated expression of autocrine mitogens.

**TGF-α**

The transforming activity of SGF on NRK-49F fibroblast cells has been found to be due to the combined action of two transforming growth factors present in the crude preparation. One of these is an EGF-like molecule (TGF-α) with a molecular weight of 6kDa (Marguardt, et al., 1983) which is able to compete for the EGF receptor (Carpenter, et al., 1983). The other is TGF-β which is reviewed in the following section.

Expression of TGF-α (see Derynck, 1988; Rosenthal, et al., 1986), first demonstrated in the conditioned medium of virally transformed neoplastic mouse cells, appears to be specific to transformed cells (Brown, et al., 1985), more specifically carcinoma cells (Derynck, 1988), with the exception of embryonic tissue (Twardzik et al., 1982), but has also been detected in vivo and in vitro in normal neonatal and adult epidermal keratinocytes (Coffey, et al., 1987). Furthermore, this peptide may serve as a useful marker in patients with hepatocellular carcinoma, since the average TGF-α concentration for these patients is significantly higher than that of healthy subjects (Yeh, et al., 1987).
TGF-α has been shown to mimic EGF in a number of biochemical events. For example, TGF-α, like EGF, significantly increases the levels of testicular ornithine decarboxylase (ODC) activity of eight day old mice and a smaller 2-7 fold increase in kidney (Nakhla & Tam, 1985, Carpenter & Cohen, 1984, Lewis & Chikaraishi, 1987). This increase is accompanied by an increase in new protein synthesis. As well, both TGF-α and EGF stimulate osteoclastic bone resorption in vitro (Takahashi, et al., 1986), and activate the receptor-associated tyrosine kinase (Pike, et al., 1982). It appears however, that TGF-α is more potent than EGF in promoting release of calcium from fetal long bones (Ibbotson, et al., 1986) and in the induction of angiogenesis (see Folkman, 1985; Schreiber, et al., 1986).

TGF-β

TGF-β belongs to a family of related proteins which include: Inhibins, Activins, Mullerian inhibiting substance, and the predicted product of the decapentaplegic gene complex (DPP-C) transcript in drosophila in which the homologies reside within the C-terminal domain with conservation of the multiple cysteins (Massague, 1987). TGF-β also has been shown to be identical with growth Inhibitor (GI) from BSC-1 cells (TGF-β/GI) (Derynck, et al., 1985). TGF-β message has a long 5' untranslated region of high G-C content similar to the structural organization of the mRNA's for c-myc and insulin-like growth factor-2 (Dull, et al., 1984). To date, three forms of TGF-β exist, 1, 2, and 12, which depend upon the combination in either a hetero or homodimeric form (B1 & B2) (Massague, 1987). The first TGF-β identified (B1B1), present in both normal and transformed cells (Derynck, et al., 1985), is composed of two 12.5 kDa polypeptide chains (Roberts,
et al., 1981) and has been isolated from human placenta (Frolik, et al., 1983), platelets (Assoian et al., 1983), and bovine kidney (Roberts, et al., 1983).

It appears that although TGF-β is secreted into the medium it may remain in an inactive form until activated, possibly by acidification (Lawrence, et al., 1984; Pircher, et al., 1984; Leboef & Kerchaert, 1986) and/or proteolytic cleavage (Lyons, et al., 1988). The mature form of TGF—β may be released from its precursor, a first step towards activation, as a result of the cleavage by a dibasic peptidase localized at the external site of the membrane (Devault, et al., 1984). Mechanistically, TGF-β's mode of action appears to be through its receptor. Three cell surface receptors for TGF-β have been identified; all are glycoproteins with molecular weights of 65, 85 and 280 kDa (Massague, 1987). Transformed cells bind only 20-50% as much TGF-β as normal cells which appears to be due to down-regulation of the TGF-β receptor similar to that seen with TGF-α and PDGF, although cell transformation can cause a large increase in TGF-β excretion (Anzano, et al. 1985). TGF-β mRNA levels are also higher in mitogen-induced lymphocytes than in in their unstimulated counterparts (Anzano, et al., 1985).

TGF-β is a potent inhibitor of normal and some tumor-derived epithelial cell lines (Massague, 1987; Shipley, et al., 1986), as well as vascular endothelial cell growth. For example it has also been shown to block adipogenesis at a step before becoming committed to differentiation (Ignotz & Massague, 1985). Two ng/ml of TGF-β inhibits DNA synthesis of primary adult rat hepatocytes (Nakamura, et al., 1985). Commonly 0.1 ng/ml (4x10^-12 M) of TGF-β is sufficient to cause a 50% inhibition of DNA synthesis (Carr, et al., 1986), and the inhibitory action of TGF-β does not appear to be caused by direct competition with EGF at the cell surface (Carr, et al., 1986), although a decrease in the number of high affinity receptors for EGF has been seen in the presence of TGF-β (Takehara, et al., 1987).
This growth factor demonstrates a bifunctional character by both inhibiting and in many cases stimulating cell growth, whereas some cells appear to be non-responsive to this factor. For example, TGF-β does not inhibit clonal growth of a squamous cell carcinoma (Shipley, et al., 1986), and had no effect on the growth of a hepatoma cell line (Nakamura, et al., 1985). Furthermore, TGF-β "alone" can induce AKR-2B fibroblasts to form colonies in soft agar (Tucker, et al. 1983). NRK fibroblasts exposed to TGF-β, which is slightly mitogenic for these cells (Van Zoelen, et al., 1986), show increased binding of radiolabeled EGF and an increased rate of amino acid uptake with very little effect on protein synthesis (Assoian, et al., 1984). Although TGF-β does not appear to effect the synthesis of secretory and intracellular proteins by certain cells directly, it may act by modulating EGF-induced secretion (Carr, et al., 1986; Nakamura, et al., 1985; Laverdure, et al., 1987). However, data demonstrates that TGF-β can affect the biosynthesis of matrix components (Ignotz & Massague, 1987; Wrana, et al., 1988). In human lung carcinoma cells TGF-β has been shown to enhance mRNA levels for fibronectin in NRK cells as well as the secretion of endothelial type plasminogen activator inhibitor (Keski-Oja, et al. 1988) - called serpin (Sporn, et al., 1987). This factor also increases the formation of a metalloprotease called transin (Sporn, et al., 1987). In chondrocytes TGF-β was able to decrease synthesis of glycosaminoglycans and collagen (Skantze, et al., 1985). In addition mouse AKR-2B cells secrete PDGF-like molecules in response to TGF-β (Leof, et al., 1986), as well as induce c-sis mRNA expression in NRK cells (Ohlsson & Pfeifer-Ohlsson, 1987) and in human renal microvascular endothelial cells (Daniel, et al., 1987). Expression of c-sis mRNA, accompanied by a 3-4 fold increase in PDGF activity [binding to specific extracellular matrix components (Ross, et al., 1986)] in the conditioned media of
media of these endothelial cells, could be blocked by forskolin - a diterpine capable of activating adenylate cyclase.

EGF

In many cell culture systems, an increase in DNA synthesis begins approximately 15 hours after the addition of EGF, a low molecular weight, heat-stable, 53 amino acid single polypeptide that has been isolated from mouse submaxillary glands (Carpenter & Cohen, 1976), and reaches a maximum at approximately 22 hours. Whereas, maximal enhancement of sugar transport is achieved 2 hours after EGF addition in 3T3 and human fibroblast cells (Carpenter & Cohen, 1979). The peak stimulatory effect of 10ng/ml EGF was found to be between 72-96 hours in primary rat hepatocytes (Carr, et al., 1986) EGF treatment induces prostaglandin E2 synthesis in embryonic palate mesenchymal cells (Greene & Lloyd, 1985) and in an osteoblastic cell line which reaches a peak after 3 hr, followed by an increase in DNA synthesis and a decrease in cellular ALP activity, not solely due to the autocrine effect of PGE2 (Yokota, et al., 1986). It has been suggested that EGF may regulate certain kinases and/or phosphatases as its major mode of action (Cohen, 1987) Early work demonstrated that phosphorylation of membrane proteins occurs once EGF is added to membrane preparations in the presence of ATP (Carpenter, et al., 1978; Carpenter & Cohen, 1984) EGF also induces phosphorylation in 8 of 11 tryptic peptides of the 40S ribosomal protein S6 (Martin-Perez, et al., 1984), which has been shown to be phosphorylated in quiescent animal cells stimulated to proliferate by serum (Chambard & Pouyssegur, 1986). Multiple serine residue phosphorylation of S6 has been closely associated with the activation of protein synthesis EGF stimulates
tyrosine phosphorylation of a number of cellular proteins in quiescent cultures of human diploid fibroblasts (Guigni et al., 1985). EGF also enhances the phosphorylation of a number of low molecular weight soluble acidic proteins in A431 epidermoid carcinoma cells (Sahai et al., 1986). In variants of these cells EGF was shown to enhance the phosphorylation of 6 cellular proteins, 2 of which contained phosphoserine (Buss et al., 1984). EGF is also able to enhance the phosphorylation of a 35 kDa calcium-dependent membrane-binding substrate in intact A-431 cells (Sawyer & Cohen, 1985) which is similar to the tyrosine phosphorylated 34KDa protein found in Rous Sarcoma Virus transformed cells (Decker, 1982).

There appears to be a remarkable parallel between EGF stimulated growth and tumor associated growth. For example addition of EGF can cause relaxation of contact-inhibition and loss of high serum requirement but does not cause significant AIG alone (Carpenter and Cohen, 1979). The induction of NRK transformation using AIG as a criteria, requires the combination of EGF or TGF-α with TGF-β (Anzano et al., 1983). Unlike many hormones and growth factors, EGF does not appear to use cAMP or Ca²⁺ as second messengers (Cohen, 1982, Besterman et al., 1986). EGF, FGF and insulin are among the very few factors that do not stimulate the breakdown of phosphoinositides and thereby do not cause the release of intracellular Ca++ and diacylglycerol (Paris et al., 1987). However, the capacity of EGF to stimulate cell proliferation is in part mediated by the binding of this growth factor to its receptor, an integral membrane protein of 170 KDA (Carpenter, 1984) The receptor itself is subject to modification by phosphorylation on tyrosine, threonine and/or serine residues (Carpenter, 1984), and appears to contain mannose phosphate, a novel modification for a membrane receptor (Toddurud & Carpenter, 1988).
RETINOIDS

Retinoids comprise a group of natural and synthetic analogs of vitamin A, whose role in the visual cycle is well known (Jetten, 1984). Retinoids are a biphasic, teratogenic compound, which in some cell systems inhibits or stimulates cell growth (Roberts & Sporn, 1984), similar to TGF-β and can promote differentiation of murine embryonal carcinoma (EC) cells (Strickland & Mahdavi, 1978). Jetten, (1984), has suggested that retinoids, in particular the formation and action of MRP (mannosyl retinyl phosphate), could result in changes in secreted proteins. It is now clear that RA can control or modulate the synthesis of specific proteins (Roberts & Sporn, 1984). For example, in bone and cartilage addition of vitamin A results in the degradation of extracellular matrix due to an increase in the synthesis of cathepsin D (Lotan, 1980). Although it has been suggested that RA and TGF-β may have some common mechanistic effects in terms of their anti-proliferative capacities, growth in monolayer of myc-1 transfected cells was consistently inhibited by TGF-β and either unaffected or stimulated by retinoic acid (Roberts, et al., 1985b).

RA has been shown to inhibit AIG (Jetten & Goldfarb, 1983), inhibit phospholipid turnover (Vanier, et al., 1988), increase the number of EGF receptors, while decreasing the mitogenicity of EGF, and decrease ODC activity (Marx, 1978). In one study, RA has been reported to inhibit AIG and restore density-dependent growth to MSV-transformed cells without increasing cellular binding of 125I-EGF (Mukherjee, et al., 1982). Therefore, some of the effects may be secondary to its mode of action.
PHORBOL ESTERS and Tumor Promotion

Tumor promoting agents, as opposed to an initiating agent which binds DNA and is mutagenic, are important in the development of tumors (Marx, 1978) and alter certain transformation related properties such as; cell morphology, cell surface glycoproteins, saturation density, plasminogen activator levels, ODC activity (Weinstein, et.al., 1979; Rifkin, et.al., 1979; Marx, 1978), as well as angiogenesis (Montesano & Orci, 1985). Phorbol esters, which have a requirement for extracellular calcium for activity (Smith, et al., 1986), activate PKC, an enzyme that is activated by the receptor-mediated hydrolysis of inositol phospholipids (Bell, 1986; Berridge & Irvine, 1984; Nishizuka, 1986). Significantly, increased PI-kinase activity has been associated with hepatocarcinogenesis (Olson, 1985). PKC then relays information via extracellular signals across the membrane to regulate many $\text{Ca}^{2+}$-dependent processes. The role of PKC can be influenced by various $\text{Ca}^{2+}$-mediated processes such as secretion and exocytosis which would be modulated by protein phosphorylation catalyzed by this kinase (Kikkawa, et.al., 1986).

TPA (12-O-Tetradecanoyl-Phorbol-13-Acetate), an extensively used tumor promoter stimulated the phosphorylation of a number of cell surface proteins in $\text{Ca}^{2+}$-deficient medium (Kleine, et.al., 1986). Phosphorylation of certain cell surface proteins appears to correlate with transformation and increased AIG (Banerjee, et al., 1986). TPA can alter gene expression and protein phosphorylation in avian cells containing c-src without affecting the pp60src protein or its 34-36kDa substrate, suggesting independent pathways present in transformation and lending support to the multistep carcinogenesis hypothesis (Laszlo, et.al., 1981). TPA has also been shown to alter the intracellular
phosphoprotein profile of a 67kDa protein with a pI of 4.5 (Sagara, et al., 1986, Bouche, et al., 1984) and a protein of 80kDa in human fibroblasts (Sagara, et al., 1986). Treatment of human eosinophils with PMA, another tumor promoter, also caused the alteration in the phosphorylation of some soluble proteins (Ramesh, et al., 1987) TPA treatment of mouse embryonic fibroblasts increased levels of 5 cellular and 34 extracellular peptides; one of which was ODC (Haarr, et al., 1986). TGF-β as well as TPA stimulate the secretion of 48-51kDa proteins in AKR-2B cells (Thalacker & Nilsen-Hamilton, 1987), where EGF appears to act synergistically.

Although some of TPAs' effects differ from those seen with growth factors (Allard, et al. 1987; Vaartjes, et al., 1986), TPA acts synergistically with most known mitogens and share common pathways in their mode of action, with the exception of the neurohypophyseal hormone vassopressin (Dicker & Rozengurt, 1980). For example, TPA stimulates the phosphorylation of the receptors for insulin and somatomedin C suggesting a role of PKC in regulating these receptors (Jacobs, et al., 1983). Thrombin which can stimulate proliferation of hamster fibroblasts, also stimulates the 32P incorporation into a number of phosphotidylinositols and stimulates recycling of these molecules (Carney et al., 1985) similar to the effect seen with TPA. TPA can act synergistically with EGF in stimulating DNA synthesis in mouse 3T3 cells (Brown, et al., 1979). As a result of TPA's activation of Ca2+-dependent C-kinase, reduction in the binding of EGF to surface receptors in intact cells occurs and may modulate EGF receptor affinity (Decker, 1984, Fearn & King, 1985; Rosner, et al., 1985), by phosphorylation (Hunter, et al., 1984) and inhibition of the EGF receptor (Davis & Czech, 1985). Although EGF does not appear to use the IP-3/DG pathway and does not activate phospholipase C in most cell lines, EGF and TPA may be related since EGF raises the Ca2+ concentration (Castagna, 1987). However, activation of the proliferation-dependent Na+/H+ exchange is not
dependent on an early rise in calcium seen to occur with many growth factors (Moolenaar, et al., 1984).

GROWTH FACTORS as Oncogenes

When links between genetic damage, such as chromosomal translocations, insertional mutagenesis, and cellular transformation were analyzed (see Bishop, 1987), and through work on virus-induced transformation, a hypothesis that a single gene of cellular origin could be involved in malignancy if inappropriately activated (Weinberg, 1984) was proposed.

There are many different retroviral as well as cellular oncogenes (Bishop, 1985; Bishop, 1987) known to date and many are thought to play a role in cellular growth control. In fact, there appear to be common elements between growth factor stimulation and oncogenic transformation (Kaplan, et al., 1987). A role for the fos and myc proto-oncogenes in cell proliferation has been suggested from the fact that activators of cell proliferation cause a rapid induction of the c-fos gene in the early G1 phase of the cell cycle, as well as govern the pattern of c-myc gene expression in a variety of cell types (Kelly, et al. 1983; Ohlsson & Pfeifer Ohlsson, 1987). It has been established that many c-onc genes code for growth factors or their receptors through work on their retroviral counterparts. For example, the N-terminal 109 amino acid residues of the B chain of PDGF (Ross, et al., 1986) are similar with the predicted sequence of the transforming protein p28sis (Waterfield, et al., 1983). Furthermore, the product of the c-fms protein product has been shown to be the receptor for colony-stimulating factor (CSF-1) (Sherr, et al., 1985). By direct amino acid sequence comparison the product of c-erbB has
been identified as the cell surface receptor for epidermal growth factor (Ohlsson & Pfeifer-Ohlsson, 1987).

Among the known retroviral oncogene products many have been shown to possess kinase activity (Heldin & Westermark, 1984). It appears that a single transforming gene coding for a tyrosine-phosphorylating protein kinase is responsible for making a tumor out of a normal chick embryo fibroblast (Racker, 1983). Furthermore, growth factors such as EGF, PDGF, and hormones such as insulin have also been shown to induce tyrosine phosphorylation (Racker, 1983). Phosphotyrosine, however, accounts for only 0.03% of all phosphorylated amino acids whereas phosphoserine and phosphothreonine account for the remaining 99.7% (Cohen, 1982). Cooper & Hunter (1982), in comparing the effects of EGF and Rous Sarcoma virus (v-src) on cells, suggest that hormones may activate oncogene phosphorylating activity related to cell proliferation. On the other hand dephosphorylation has been shown to stimulate pp60\(^{c-src}\) (Cooper & King, 1986).

It has also been shown that phosphorylation of serine and threonine residues appears to activate c-src during fibroblast mitosis (Chackalaparampil & Shalloway, 1988). Cooper and Hunter's suggestion that hormones may activate oncogene phosphorylating activity is further exemplified by the membrane protein p21. This protein is coded for by c-ras, possesses GTP-binding activity, becomes phosphorylated on a threonine residue in the presence of GTP (Racker, 1983), and is known to vary through the cell cycle (Marshall, 1984). It has been suggested that p21 is involved in the transduction of the growth factor signal and may be an obligatory intermediate in this pathway (Mulcahy, et al., 1985). In support of this hypothesis, p21 appears to interact with the receptor for EGF. EGF itself increases the binding of GTP to p21 and causes activation of certain receptors (Kamata & Feramisco, 1984).
Numerous morphological and biochemical differences exist between normal and cancer cells. Principal among these differences is the ability of cancer cells to grow in vitro in semi-solid media (AIG) and to grow with reduced growth factor requirements (Macpherson & Montagnier, 1964; Giguere & Gospodarowicz, 1983; Freedman & Shin, 1974). The latter provides one of the most consistent in vitro tests for cellular malignancy (Mukherjee, et al., 1982, Steuer & Ting, 1977) and an important system for looking at cell proliferative control mechanisms. AIG is a long term phenomenon requiring 3-7 days of treatment and is thought to be due to the amplification of an original signal transduction mechanism (Assolan et al., 1984a) which also requires the cooperation of numerous pathways, similar to multistep carcinogenesis involving cooperation and synergism between oncogenes (Land, et al., 1983).

AIG induced by growth factors and hormones has been roughly separated into three classes, 1) activity independent of EGF, 2) activity dependent on EGF and 3) activity potentiated by EGF (McClure, 1983). The synergism between EGF and TGF-β leading to AIG has been extensively studied using NRK cells (Sporn, et al., 1987; Rizzino, et al., 1986, Roberts, et al., 1985a). Although NRK cells are considered to be a unique system requiring both growth factors for AIG (Tucker, et al., 1983), there has also been shown to be a [TGF-β/EGF] dose-dependent increase in the DNA content of rabbit articular chondrocytes, a system in which neither compound alone was mitogenic (Skantze, et al., 1985). The combination of EGF (10ng/ml) and TGF-β (0.1 ng/ml) was able to increase the tritiated thymidine incorporation in adult rat hepatocytes, to levels higher than those in cells treated with EGF alone (Carr, et al., 1986). Pre-treatment of NRK-49F cells with both EGF and TGF-β causes
an increase in glycolysis as well as amino acid uptake (Boerner, et al., 1985) Using a partially transformed rat kidney cell line it has been suggested that TGF-β induced qualitative responses which may be an intermediate stage in the spontaneous transformation of NRK cells (Newman, et al., 1986), and further, that TGF-β modulates the effects of EGF in soft agar (Roberts, et al., 1985a, Van Zoelen, et al., 1986).

Other synergistic models have been explored in order to understand the complex interactions that lead to AIG. It now appears that at least four different growth factors may regulate AIG (Rizzino, et al., 1986) Furthermore, TGF-β as well as ligand binding of TGF-β to its receptor may not be essential for AIG since cells can also be induced to grow in semi-solid media with the combined effects of EGF, PDGF and RA (Van Zoelen, et al., 1986) RA, which is mitogenic for NRK cells, also markedly enhances the AIG of these cells in the presence of TGF-β and EGF, (Van Zoelen, et al., 1986), but cannot simulate TGF-β induced transformation (Assolano, et al., 1984a).

Synergistic models of AIG have been simplistic in scope due to the addition of 10% serum which is still a normal component of the AIG assay. Prior to studies with purified PDGF, serum provided the necessary PDGF, as well as a number of other components required for AIG (Assolano, et al., 1984a) Wharton, et al., (1983) observed that PDGF, which appears to act as a "competence" factor (Ross, et al., 1986), modulates the mitogenic response of cells to EGF by increasing the sensitivity of fibroblasts to EGF. Primary rat embryo cells and established NIH/3T3 cell lines grow in soft agar in the presence of PDGF (Rizzino, et al., 1986). The PDGF-induced growth of these primary rat embryo cells in soft agar, can be blocked by TGF-β but is not affected by EGF (Anzano, et al., 1986)
Tumor promoters such as TPA, which have been shown to cause a decrease in EGF receptor levels (Kraft, 1986), also appears to enhance the activity of the combined action of EGF and TGF-β in a concentration dependent fashion but cannot stimulate AIG by itself (Kraft, 1986). As well, TPA combined with EGF was able to increase 3-5 fold the AIG ability of rat embryo fibroblasts cells transformed by a ts mutant of adenovirus (Fisher, et.al., 1979).

SECRETED PROTEINS -and Cell Growth

Normal fibroblastic cells in culture secrete into their extracellular environment a wide variety of proteins which are required for the formation of the extracellular matrix or in regulating growth. These include, amongst others, albumin, transferrin, apolipoprotein, fibronectin, complement c3 and alphal-anti-trypsin (Nakabayashi, et al., 1985, Celis, et.al., 1987).

The mechanism of secretion and regulation of secreted proteins has been investigated quite extensively (Bienkowski, 1983; Blobel, 1980; Davis & Tai, 1980). The basic components of protein secretion involve; synthesis, segregation, transport, and in some cases concentration and storage, as well as discharge (Palade, 1975; Nussdorfer, 1984). In recent years the focus in growth regulatory peptides has shifted to the extracellular environment and the importance of these secreted proteins in relation to cell growth are now becoming apparent. Racker, (1983) has suggested that an intermediate protein which is bound externally to the plasma membrane that could influence growth of cells externally is important for activation of proteins secreted in response to TGFs. Human hepatoma cells treated with TPA for 6 hr induced marked secretion of a non-phosphorylated 46kDa polypeptide inhibitable by actinomycin D (Yoneda, et.al., 1985). In addition, the
secretory protein profiles of early and late passage cultures were compared in human fibroblasts; a heparin binding protein with a molecular weight of approximately 55 kDa increased in senescent cultures (Sottile, et al., 1987). MCIF is a 160 kDa glycoprotein which appears to be released into the medium which restores contact inhibition of growth to malignant melanocytes (Lipkin, et al., 1978). Mitogen-regulated protein (MRP) was described as a family of glycoproteins with a molecular weight of 30-38 kDa which are released by Swiss 3T3 cells 18-24 hours after TPA or growth factor stimulation and NH₄Cl treatment (Nilsen-Hamilton, et al., 1980; Nilsen-Hamilton, et al., 1981).

LeCam, et al., (1985) have found that hepatocytes secrete a major serine-phosphorylated 63kDa (pp63) glycoprotein with a pI of 4.8-5.3 and with extensive charge heterogeneity. Unlike albumin which is present in the media of cultured hepatocytes after 15 min., this protein appears in the media after 40-60 min. Although pp63 appears to be secreted in an unphosphorylated form, the protein was originally produced by liver cells in a phosphorylated form. PP63 is insensitive to endo H and sensitive to neuraminidase indicating that it is terminally glycosylated in the Golgi. Furthermore, secretion of this phosphoprotein was altered by tunicamycin (LeCam, et al., 1985).

Angiogenin is a secreted protein with a large sequence identity to pancreatic ribonuclease (Kurachi, et al., 1985) and a molecular weight of 14.4 kDa and a pI > 9.5. It is a human tumor-derived protein from a human adenocarcinoma cell line (HT-29). It binds heparin and augments the growth of a vascular network by invasion of the perivascular extracellular matrix by sprouting endothelial cells in such processes as wound healing, embryonic development, and progressive tumor growth (Fett, et al., 1985; Strydom, et al., 1985; Liotta, et al., 1983). TPA and vanadate, a well known tyrosyl-phosphatase inhibitor, can induce angiogenesis.
As well, cells treated with these agents mimic tumor cell invasion (in three-dimensional collagen gels) (Montesano & Orci 1985).

EGF stimulates secretion of a glycoprotein hormone, human chorionic gonadotrophin, from human trophoblastic tumor cells (Hirata, et al., 1982). It has also been shown to stimulate synthesis of prostglandins in canine kidney cells (Lovine & Hassid, 1977). Stimulation of quiescent 3T3 cells with serum or EGF also induces a group of 45kDa proteins which may be components of the extracellular matrix (Santaren & Bravo, 1987). In non-transformed rat kidney cells (NRK-49F) EGF is able to enhance levels of a secreted, phosphorylated 69kDa protein (Laverdure, et al., 1987). PP62 is a major phosphoprotein secreted by both epithelial and fibroblastic transformed cells and is not antigenically related to pp60-src (Senger, et al., 1983; Senger & Perruzzi, 1985), but is antigenically related to pp69 (Laverdure, et al., 1987), and does not appear to be related to exponential growth.

Many of these secreted proteins share various similarities. For example, a 58kDa protein which appears to behave similarly to the NRK-49F-derived pp69 has also been described (Binas & Gross, 1987). EGF and TPA enhance the amount of phosphate found in pp58, while TGF-β reduced the amount and NH₄Cl, a lysosomotropic amine, did not increase its secretion. However, unlike pp69 EGF did not influence the level of this protein. A 69kDa oncofetal protein has also been seen in the plasma of tumor-bearing rats (Hanousek-Walaszek, et al., 1984). Two secreted peptides with molecular weights of 64 and 68 kDa proteins derived from three intracellular peptides 60, 63, 66 kDa peptides from temperature-sensitive MuSV-transformed NRK cells (6M2), termed TAPs (transformation associated protein) (Chan, et al., 1986; Li, et al., 1987) have also
been identified. Purified TAP's have been shown to bind specifically to NRK-2 cells and stimulate their cellular proliferation (Li, et.al., 1987).

Much attention has recently been placed on a what is probably a family of related proteins - certain phosphoproteins which are synthesized and secreted by osteoblast-like cells (Prince, et.al., 1987). A 45kDa glycoprotein otherwise known as osteopontin or sialopontin-I can be found in the convoluted tubules of kidney (Mark, et.al., 1987a) and can be immunologically related to pp69 (Chackalaparampil, et.al., 1985) secreted by NRK-49 cells (Craig, et.al., 1988). The discrepancies in molecular weight are likely due to post-translational modification and to a high content of negatively charge amino acid residues. Carbohydrate composition analysis indicates it is rich in sialic acid (Prince, et.al., 1987). This protein appears to enhance cell attachment (Somerman, et.al., 1987). As well, osteonectin (38 kDa) and another non-collagenous bone protein with a molecular weight of 62kDa (Mark, et.al., 1987; Somerman, et al., 1987) have been described. The role of these secreted molecules in the regulation of the extracellular matrix and whether this is coupled with cell proliferation is unclear.

Many secreted proteins are in fact proteases which may regulate the breakdown of other secreted proteins including the extracellular matrix. A 52kDa estrogen-induced protein which is a lysosomai acid protease is similar to procathepsin D, an aspartyl protease with mannose-6-phosphate signals able to degrade basement membrane (Morisset, et.al., 1986; Rochefort, et.al., 1987). It has been well established that excretion of plasminogen activator is enhanced in transformed cells (Racker, 1983). In addition, PDGF stimulates density-arrested Balb/c-3T3 cells to synthesize MEP, a lysosomal protein similar to cathepsin L (Frick, et.al., 1985; Doherty, et.al., 1985; Gal, et.al., 1985; Rochefort, et al., 1987).
EXTRACELLULAR MATRIX - Control of Formation/Degradation

The nature of the matrix on which cells anchor themselves is probably dependent on parameters such as: cell type and growth factor concentration. For example, rat-1 fibroblast cells show improved growth in dishes that had been coated with fibronectin as compared to growth in dishes coated with collagen (Giguere & Gospodarowicz, 1983). It is also clear that secreted proteins may interact with extracellular matrix components to affect growth, and migration of cells. For example, synovial fibroblasts secrete a protein which in turn regulates collagenase production (Brinckerhoff, et al., 1985). In fact, fibroblasts have been shown to modify the extracellular matrix during migration under several different growth conditions (Kalebic, et al., 1983).

It appears as a necessary caveat that alteration of the extracellular matrix must take place for a growth or migratory response to take place. Primary tumors show extensive degradative activity toward the basement membrane matrix (Starky, et al., 1987) and transformed NIH/3T3 cells lose their migratory ability in response to PDGF (Massague, 1987) which may inhibit this degradation. Normally, cells that respond to PDGF show a chemoattractant response to this hormone (Grotendorst, 1984). This may in part be explained by the hypothesis that in transformed cells where collagen levels are drastically reduced fibronectin may be more accessible to degradation (Chan, et al., 1987).

Ignotz & Massague, (1986) showed that TGF-β increases the synthesis of fibronectin and collagen into the ECM of cultured chicken embryo fibroblasts. Prior to this, TGF-β causes an increase in the assembly of exogenous fibronectin and binding of fibronectin to the cell surface (Allen-Hoffman, et al., 1983). EGF has also been shown to increase the synthesis of extracellular matrix components
such as fibronectin and collagen, as well as decrease the chemoattractant response to PDGF (Massague, 1987; Carpenter & Cohen, 1979). In the presence of EGF quiescent human fibroblasts show an increase in the synthesis and extracellular accumulation of hyaluronic acid which can be enhanced by a mEGF-arginine binding esterase (Lembach, 1976).

PHOSPHORYLATION-Regulatory Mechanisms

The importance of this ubiquitous phenomena (Greengard, 1978; Alix & Hayes, 1983) has been known since the discovery that synthesis and breakdown of glycogen was regulated by the contractile state of the tissue as well as by adrenalin and insulin, through multi-site phosphorylation. In particular, changes in the phosphorylation state of glycogen phosphorylase and synthase [where there is a possibility of 7 serine sites which can be phosphorylated to inactivate this protein]. In many cases phosphorylation of one residue could amplify or antagonize phosphorylation at other residues (see Cohen, 1982). These interconversions involve 5 protein kinases and 4 protein phosphatases and the regulatory molecule calmodulin (Cohen, 1982; Lipmann, 1983). Significantly, differences in calmodulin levels develop as cultures become more densely populated, and appear to depend on the differing abilities of normal and transformed cultures to respond to cell-cell contact and cellular recognition signals which may in turn affect the calmodulin-dependent protein kinases (Veigl, et.al., 1984).

Does phosphorylation mediate events such as protein secretion? Some evidence suggests that it does. For example, parathyroid hormone and fibrinogen are excreted in a phosphorylated form (Racker, 1983). Phosphovitin uptake of the
dephosphorylated protein into oocytes is decreased by 79% as compared to the native form (Racker, 1983), while removal of the first eight phosphates attached to serine residues decreased transport of the protein into oocytes by 60%. It is the phosphate attached to mannosyl residues which is thought to be important for the recognition and uptake of lysosomal hydrolases into human fibroblasts (Kaplan, 1977).

Differences in residue preference as well as multi-site phosphorylation are clearly important regulatory mechanisms for controlling cell proliferation. It has been shown, for example, that extracts from normal cells prefer the NH2-terminal of histone-1 as phosphorylating substrate while transformed cells prefer the COOH-terminal (Sykes & Hohmann, 1985). The phosphorylation at multiple sites of acetyl CoA carboxylase decreases its activity and increases the Ka for the activator citrate (Cohen, 1982). As well, phosphorylation also appears to be one of three mechanisms that regulate cholesterol biosynthesis (Beg, et al., 1987). It is now thought that inactivation of certain enzymes such as isocitrate dehydrogenase by phosphorylation, which is considered to be a short-term mechanism by which enzyme activity can be regulated, is mediated by the negative charge on the phosphate group (Thorsness & Koshland, 1987).

Molecules which appear to be involved in matrix organization are also subject to phosphorylation control. For example, a 47 kDa phosphorylated protein which is a membrane associated collagen-binding protein of fibroblasts is phosphorylated and altered 5-7 fold higher in both quantity and degree of phosphorylation after malignant transformation (Nagata & Yamada, 1986). A 58kDa subunit protein of fibroblast intermediate filament which appears necessary in the regulation of matrix organization is phosphorylated when CHO cells are arrested in mitosis (Robinson, et al., 1981). The unphosphorylated MAP2
protein has been shown to bind and bundle actin filaments whereas phosphorylated MAP2 does not (Seldon & Pollard, 1986, Sattlaro, et al., 1981).

Phosphorylation of synapsin I which contains three potential phosphorylation sites, one site phosphorylated by a cAMP-dependent kinase and the others by Ca²⁺/calmodulin-dependent kinase, appear to cause the stimulation of microtubule-binding activity while reducing this protein's ability to bundle actin filaments. (Petrucci & Morrow, 1987).

POLYAMINES

The polyamines (Tabor & Tabor, 1976; Tabor & Tabor, 1984) putrescine, spermine and spermidine, are aliphatic, nitrogenous molecules of a cationic nature which appear important in a number of cellular processes, including cell cycle transition (Marchesini, et al., 1987), and differentiation (Ewton, et al., 1984). Significantly, transfer RNA's isolated from a variety of cells has been found to contain one or two moles polyamine per mole of tRNA. In fact tRNA can be converted from their active to inactive forms by addition of polyamines (Cohen, 1978). Elevated levels of polyamines have been found in cells transformed by retroviruses whereas ODC levels, a key enzyme in the biosynthesis of polyamines, are low in quiescent tissue (Nakhla & Tam, 1985). Spermine, which must first be "activated" by serum polyamine oxidase, has been shown to inhibit SGF-induced AIG with an LD₅₀ of 0.7-1.1 uM (Frolik, et al., 1984). Various cyclic nucleotide-independent protein kinases can be enhanced by polyamines (Rose, et al., 1981; Leiderman, et al., 1985). Polyamines appear to stimulate the phosphorylation of phosphatidylinositol through activation of PI kinase (Vogel & Hoppe, 1986). Although polyamines can activate certain kinases, spermine has also been
implicated in the inhibition of PKC (Kikkawa & Nishizuka, 1986; Thams, et al., 1986) possibly by inhibiting protein kinase C's association to the membrane (Moruzzi, et al., 1987). Polyamines can also inhibit certain calmodulin-dependent protein kinases (Kikkawa & Nishizuka, 1986). Furthermore, they have been shown to decrease the level of phosphorylation of proteins in hepatocytes mainly through activation of a protein phosphatase (Auberger, et al., 1984). As well, polyamines have been shown to activate protein phosphatase activity in HeLa cell nuclei (Friedman, 1986).

PHOSPHATASES - Dephosphorylation

The importance of phosphatases in the control of metabolic processes became evident when their role in the regulation of the enzyme glycogen phosphorylase was demonstrated (Ballou & Fisher, 1986). Both protein kinases and phosphatases display broad specificity. In other words, residues at different positions may be phosphorylated by a single kinase, yet are dephosphorylated by a number of phosphatases and vice versa (Sparks & Brautigan, 1986, Parker, et al., 1986). In several studies using synthetic peptide substrates it appears that ser/thr specific kinases react on the basis of a protein's primary structure, a residue preceded by basic (hydrophobic) side chains while tyrosine specific kinases require residues preceded by several acidic side chains (Sparks & Brautigan, 1986). As well, kinases recognize secondary structure which often appear as beta bends, whereas phosphatases appear to have the ability to recognize both acidic and basic proteins with diverse sequences in which tertiary structure may play a more prominent role (Sparks & Brautigan, 1986).
Phosphatases fall into two major classes which are partially distinguished by pH optimum. These two classes are, the protein phosphatases with a pH optimum of pH 7.4, and the alkaline (ALP) or acid phosphatases with pH optima of pH 9.0 and 4.8 respectively (Sparks & Brautigan, 1986) Purification of alkaline phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.1) from rat intestine has shown that there exists two forms, a membrane bound and a soluble form. The latter is secreted into the bloodstream (Yedlin, et.al, 1981), and both are likely coded from different mRNA's (Sussman, et al., 1986) Three isoenzyme forms of ALP have been described; placental, intestinal and tissue non-specific (bone/liver/kidney) (Whyte & Vrabel,1987) Alkaline phosphatases can be inhibited by divalent cation chelators such as EDTA (Fernley, 1971) and specifically inhibited by levamisole (L-(-)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole (Chan & Stinson, 1986; VanBelle, 1972)

The relationship between ALP activity and growth rate remains unclear and may depend on the differing effects of the soluble versus the membrane bound forms of ALP. Hamster embryo cells which have been transformed by different carcinogens have no detectable ALP activity (Sela & Sachs, 1974) Prior to transformation ninety percent of cells were positive in culture for ALP. Transformed cells that were constitutively ALP+ showed decreased DNA synthesis, cell multiplication, decreased ability to form colonies in soft agar, and decreased tumorigenicity in animals compared to transformed cells that were ALP- On the other hand, Whyte & Vrabel, (1987) using cells from patients with infantile hypophosphatasia, have suggested that the tissue non-specific isoenzyme form of the membrane bound ALP may not have a role in cell growth and differentiation in a number of systems ALP activity, which has been implicated in bone calcification and phosphate transport in epithelial cells of the intestine (Chan &
Stinson, 1986), appears to be hormonally controlled and may somehow be involved in cell growth (Wergedal, et al., 1988). Since many kinases such as those dependent on cAMP become activated in response to growth factors, it seems logical to assume that these same factors could control protein phosphatase activity. For example, parathyroid hormone, itself a phosphorylated protein, in turn appears to inhibit the rise in alkaline phosphatase activity during growth of ROS 17/2 osteoblast-like cells in culture (Majewska & Rodan, 1982). Exposure of cultured human choriocarcinoma cells to methotrexate which causes the formation of "giant cells" in a density-dependent fashion appears to correlate with an increase in alkaline phosphatase activity, while high population densities caused reduced ALP expression (Burres & Cass, 1986). Corticosteroids induce the expression of ALP in several human cell lines (0x, et al., 1971). Vitamin D also induces intestinal ALP in chicks in vivo, while in bone cells vitamin D3 inhibits this activity (Wong et al., 1977). RA which causes changes in growth and morphology of 9-1C cells also induces ALP activity (Reese, et al., 1985). Bovine bone extract treatment of chick bone and skin fibroblasts stimulates thymidine uptake and decreases ALP activity (Wergedal, et al., 1988).

The protein phosphatases (1, 2A, 2B, and 2C), with a pH optimum of 7.4, constitute another important class of regulatory molecules whose role in the cell proliferative response is becoming clear. Protein phosphatases, 1, 2A and 2C have broad substrate specificity (Cohen, 1982). Protein phosphatases can be inhibited by phenyl phosphonic acid (PPA) (Chan & Stinson, 1986). Protein phosphatase-1 and 2A appear to be major phosphatases acting on protein synthesis initiation factor elf-2 and may function as stimulators of protein synthesis (Cohen, 1982; Olivier, et al., 1988). Microinjection of Inhibitor-1 blocks the progesterone-induced division of Xenopus oocytes, suggesting that protein phosphatase-1 is
vanadium is a transition metal required for normal growth (Swarup, et al., 1982). Orthovanadate alone can affect morphology, density dependent growth as well as soft agar growth in a dose dependent manner (Klarlund, 1985), possibly linked to the insulin-like effect of vanadate (Ballou & Fisher, 1986). Protein tyrosine phosphatases (Foulkes, 1983) present at high levels in kidney tissue are inhibited 50% by 1 μM orthovanadate or by Zn$^{2+}$ in μM quantities, whereas ALP can also be inhibited by orthovanadate but require Zn$^{2+}$ for activity (Sparks & Brautigan, 1986; Sargent & Stinson, 1979). This suggests that certain effects attributed to tyrosine phosphorylation may not be restricted to tyrosine-phosphatase inhibition but may in part represent overlapping substrate specificities. In fact, there does appear to be an ALP substrate specificity for P-tyr (Swarup, et al., 1981) over P-ser in a two substrate system (Takahashi, et al., 1987). Insulin treatment induced the activation of several protein phosphatases, specifically phosphatase 1 (Olivier, et al., 1988), 2A, and 2C, that catalyze the dephosphorylation of the regulatory phosphorylation site of the hormone-sensitive lipase, resulting in reversed nor-adrenaline induced lipolysis by decreasing the cAMP-dependent protein kinase (Olsson & Belfrage, 1987; Londos, et al., 1986). Inactivation of inhibitor 1 (i.e., activation of protein phosphatase-1) by phosphorylation is decreased by insulin and increased in the presence of isoproterenol and adrenalin (Ballou & Fisher, 1986). Insulin at low μM concentrations activates phosphatase-2A, which can also be activated by polyamines, 4-fold by binding to it. As a result it has been suggested that polyamines could act as second messengers of insulin action (Huang & Chang, 1980).
MATERIALS and METHODS

Cells and Culture Conditions

The normal rat kidney cell line NRK-49F p19 (DeLarco & Todaro, 1978), Balb/3T3 clone A31, Kirsten murine sarcoma virus transformed NRK cells (KNRK) and Schmidt-Rupin RSV-transformed rat cells (RR1022) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Rat-1, an embryonic rat fibroblast cell line derived as an independent subclone of the parental F2408 line was obtained as a gift from Dr. J. Hassel's Laboratory (McGill University). The FaO cell line derived from rat hepatoma cells was a gift from Dr. J. Fuks (McGill Cancer Centre, Montreal, Canada). LA-23 cells, NRK cells transformed by a ts-mutant of RSV (Prague strain) were obtained from Dr. J.F Whitfield (Cell Physiology Group, NRC of Canada, Ottawa). Spontaneously transformed NRK cells were obtained by subculturting NRK cells for >35 passages. All cells were grown in DMEM (GIBCO) supplemented with streptomycin, ampicillin, gentamycin and 10% NCS. Cells were subcultured 24-48 hr before labeling.

Cell Growth & Soft Agar Assays

Cells (1x10^4) were seeded in 35mm petri dishes (Falcon) and refed every 24 hr. Stock solutions of 2ug/ml TGF-β, stored in 4mM HCl with 1 mg/ml BSA (a gift from Dr. Anita Roberts, NIH, Bethesda), and 10mM levamisole, 10mM PPA, and 10µM spermine (Sigma) were diluted with DMEM prior to use. Stock solutions of insulin and EGF (Sigma) were prepared in PBS and stored at -20°C and then diluted with DMEM to concentrations of 10ug/ml and 5-50ug/ml respectively and stored at 0°C.
for no more than 1 week. RA (all-trans-B-retinoic acid) and TPA were kept as 10^{-2} M stock solutions in DMSO and stored at -70^\circ C for not more than two weeks.

Dilution of the RA and TPA stock solutions was made with DMEM to yield a final concentration of 10^{-7} M and 2 \times 10^{-7} M respectively. The final DMSO concentration in the RA and TPA containing media was 0.001%. After 48 hr of incubation, replicate cultures were harvested and total cell counts were carried out in a Coulter Counter. All assays were done in triplicate.

A crude preparation of transforming growth factor (cTGF) extracted from a Moloney murine sarcoma virus transformed 3T3 cell line (3B11-1C) was kindly supplied by Dr. J. E DeLarco (NIH, Bethesda, Md.). This crude TGF was concentrated by resuspension in 1M acetic acid, clarified by ultracentrifugation at 100,000xg for 30 min, dialyzed in excess of 1% acetic acid with 2 changes followed by lyophilization. The lyophilized material was resuspended in PBS to give a final protein concentration of 0.5 mg/ml. All aliquots were heated at 60^\circ C for 2 min and tested for their ability to affect the cells ability to be subcultured, DNA synthesis, tritiated thymidine uptake in Rat-1 cells, as well as growth in soft-agar using NRK-49F and Rat-1 cells.

The assay for colony growth in soft agar (Macpherson & Montagnier, 1964) was carried out. Two ml of 0.3% agar in DMEM + 10% NCS containing 1 \times 10^3 cells was layered on top of 1 ml of 0.5% agar (base layer) in DMEM containing 10% NCS pipetted onto a 35mm petri dish. The cultures were incubated at 37^\circ C in a humidified CO_2 incubator and refed at 48 hr intervals. Supplements such as, TGF-β, EGF, RA were added every 24 hr. Colonies were grown for 7 days and counted on grid using an ocular micrometer. Colonies greater than or equal to 30um in diameter as well as colonies greater than or equal to 60um in diameter were scored. Photographs were taken with an inverted Zeiss microscope.
Treatment & Metabolic labeling of NRK-49F Cells

Cells, at passage 19-24 which demonstrated no incorporation of $^{32}\text{p-orthophosphate}$ into low molecular weight membrane proteins (Banerjee, et.al., 1985), were seeded into 35mm dishes at a density of approximately $2 \times 10^4$ cells and pretreated with: cTGF (10 ug/ml), purified TGF-$\beta$ (0.01-10 ng/ml), EGF (5 ng/ml), insulin (10 ug/ml), levamisole (0.1 mM), PPA (0.1 mM), spermine (1-5 uM), cycloheximide (25 ug/ml), colcemid (0.1-1 ug/ml), cytochalasin B (10 ug/ml), NH$_4$Cl (20 mM) or actinomycin D (2 ug/ml) for the desired length of time. Monolayers were incubated with phosphate free media with or without supplements for 1 hr before incubation for 3.5 hr in 1 ml growth medium containing 0.5%-1.0% serum and 0.4 mCi/ml of $^{32}$P-orthophosphate (Amersham).

Thymidine Incorporation

Crude TGF growth promoting activity was determined by the incorporation of tritiated thymidine into Rat-1 cells. After 96 hr of treatment, triplicate cultures were labeled for 5 hr with tritiated thymidine. At the end of the labeling period cells were incubated for 30 min in 1 ml/plate DMEM with 100 ug of unlabelled thymidine. The plates were then washed 5x with PBS Cells were lysed by adding 1 ml of cold RIPA buffer (10% Triton X-100, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 2.0 mM phenyl methyl sulfonyl fluoride, 0.05 M Tris-HCl, pH 7.2). After 10 min on ice, the slurry was collected and an additional 250 ul of RIPA was added to each plate, and any remaining material removed from the plate with a rubber policeman The lysate was treated with 3 volumes of cold 10% TCA. Precipitated material was removed by filtration (Millipore 0.45 um) Filters were
washed 3x with cold 5% TCA, dried and placed in 10ml of scintillation fluid (New England Nuclear, Omnifluor) and counted (Intertechnique SL-10).

Analysis of Secreted pp69 from the Conditioned Media

For the analysis of secreted proteins, conditioned media was removed and centrifuged in an Eppendorf microfuge for 3 min. PMSF (2mM) was added. Secreted phosphoproteins were frozen and remained stable for 1-2 weeks provided freeze-thaw was minimized. 100ul aliquots of conditioned media were precipitated with 4 vol. of cold acetone and pelleted by centrifugation. The pellet was washed with a small volume of cold acetone, dried, and solubilized in 50ul sample buffer (0.07M Tris HCL, pH 6.8, 10% glycerol, 5% B-mercaptoethanol, 3% SDS, 0.01% bromophenol blue) and samples containing equal cpms were applied to a 10% SDS-PAGE gel based on the procedure of Laemmli, (1970) and analyzed. After electrophoresis, gels containing [35S]-methionine labeled material were soaked in 1M sodium salicylate, pH 5.0-6.0 (Chamberlain, 1979) and gently shaken for 30 min The gel was then rinsed briefly with distilled water and placed on a 3mm Whatman filter paper and dried. The dried gel was exposed to a Fuji-XR film for an appropriate time interval. Radioactivity present was analyzed and quantitated either by direct radioactive measurement of gel slices and/or quantitated using an LKB densitometer.
Preparation of Cell Lysates

Cells, either metabolically labeled or unlabeled were washed 3x with cold PBS, harvested by using a rubber policeman and stored at -20°C or were lysed immediately in RIPA buffer, clarified by centrifugation and stored.

Immunoprecipitation of pp69 from Conditioned Media and Cell Lysates

Polyclonal antibodies directed against pp69 were prepared in our lab by Dr. 1. Chackalaparampil using concentrated conditioned medium or pp69 eluted from gel slices. The samples were mixed with Freunds adjuvant and injected into rabbits as previously described (Senger, et.al., 1979). For immunoprecipitation of radiolabeled proteins, cell lysates containing equal number of cpm's were incubated for 2 hr at 4°C with 30-50ul of antibody and equal volumes of RIPA. Normal rabbit serum was used in control experiments. The resulting immune complexes were collected by adding an excess (35ul of a 50% slurry in RIPA buffer) of protein A-Sepharose (Pharmacia) to the reaction mixture. The adsorbed complexes were washed 3x with RIPA buffer, then washed 2x with PBS. The complexes were subsequently suspended in 50ul of sample buffer and analyzed by SDS-PAGE followed by fluorography. Gels were rehydrated in ddH2O. To ensure that equal amounts of protein were loaded in each lane, gels were stained with Coomassie Brilliant Blue (see Fig. 1.7.A,R) (0 125% Coomassie Blue, 50% methanol, 10% acetic acid) for 12hr and destaining was performed in 50% methanol and 10% acetic acid for one hr, followed by destaining in a solution of 5% methanol and 7% acetic acid. Dowex basic ion exchange beads were used throughout the second destaining procedure.
Phospholipid Extraction and Analysis

This method was essentially carried out according to the method described by Vanier, et al., (1988). Two ml of methanol was added to each 1 ml of cells resuspended in PBS and vortexed vigorously. One ml of 2.4M HCl and 3 ml chloroform were added to the PBS: methanol solution vortexed and centrifuged at 1500 rpm for 5 min. The lower chloroform phase was removed. The upper aqueous phase was treated with an additional 2 ml of chloroform, vortexed and spun again. The chloroform phases were combined and re-extracted with 0.5 ml 2.4M HCl and 4 ml methanol-water (1:1 v/v). This was vortexed and spun as before. The lower chloroform phase was removed, placed in a test tube and evaporated in a warm water bath under a stream of nitrogen gas until 10 ul remained. Samples containing equal cpm were spotted on a 0.25 mm silica gel plate which was developed in chloroform/methanol/acetic acid/water (65:43:1:3 v/v) saturated chamber. Iodine staining and exposure to Fuji-XR film showed the position of the separated phospholipids. Areas corresponding to various known phospholipids were scraped into scintillation vials and counted.

Phosphatase assays

NRK-49F cells were harvested either by lysis in RIPA or by scraping into 1 ml of PBS and the resulting mixture was stored at -20°C for no more than 2 days. Protein content of extracts was determined with the Coomassie blue dye-binding assay (Bradford, 1976). Cellular and extracellular phosphatase activity was measured by combining 100 ul extracts and 250 ul of substrate solution (10 mM p-nitro-phenylphosphate, 1 mM MgCl2 in 0.5 M Tris-HCl; pH adjusted to either 4.5, 7.4, or 10), and after 30 min at 37°C reactions were stopped by addition of 0.75 ml 0.25M
NaOH. The amount of p-nitro-phenol produced was determined by absorbance at 410nm. For comparison, alkaline phosphatase activity was also measured using the Sigma ALP assay containing 2-amino-2-methyl-1 propanol buffer, pH 10.3 (Sigma, diagnostics).

Dephosphorylation of pp69

Fifty ul of media containing $^{32}$P-labeled pp69 secreted from cells after various treatments were exposed to 1 unit of alkaline phosphatase (dissolved in phosphatase assay buffer containing 50mM Tris/HCl pH 7.4, 50mM NaCl, 2mM PMSF, 1 mg/ml trypsin inhibitor, and 100 Kallikrein inactivator units/ml of aprotinin) in an Eppendorf tube. The mixture was incubated at room temperature for the required lengths of time. Reactions were stopped by addition of 4 vol of cold (0°C) acetone and placing them on ice on a shaking platform. Precipitates were processed as previously described.

Two dimensional Analysis of Proteins

Analysis of secreted proteins in two dimensions was carried out according to the procedure of O'Farrell, (1975). To prepare isoelectric focussing, 5.5g urea, 1.33 ml of a 30% bis-acrylamide stock, 2ml of a 10% NP-40 stock, 1.97 ml H2O, 0.4ml ampholines pH 5-7 and 0.01ml ampholines pH 3-10 were added to a side arm flask. 10ul of ammonium persulfate was added and the mixture degassed under vacuum for 3 min followed by the addition of 7ul TEMED. The final solution was loaded into tubes and overlayed with 8M urea for 1-2 hr. The urea was replaced with lysis buffer (9M urea, 2% NP40, 1.6% and 0.4% pH 4-6, pH 3-10 ampholines respectively,
5% B-mercaptoethanol) and then overlayed with water. Water and lysis buffer were replaced with fresh lysis buffer. Tubes were filled with 0.02M NaOH. The lower buffer chamber was filled with 0.01M H₃PO₄. A pre-run at 400v for 15 min was carried out. Followed by a pre-run of 200v for 15 min, 300v for 30 min, and 400v for 30 min. Lysis buffer and NaOH were removed. Samples were loaded and overlayed. Then 0.02M NaOH was used to fill the chamber and the run was carried out at 800v for approximately 12-20 hr.

**Phosphoamino acid Analysis**

Phosphoamino acid analysis was carried out according to the method of O'Farrell, (1975) with some modifications. This analysis was done either by using [³²P]-labeled proteins from gel slices, or from acetone precipitated conditioned media. Proteins were hydrolyzed in 6N HCl at 110°C for 1 hr and the hydrolysates were resuspended in small volumes of distilled water, mixed with unlabelled phosphoamino acids, and then separated either by ascending thin layer chromatography using 0.05M NH₄OH, isobutyric acid (5:3), v/v, or by high voltage electrophoresis in pyridine, acetic acid and water (1:10:189).
Tryptic peptide analysis

Tryptic peptides were analyzed based on the methods of Beeman & Hunter, (1978) and Carlin & Knowles, (1984) with modifications. Acetone precipitated pp69 protein was washed with acetone. Proteins were pelleted by centrifugation in an Eppendorf microfuge for 3 min at 4°C and subsequently air dried. The dried pellet was dissolved in 150ul of chilled performic acid (30% H2O2, 98% formic acid [1:9] incubated for 1 hr at room temperature) and incubated for 2 hr at 0°C. The performic acid solution was diluted with 3 ml of water, frozen and lyophilyzed. The oxidized protein was digested with 30ug of L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) in 0.5ml of 0.05M NH4HCO3 for 18 hr at room temperature. A further 20ug of TPCK-trypsin was added for an additional 6 hr. The resulting soluble peptides were diluted with water, and lyophylized. This procedure was repeated twice. The peptides were then dissolved in 10ul of 1% ammonium carbonate pH 8.9 and applied onto 20x10cm cellulose plates. Electrophoresis was performed toward the anode in the first dimension in the presence of 1% ammonium carbonate at 800V for approximately 1 hr and was followed by ascending chromatography in the second dimension in n-butanol:pyridine:glacial acetic acid: H2O (75:50:15:60 v/v). The positions of radiolabelled tryptic peptides were determined by autoradiography of the dried plates. Plates were subsequently stained with ninhydrin. Isolated peptides were also run in one dimension on DEAE-cellulose plates in the presence of 1% ammonium carbonate pH 8.9 or pH 7.0 at 800V for approximately 30 min.
CHAPTER 1

Effect of Crude TGF (SGF), EGF, TGF-β, RA, and TPA on the Secreted and Phosphorylated Levels of PP69
Abstract

Our study shows that the secretion of a major glycosylated, phosphoprotein with a molecular weight of 69kDa (pp69) is a specific marker for non-transformed NRK-49F cells. Antibody raised against pp69 recognizes, in addition to pp69, another major phosphoprotein with a molecular weight of 62kDa (pp62) secreted by RR1022 and spontaneously transformed NRK-49F cells (spt-NRK-49F). Increased concentrations of TGF-β appears to eliminate the presence of pp62 in the secreted media of these spt-NRK-49F cells. Immunoprecipitation of total cell lysates from both [32P]-labeled NRK-49F and RR1022 cells with anti-pp69 antibody detected only pp69, suggesting that the presence of pp62 is a late event taking place either at the cell surface or in the media. Treatment of NRK-49F cells with EGF alone or with TGF-β, as well as addition of TPA or RA in combination with EGF and TGF-β, modulates the secretion and phosphorylation of pp69. TPA and EGF induce 3 internal peptides with molecular weights of 58, 54, and 44 kDa which may be pre-processed forms of phosphorylated (pp69) and unphosphorylated (np69) 69kDa protein. These results suggest that there is a relationship between pp69 and cellular proliferation.
INTRODUCTION

Mammalian fibroblast cells in culture secrete into their extracellular environment a wide variety of proteins (Nilsen-Hamilton & Hamilton, 1982, including a few major phosphoproteins (Gal & Gottesman, 1986, Hirata, 1981, Lee, et al., 1984). One of these phosphoproteins is a polypeptide with a molecular weight of approximately 62kDa, which has been shown to be associated with cell transformation (Senger, et al., 1979). We have previously reported that non-transformed normal rat kidney (NRK-49F) fibroblasts, as well as retinoic acid-treated RR1022 and SR-1T cells which reversibly acquire non-transformed phenotype, predominantly secrete a 69kDa phosphoprotein (pp69) (Chackalaparampil et al., 1985). Recently pp69 has been found to be immunologically related to osteopontin (Craig, et al., 1988) and is also immunologically related to the 62kDa phosphoprotein secreted by many transformed cells. In addition, we show that pp69 phosphorylation and secretion by NRK-49F cells is modulated upon treatment with various combinations of EGF, TGF-β, RA, and TPA.
RESULTS and DISCUSSION

Effect of SGF (cTGF) Treatment on the Secreted Protein Profiles of Normal Cells

In the initial stages of this work SGF (cTGF) - treatment was used to determine if this growth factor would have any effect on the secreted protein profile of mammalian cells in culture. More specifically, if cTGF would induce the secretion of a transformation-associated 62kDa phosphoprotein (Senger, et al., 1983; Senger & Perruzi, 1985)

Both NRK-49F cells and rat-1 (Giguere & Gospodarowicz, 1983) cells were treated with 10ug/ml heat-treated cTGF as described in Materials & Methods. This growth factor was able to affect both the morphology (Fig 1.1 1 A,B; Fig.1.1.2 A,B) and AIG (Fig.1.1 1 C,D, Fig 1.1.2 C,D) of these two cell lines analogous to the growth patterns seen with retrovirally transformed cells (Fig.1.1 3 A,B,C) (Delarco & Todaro, 1978). Untreated NRK-49F cells and Rat-1 cells were labeled with [35S]-methionine and the secreted proteins were isolated and run on an SDS-PAGE (Fig.1.2 A, lanes 1,2) for comparison with those from transformed cells (Fig.1.2 A, lanes 3,4,5,6). Among the several changes which can be seen, one alteration is readily identified: a 2-3 fold increase, as determined by densitometric tracings, of a 69kDa protein. This protein corresponds to the previously identified pp69 protein secreted by NRK-49F cells (Chackalaparamil, et al., 1985) As a result of the above observations, cTGF treatment was used in the presence of either [32p]-orthophosphate or [35S]-methionine and demonstrated that an approximately
Figure 1.1 Crude TGF (10ug/ml ) Treatment Alters the Cell Morphology and AIG of NRK-49F and Rat-1 cells.

Cells were treated with 10ug/ml of crude TGF as described in Materials and Methods. Treated cells were compared to normal and transformed controls to ascertain whether our cTGF samples were effective.

1 Panels; A) NRK-49F cells in monolayer, B) NRK-49F + 10ug/ml of cTGF,
C) NRK-49F cells in soft agar D) NRK-49F + 10ug/ml cTGF

2 Panels; A) Rat-1 cells in monolayer, B) Rat-1 + 10ug/ml cTGF
C) Rat-1 cells in soft agar, D) Rat-1 + 10ug/ml cTGF

3 Transformed controls; Panels; A) KNRK, B) KA31, C) B77-NRK
transformed controls demonstrate the similarity between AIG induced by cTGF with AIG of cells transformed by retrovirus
Figure 1.2.A Secreted protein changes in normal vs. transformed cells

Normal and transformed cells were labeled with $[^{35}S]$-methionine. Acetone precipitated secreted proteins were isolated and run on a 10% SDS-PAGE for comparison.

Lanes: 1) NRK-49F, 2) Rat-1, 3) B77-NRK, 4) RR1022, 5) KA31, 6) 3B11-1C

Figure 1.2.B Effect of 96 hr cTGF (10μg/ml) treatment on the secreted protein profiles of NRK-49F and Rat-1 cells

Both $[^{32}P]$ (lanes 1, 2, 3, 7, & 8) and $[^{35}S]$-labeled peptides (lanes 4, 5, 6, 9, & 10) were analyzed.

Lanes: 1) FaO, 2) NRK-49F, 3) NRK-49F +10μg/ml cTGF, 4) NRK-49F, 5) NRK-49F +10μg/ml cTGF, 6) FaO, 7) Rat-1, 8) Rat-1 +10μg/ml cTGF, 9) Rat-1, 10) Rat-1 +10μg/ml cTGF, M=$[^{14}C]$-labeled markers

Figure 1.2.C Effect of short and long term cTGF (10μg/ml) treatment on the secreted protein profiles of NRK-49F cells

$[^{35}S]$-methionine labeled proteins were run on a 7.5% SDS-PAGE.

Lanes: 1) NRK-49F, 2) NRK-49F +cTGF -24hr, 3) NRK-49F +cTGF -96hr, 4) NRK-49F +cTGF -120hr, 5) KNRK
3-fold increase in phosphorylated pp69 can be seen (Fig 1 2 B, lane 3) with the concomitant increase in unphosphorylated pp69 (np69) (Fig.1.2 C, lane 2,3,4). Although no induction of pp62 could be detected in the media of [32p]-orthophosphate labeled cTGF-treated NRK-49F and Rat-1 cells (Fig.1 2 B, lanes 3,8 respectively), the pp62 protein is seen in the secreted media of transformed control FaO cells (Fig.1.2.B,lane 1). Since no induction of pp62 could be observed with the addition of cTGF, the above observations suggest that induction of pp62 may take place prior to or at a point where the cell is irreversibly transformed or malignant (see Fig.2.7). It is well known that AIG changes and monolayer changes induced with cTGF are in fact reversible (Todaro & Delarco, 1980) and may explain the reason for the lack of pp62 induction. It can also be suggested that an increase in pp69 is an event prior to the induction of pp62 and may be a phenomena occurring as a result of growth stimulation.

Crude TGF-treatment of [35S]-methionine labeled NRK-49F cells for 24, 76 and 120 hr (Fig.1.2.C lanes 2,3,4) clearly increases the levels of np69 of these cell lines which, in comparison, does not appear to be induced by the addition of fresh serum (Fig.1.2. A,B,C, lane 1,4,1 respectively). However, a lower molecular weight protein (64-68kDa) was induced. Although the significance of this is not clear at present, it appears that this lower molecular weight protein may be another form of pp69 (np69) (Fig.1.2 C lanes 2,3,4)

In response to these observations and suggestions, the increase in pp69 and np69 protein as a result of cTGF treatment of NRK-49F cells was analyzed in more detail by treatment of NRK-49F cells\footnote{Although these observations are applicable to both NRK-49F cells and rat-1 cells, the results obtained with NRK-49F cells appear to be cleaner. Therefore this system was used, predominantly, for all future work} with two purified components known to be present in cTGF: TGF-β (Roberts, et al., 1983) and EGF (as a substitute for the EGF-1
like molecule TGF-α) (Marquardt, et al., 1983). This was done in an attempt to determine the roles of each of these components, as well as their interaction with respect to pp69 and growth control.

Epidermal and Transforming Growth Factors Modulate Secretion of a 69kDa Phosphoprotein In Normal Rat Kidney Fibroblasts

One problem with working with rodent cells in culture is that they undergo spontaneous transformation at an extremely high rate (Marsh, 1984). Therefore, non-transformed NRK-49F cells were characterized by their inability to grow in soft-agar (Macpherson & Montagnier, 1964), and their lack of phosphorylation of a group of low molecular weight membrane proteins (Banerjee, et al., 1986). These non-transformed cells secrete a major phosphoprotein with a molecular weight of 69kDa (pp69) (Fig. 1.4 1.A, lane 1; Fig. 1.4 2.B, lane 1). Densitometric tracings of the autoradiograms revealed that treatment of NRK-49F cells with 5ng/ml of EGF, which was found to be mitogenic for these cells (Van Zoelan, et al., 1985) (Fig. 1.3.B), caused an approximately 3-fold increase in the levels of [32P]-labeled pp69 (Fig. 1.4 1.A, lane 4), as compared to untreated cells. On the other hand, TGF-β, a known inhibitor of cell proliferation (Roberts, et al., 1985a), at concentrations of 0.1 and 1 ng/ml caused 2- and 4-fold decrease respectively, of the [32P]-labeled pp69 (Fig. 1.4 1.A, lane 2; Fig. 1.4 1.B, lane 2) relative to levels observed for untreated cells.

This putative growth related modulation of the levels of [32P]-labeled pp69 was further tested by treating NRK-49F cells with both TGF-β and EGF. Simultaneous treatment with 0 1 ng/ml of TGF-β plus 5 0 ng/ml of EGF, which induced colony formation of NRK-49F cells in soft agar (Fig. 1.3.A) caused a 3-fold
**Figure 1.3** Synergistic interaction between EGF and TGF-β: differences in AlGand monolayer growth.*

A) The colony forming ability of various combinations of EGF and TGF-β was analyzed. The % NRK-49F cells forming colonies refers to the % of colonies greater than or equal to 30μm² in relation to B77-NRK or KA31 transformed controls which under the conditions of these experiments achieve approximately a 30% colony forming efficiency.

[Insert] = Colony formation: NRK-49F cells treated with 0.1ng/ml TGF-β + 5ng/ml EGF.

**symbols:** small black diamonds, d10MEp8; open circles, TGF-b treated NRK-49F cells; open diamonds, EGF treated NRK-49F cells; closed triangles, 10ng/ml EGF + x[TGF-β]**; open triangles, 50ng/ml EGF + x[TGF-β]; closed star, 5ng/ml EGF + x[TGF-β].

B) Monolayer growth analysis of NRK-49F cells in the presence of purified EGF and TGF-β.

**symbols:** open circles, NRK-49F; open diamond, 1ng/ml TGF-β; open square, 0.1ng/ml TGF-β; closed diamond, 0.1ng/ml TGF-β + 5ng/ml EGF; small closed circle, 50ng/ml EGF; open triangle, 5ng/ml EGF; closed triangle, 10ng/ml EGF.

* open circles at the 0 time point are the NRK control samples

** constant EGF concentration with variable TGF-β concentrations x[TGF-β].
Figure 1.4.1 Analysis of $^{32}\text{P}$-labeled secreted pp69 by SDS-PAGE.

A and B, acetone precipitated secreted proteins; C, immunoprecipitated secreted proteins and total cell lysates. All treatments were for 96 hr.

A) lanes: 1) NRK-49F untreated, 2) NRK-49F +1ng/ml TGF-β, 3) NRK-49F +1ng/ml TGF-β + 5ng/ml EGF, 4) NRK-49F +5ng/ml EGF.

B) lanes: 1) NRK-49F untreated, 2) NRK-49F + 0.1ng/ml TGF-β,

3) NRK-49F + 0.1ng/ml TGF-β + 5ng/ml EGF.

C) lanes: 1) NRK-49F untreated, 2) NRK-49F + 0.1 ng/ml of TGF-β + 5 ng/ml EGF.

3) NRK-49F + 0.1 ng/ml TGF-β + 50 ng/ml EGF, 4) RR1022 cells

Figure 1.4.2 Analysis of $^{35}\text{S}$-methionine labeled secreted pp69 by SDS-PAGE.

A, acetone precipitated secreted proteins; B, immunoprecipitated secreted proteins. All treatments are for 96 hr.

A) lanes: 1) NRK-49F +50 ng/ml EGF, 2) NRK-49F +0.1 ng/ml TGF-β + 50 ng/ml EGF,

3) NRK-49F +0.1 ng/ml TGF-β + 5ng/ml EGF, 4) NRK-49F +0.1 ng/ml TGF-β,

5) NRK-49F untreated.

B) lanes: 1) NRK-49F untreated , 2) NRK-49F +0.1 ng/ml TGF-β +5 ng/ml EGF, 3) NRK-49F +0.1 ng/ml TGF-β +50 ng/ml EGF.
increase in the levels of $^{32}\text{P}$-labeled pp69 (Fig.1.4.1.B, lane 3) as compared to untreated cells. When the concentration of TGF-β was increased 10-fold (1.0 ng/ml) but the EGF concentration remained the same (5.0 ng/ml), an 18-fold decrease of $^{32}\text{P}$-labeled pp69 was observed (Fig.1.4.1.A, lane 3). These observations were further confirmed by immunoprecipitation of $^{32}\text{P}$-labeled secreted proteins with a polyclonal anti-pp69 rabbit antibody (Fig.1.4.1.C). Phosphoamino acid analysis of pp69 secreted by both untreated and EGF plus TGF-β treated NRK-49F cells as well as of pp62 secreted by RR1022 and KNK cells revealed that only serine residues are phosphorylated (not shown).

Immunoprecipitation of secreted phosphoprotein from RR1022 cells with anti-pp69 antibody showed two major phosphoprotein bands, one with a molecular weight of 69kDa (pp69) and the other with 62kDa (pp62) (Fig.1.4.1.C, lane 4). This lower molecular weight phosphoprotein has been shown to be a specific marker for most transformed cells (Senger, et al., 1979; Chackalaparampil, et al., 1985; Senger & Perrizi, 1985). It is apparent, therefore, that the 62kDa transformation associated phosphoprotein is immunologically related to pp69. Peptide mapping of partially digested pp69 and pp62 with V8 protease, carried out in a previous study, showed considerable fragment homology (Chackalaparampil, et al., 1985). Immunoprecipitation of $^{32}\text{P}$-labeled total cell lysates from both non-transformed NRK-49F and transformed RR1022 cells showed only pp69 (not shown).

To determine whether the increased levels of $^{32}\text{P}$-labeled pp69 found in the conditioned media of EGF and TGF-β treated cells was due to increased secretion or phosphorylation of this protein, secreted proteins were labeled with $^{35}\text{S}$-methionine and analyzed (Fig.1.4.2.A). It can be seen that treatments of NRK-49F
Figure 1.5. Time-course study of the effects of EGF treatment (50 ng/ml) on the secretion of S^{35} labelled pp69 by NRK-49F cells.

A) lanes: 1-5 acetone precipitated secreted proteins; lanes 6-10, immunoprecipitated (autoradiograms shown) secreted proteins. Lanes: 1) 0 hr, 2) 4 hr, 3) 48 hr, 4) 96 hr, 5) 120 hr, M) markers, 6) 0 hr, 7) 4 hr, 8) 48 hr, 9) 96 hr, 10) 120 hr

B) radioactivity (cpm) present in pp69 band from samples taken at various time points after EGF treatment.

C) typical SDS-PAGE pattern of immunoprecipitated pp69 taken at various time points after EGF treatment. The numbers under each band represents hours of EGF treatment. Arrows indicate the position of pp69.
cells with 50ng/ml of EGF, 0.1ng/ml TGF-β +50ng/ml of EGF, and 0.1ng/ml of TGF-β +5ng/ml of EGF, showed significant increase in the amount of labeled pp69 (Fig 1.4.2 A, lanes 1, 2 & 3 respectively).

Treatment of NRK-49F cells with 0.1ng/ml of TGF-β alone caused no apparent change in the radioactivity associated with pp69 (Fig 1.4.2 A, lane 4) as compared to untreated cells (lane 5). This is in conformity with the results obtained by Nakamura, et al., (1985) using primary adult rat hepatocytes.

Immunoprecipitation of [35S]-methionine labeled pp69 with an anti-pp69 antibody which has been shown to also bring down fibronectin (Nemir & Mukherjee, 1988) (see Fig 1.4.2 B lanes 1-3) confirmed that treatment of NRK-49F cells with EGF and TGF-β significantly increases the secretion of pp69 (Fig 1.4.2 B). These observations suggest that EGF and TGF-β treated cells secrete more pp69 than their untreated counterparts. Although there is an increase in secretion, phosphate site alterations can also be seen using 2-D tryptic digests as well as mobility changes on DEAE-cellulose of pp69 peptides from NRK-49F cells treated with 5ng/ml EGF and 0.1 ng/ml TGF-β (Fig 1.8 A,B). Only a small number of sites within pp69 are phosphorylated which resembles a characteristic seen with osteopontin (Mark, et al., 1987).

Important, since EGF treatment alone caused increased secretion of pp69 by NRK-49F cells (Fig 1.4.2 A, lane 1), we determined the length of EGF treatment needed for such an increase. Initially, long- and short-term treatments were carried out. Treatment of NRK-49F cells with EGF for 48hr, 96hr and 120hr significantly increased the secretion of pp69 (Fig 1.5 A, lanes 3-5). A 4hr EGF treatment did not increase secretion of pp69 (Fig 1.5 A, lane 2). Analysis of immunoprecipitated pp69 confirmed the above results (lanes 6-10). In order to determine the exact length of EGF-treatment needed for the increased secretion of
pp69, the time course study was extended to include time points between 4 hr and 48 hr. As can be seen in Fig. 1.5.B and 1.5.C, a significant increase in the levels of pp69 could be detected between 4 hr and 6 hr of EGF treatment, and a maximal level was reached at approximately 48 hr. Estimation of radioactivity in pp69 bands taken from four gels from two separate experiments confirmed the above observation (Fig. 1.5.B). Frequently two bands can be resolved in these gels which are thought to represent either the phosphorylated and non-phosphorylated forms of the protein or unphosphorylated pp69 and pp62. Some of the above results were again duplicated using rat-1 cells (Fig. 1.7.C). Matrisian, et al., (1985a,b) have found a secreted 60kDa doublet in EGF-induced rat-1 cells which appears to resemble the pp69 and np69 profile and clearly resembles the Rat-1 EGF-induced pp69 doublet (Fig. 1.7.C, lane 1,2).

Two-dimensional electrophoretic analysis of [35S]-methionine labeled secreted proteins demonstrated that two distinct 69kDa proteins are in fact excreted by NRK-49F cells, the phosphorylated form (pp69) which has a pI of approximately 3.8, and a less acidic non-phosphorylated protein (np69) (Fig. 1.6.A). Secretion of both of these proteins were increased upon treatment with 5 ng/ml of EGF plus 0.1 ng/ml of TGF-β. This secretion could not be inhibited with 4 hr actinomycin D treatment, but could be completely inhibited by 4 hr cycloheximide treatment (Fig. 1.9.B, lanes 5,6). Correspondingly, phosphorylation of pp69 could also be inhibited by cycloheximide (not shown). Treatment with ammonium chloride which inhibits degradation of lysosomal proteins affected a 39kDa protein thought to be MEP (Gal, et al., 1985) (Fig. 1.9.B, lane 7) but had no effect on pp69 suggesting that it is not a lysosomal protein.
Figure 1.6.A Two-dimensional gel analysis of $[^{35}\text{S}]$-methionine (a and b) and $[^{32}\text{P}]$-orthophosphate (c and d) secreted proteins;

Panels a, c) NRK-49F untreated, b, d) NRK-49F +0.1 ng/ml TGF-β +5 ng/ml EGF. Small arrow indicates pp69 and large arrow head indicates the non-phosphorylated 69kDa protein.

Figure 1.6.B Analysis of immunoprecipitated $[^{32}\text{P}]$-labelled secreted proteins from partially transformed NRK-49F cultures and RR1022 cells.

Lanes: 1) NRK-49F untreated, 2) NRK-49F +0.1 ng/ml TGF-β +5 ng/ml EGF, 3) NRK-49F +1 ng/ml TGF-β +5 ng/ml EGF, 4) RR1022, acetone precipitated control
Figure 1.7 Coomassie blue stained gels

Gels which were used for fluorography were rehydrated and stained with coomassie blue as described in Materials and Methods to demonstrate that lanes were loaded with equal amounts of protein. Portions of A & B correspond to the gels used for previous figures (Fig.1.4, 1.5, 1.9C).

A) Immunoprecipitated intracellular pp69 after 50ng/ml EGF treatment. lanes, 1) 120hr, 2) 96hr, 3) 48hr, 4) 4hr, 5) 0hr, n=normal rabbit serum, Immunoprecipitated secreted pp69 lanes, 6) 120hr, 7) 96hr, 8) 48hr, 9) 4hr, 10) 0hr, m=markers, Acetone precipitated pp69 lanes, 11) 120hr, 12) 96hr, 13) 48hr, 14) 4hr, 15) 0hr

B) Immunoprecipitated secreted pp69 lanes, 1) NRK-49F, 2) NRK-49F +0.1ng/ml TGF-β +5ng/ml EGF, 3) NRK-49F +1ng/ml TGF-β +5ng/ml EGF, 4) KNRK, 5) NRS, 6) KNRK.

C) Effect of 5ng/ml EGF treatment on Rat-1 secreted pp69. 

$[^{32}P]$-orthophosphate labeled lanes 1) Rat-1, 2) Rat-1 +5ng/ml EGF, $[^{35}S]$-methionine labeled; (immunoprecipitated) lanes, 3) Rat-1, 4) Rat-1 +5ng/ml EGF, (acetone precipitated) lanes, 5) Rat-1, 6) Rat-1 +5ng/ml EGF.
Figure 1.8 Tryptic digests

A) Tryptic digests were run on cellulose acetate sheets in two dimensions for a comparison between pp69 secreted by untreated NRK-49F cells and pp69 secreted by these cells treated for 96 hr with 5 ng/ml EGF + 0.1 ng/ml TGF-β.

B) Tryptic digests were also run on DEAE cellulose and the corresponding Rf values analyzed. A change in the sites of phosphorylation and/or the number of phosphate groups attached corresponds to a change in Rf.
Mobility - DEAE cellulose TLC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rf (cm)</th>
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<tbody>
<tr>
<td>NRK</td>
<td>11.5</td>
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<tr>
<td>+ 5ng/ml EGF, 0.1ng/ml TGF-b</td>
<td>10.4</td>
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Following prolonged culture of NRK-49F cells, a proportion of the cell population undergoes spontaneous transformation. As a result, significant levels of pp62 could be detected in the conditioned media of these cultures (Fig.1.6 B, lane 1). It is clear, therefore, that the secretion of pp69 can be used as a specific marker for non-transformed NRK-49F cells. Treatment of NRK-49F cultures containing spontaneously transformed cells with 5ng/ml EGF + 0.1ng/ml of TGF-β, caused an increase in the amount of both [32P]-labeled pp69 and pp62 (Fig.1.6.B lane 2). When these cultures were treated with 1ng/ml of TGF-β +5ng/ml EGF, the level of radioactivity in the pp69 band significantly decreased, and pp62 was undetectable (Fig.1.6.B lane 3). Ne\man, et al., (1986) determined that NRK-PT14, a partially transformed cell line, also exhibited an altered response to TGF-β as compared to normal cells. These results show that EGF and TGF-β modulate the secretion of both pp69 and pp62.

RA and TPA Act in Combination with TGF-β and EGF in Affecting the Secretion and Phosphorylation of pp69

Cellular transformation has been shown to be coordinated by the action of several peptide growth factors in NRK cells (Assoian, et.al., 1984b). Observations by Van Zoelan, et al., (1986) suggested that RA (Jetten, 1984), which by itself is mitogenic for NRK-49F cells, can induce AIG in the presence of EGF without the addition of TGF-β. TPA has also been shown to stimulate AIG and arachidonic acid metabolism (Levine, et al., 1985) in the presence of EGF and TGF-β of NRK-49F cells synergistically (Kraft, et al., 1986). Therefore, it is of interest to determine whether our previous observations involving pp69 secretion and phosphorylation can also be extended using RA, which appears to have TGF-β like
properties (Roberts & Sporn, 1984), and TPA. This may help to further elucidate the roles of EGF and TGF-β in relation to growth, AIG and pp69.

Prior to work on the secreted form of pp69, it was of interest to determine whether any intracellular peptides could be induced by TPA (a potent phorbol ester) and EGF which would be common to both. Both TPA (Fig. 3 I C lane 3,4) and EGF (Fig. 3 I C lane 6-9) did induce three major intracellular peptides with molecular weights of 58, 54 and 44 kDa. It can be suggested that since anti-pp69 Ab cross-reacts strongly with these three internal peptides that they could be pre-processed forms of pp69 as well as np69 and further that these may somehow also give rise to pp62. Subsequent to this study two secreted proteins with molecular weights of 68 & 64 kDa (Chan, et al., 1986) called transformation associated proteins (TAP,s) were described using an NRK subclone. Using a monoclonal Ab to these two proteins it was found that this Ab cross-reacted with three internal peptides of lower molecular weights, 66, 63 & 60 kDa. It was suggested that these internal peptides were preprocessed forms of the TAP proteins. Preliminary work using a monoclonal Ab obtained from Dr. Chan does not cross-react with either pp69 or pp62 (not shown).

Phorbol esters have been shown to alter morphology, increase saturation density, enhance growth of certain tumor cells in soft agar (Weinstein, et al., 1979), and cause the disassembly of the cytoskeleton (Reskin, et al., 1979, Antecol & Mukherjee, 1982). TPA (2 x 10^-7 M) in combination with EGF and TGF-β enhances approximately 3-fold the secreted levels of pp69 (Fig 19 B, lane 3). However, no increase in unphosphorylated levels of pp69 can be seen upon treatment with TPA alone (Fig. 19 B, lane 4). This appears to correspond to the observation made by Kraft, et al. (1986) that TPA alone could not induce AIG unless cells had previously been treated with a transforming agent (Fisher, et al., 1979). In
contrast, TPA treatment alone increases the phosphorylated levels of pp69 and in some cases induce pp62 (Petney, unpublished observations).

RA treatment of NRK-49F cells, unlike TGF-β, can be shown to induce enhanced phosphorylation of pp69 (Fig.1.9.A lane 2) but does not induce the secreted form of np69 (Chackalaparampil, unpublished results). As well, addition of RA, in combination with EGF and TGF-β, to cells significantly increases (4-fold) the phosphorylated levels of this protein (Fig.1.9.A, lane 5) over control levels (Fig.1.9.A lane 3) and 2-fold over levels induced by EGF and TGF-β (Fig.1.9.A lane 4). In contrast, the unphosphorylated levels of pp69 are non-cooperatively modulated by RA in the presence of EGF and TGF-β (Fig.1.9.A, lane 8) therefore one would not expect any induction of any intracellular peptides related to pp69.

It has recently been shown that transformation of NIH-3T3 cells with H-ras results in a large increase in 2AR/pp69 protein and mRNA expression (Craig, et al., 1988). Since pp69 is now considered to be a fibronectin binding osteopontin-like protein, it is possible that dephosphorylation of pp69 in the presence of TGF-β is a prerequisite for this molecules (pp69) ability to assemble exogenous fibronectin into fibrils and increase cell surface binding (Ignatzi & Massague, 1986; Allen-Hoffman, et al., 1988). My preliminary work with the addition of exogenous fibronectin to the media of NRK-49F cells suggests that the addition of fibronectin induces a reduction in the phosphorylated pp69 protein (not shown). It is possible that the above results are somehow related to TPA and RA's ability to cause breakdown of the extracellular matrix (Lotan, 1980) which in turn could influence the rate of proliferation of these cells.

Despite earlier observations that an increase in phosphorylation of pp69 may simply represent an increase in the amount of pp69 present in the media these results suggest that phosphorylation of pp69 itself may be an important...
Figure 1.9 RA and TPA Act in Combination with TGF-b and EGF in Affecting the Secretion and Phosphorylation of pp69

A) Analysis of $^{32}$-labeled (1-5) and $^{35}$-labeled (6-9) pp69 as a result of RA's $(1 \times 10^{-7} \text{M})$ interaction with $5 \text{ng/ml EGF} + 0.1 \text{ng/ml TGF-β}$


B) Analysis of $^{35}$S-labeled pp69 as a result of TPA's $(2 \times 10^{-7} \text{M})$ interaction with $5 \text{ng/ml EGF} + 0.1 \text{ng/ml TGF-β}$

lanes, 1) NRK-49F, 2) NRK-49F +EGF +TGF-β, 3) NRK-49F +EGF +TGF-β +TPA, 4) NRK-49F +TPA, 5) NRK-49F +EGF +TGF-β +2ug/ml actinomycin D (4hr), 6) NRK-49F +EGF +TGF-β +25ug/ml Cycloheximide (4hr), 7) NRK-49F +EGF +TGF-β +20mM NH₄Cl

C) Immunoprecipitated pp69 from $^{35}$S-labeled lysates after TPA (1-4) and EGF (5-9) treatments.

lanes, 1) NRK-49F, 2) NRK-49F +EGF +TGF-β, 3) NRK-49F +EGF +TGF-β +TPA, 4) NRK-49F +TPA, 5) NRK-49F, 6) 4hr, 7) 48hr, 8) 96hr, 9) 120hr
regulatory mechanism involved in the coordinate response of these cells to various growth stimuli.\(^2\)

\(^2\) The work with TGF-β, EGF, RA, and TPA have raised several interesting points. However, it is not feasible to cover all of these aspects and remain within the scope of a Masters thesis. Therefore, I have chosen to focus exclusively on the effects of EGF on the phosphorylation of pp69 for the latter part of this study (Chapter 2).
CHAPTER 2

Cell Proliferation and Extracellular Phosphatase Activity EGF-Induced
Regulation of the Phosphorylation of a Secreted 69kDa Osteopontin-like
Phosphoprotein
ABSTRACT

Non-transformed NRK-49F cells secrete a major, 69kDa phosphoprotein, which is phosphorylated at serine residues and is increased approximately 3-fold after 96 hr of EGF treatment (Laverdure, et al., 1987). In providing further support for pp69's involvement as an extracellular growth regulator, we report that EGF-induced increase of secreted [32P]-labeled pp69 reaches its highest level at approximately 12 hr post-EGF treatment, with no demonstrable change in the pattern of pp69 secretion after EGF treatment. This peak is accompanied by a pronounced 66% (p<0.02) reduction in secreted ALP activity as well as a reduction in secreted protein phosphatases. Intracellular ALP and protein specific phosphatase activity do not correspond with the intracellular pp69 peak reached at 6 hr post-EGF treatment, but do correspond to the secreted pp69 peak. Treatment of NRK-49F cells with 5uM spermine, known to activate phosphatase activity, inhibit SGF-induced AIG (Frolik, et al., 1984), and decrease certain phosphoproteins in hepatocytes (Auberger, et al., 1984) inhibits 2-fold the phosphorylated pp69 species and also causes a 50% reduction in cell proliferation. Insulin (20ug/ml) which activates protein phosphatases (Londos, et al., 1986) also causes a 50% reduction in the phosphorylation of pp69. Levamisole (0.01 & 0.1mM), an ALP inhibitor and PPA (0.1mM), a specific protein phosphatase inhibitor enhance cell proliferation by 25% and 50% respectively, similar to levels of enhanced cell proliferation obtained with EGF alone EGF and levamisole treated pp69 protein demonstrate increased ALP sensitivity which is likely due to altered protein conformation as compared to pp69 from insulin and untreated NRK-49F cells. We suggest that EGF induces growth and affects morphology in part by affecting the interaction between secreted phosphatases and the pp69 osteopontin-like protein.
INTRODUCTION

The focus in growth regulatory peptides has shifted to the extracellular media (LeCam, et.al., 1985; Kurachi, et.al., 1985) and to the identification of proteins whose phosphorylated pattern is altered by various agents (Adlakha, et al., 1985). For example, EGF affects both the phosphorylation of a number of proteins (Carpenter & Cohen, 1984, Martin-Perez, et.al., 1984, Guigni, et al., 1985, Sahai, et.al., 1986) and stimulates the secretion of a wide variety of proteins which may have roles in forming the basis of an extracellular matrix or in regulating growth (Hirata, et.al. 1982, Matrisian, et al., 1985a, Massague, 1987)

Growth control of animal cells remains poorly understood and is an important link in understanding the pathogenesis of neoplasia. Although the role of soluble and membrane bound phosphatases in growth regulation is also unclear, this class of enzymes do appear to be hormonally controlled (Olsson & Belfrage, 1987, Toth, et al., 1988). For example, ALP activity in ROS 17/2 cells is affected by PTH and isoproterenol (Majeska & Rodan, 1982). It also appears likely that phosphatases such as the secreted forms of ALP (Orthophosphoric monoester phosphohydrolase E.C. 3.1.3.1) and protein phosphatase (more specifically, serine specific phosphatase) could affect the regulation of key extracellular phosphoproteins (Ballou & Fisher, 1986; Cohen, 1982). Prior to this study, analysis of phosphatase activity involved the use of cell extracts which would contain membrane and some soluble forms of ALP. A relationship between secreted phosphatases and any secreted peptides, with respect to proliferative control have yet to be analyzed

We have previously reported that secretion of a major 69kDa phosphoprotein, identified as an osteopontin-like molecule (Craig, et al., 1988), is a
specific marker for non-transformed NRK-49F cells and that treatment of these cells with EGF modulates the secreted levels of this protein; suggesting a relationship with cellular proliferation (Laverdure, et al., 1987) Here we describe an effect of EGF on secreted phosphatases which in turn appear to affect the phosphorylation and conformation of pp69, a key extracellular peptide.
RESULTS

EGF-induced $[^{32}]$-Orthophosphate incorporation into pp69

Non-transformed NRK-49F cells, characterized by their inability to grow in soft agar, and lack phosphorylation of a group of low molecular weight membrane proteins (Banerjee, et.al., 1986), secrete a major phosphoprotein of 69kDa (pp69) pI 3.8 (Laverdure, et.al., 1987) which accounts for approximately 30% of the phosphate (Beckman, 1988) incorporated into all other precipitable material. This protein is presumably also present in normal rat and human plasma (Senger, et.al., 1988). It has previously been demonstrated that EGF treatment of NRK-49F cells for 96 hr with concentrations known to be mitogenic caused a 3-fold increase in $[^{32}]$P-labeled pp69 present in the secreted media which is accompanied by an increase in $[^{35}]$S-methionine labeled pp69 (np69) (Laverdure, et al., 1987).

Although phosphorylation of pp69 can largely be accounted for by an increase in secretion with a constant ratio of approximately 2, the $[^{32}]$P/$[^{35}]$S ratio (Nagata & Yamada, 1986) demonstrates that phosphorylation of pp69 reaches a maximum at 12hr post-EGF treatment (Table 1). Therefore it appears that pp69 is also regulated by a phosphorylation and dephosphorylation type mechanism. A time-course analysis of EGF treated NRK-49F (passage 23) cells was carried out (Fig 2.1A) and no increase in $[^{32}]$P-orthophosphate could be detected within the first 6 hours of treatment. However, a peak of phosphorylated pp69 occurred at 12 hr post-EGF treatment (Fig. 2.1A) in accordance to the data presented in Table 1. This is not likely due simply to transition through the cell cycle since the same peak did not occur again either at 24 or 48 hr post-treatment. Subsequent to the 12 hr peak, phosphorylated pp69 levels decreased and reached an apparent steady
Table 1 Time-course of EGF-induced pp69 Secretion: $^{32}\text{P}/^{35}\text{S}$ Ratio.

Areas of dried gels corresponding to pp69 from no less than 3 gels for any one time point were counted. The resulting counts were compared and the ratios tabulated. The ratios presented for the 0hr time point represents the steady state levels of pp69 and np69 in NRK-49F cells. All time points analyzed show similar steady-state ratios (no significant deviation from stated values were found). 12hr post-EGF treatment of these cells clearly show an increase in the steady-state ratio. This indicates that an alteration in the incorporation of phosphate into pp69 has occurred possibly due to a reduction in secreted phosphatase activity.
Table 1: Time-course of EGF-induced pp69 Secretion

<table>
<thead>
<tr>
<th>EGF treatment (5ng/ml)</th>
<th>0</th>
<th>4</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>32P/35S ratio</td>
<td>2.1</td>
<td>2.1</td>
<td>1.7</td>
<td>3.4</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Figure 2.1. Time-course study of the effects of 5ng/ml EGF treatment on the phosphorylation of pp69

A) cpm recovered from gel slices versus EGF treatment. Average of three experiments.

(Inset) - autoradiogram representation of time-course analysis of [32P]-labeled pp69 as a result of EGF treatment.

B) Bar graph representation of secreted protein phosphatase (light-hatched) and secreted ALP (dark-hatched) activity: analysis of EGF treated NRK-49F cells. Average of three experiments.

C) Bar graph representation of secreted ALP activity. Comparison between untreated (white) and 12hr (hatched) EGF treated NRK-49F cells, n=9, p<0.02.
state level similar to that seen after 96 hr EGF treatment (Laverdure, et al., 1987).

Effects of EGF on Phosphatase Activity in the Secreted Media

Chan & Stinson's (1986) previously observed that increased $[^{32}\text{P}]$-orthophosphate incorporation into membrane proteins is due to inhibition of ALP. As well, Canalis, (1983) observed that in fetal rat calvarial cells 24 and 96 hr EGF treatment resulted in the inhibition of cellular ALP. Since EGF treatment is able to induce a relative increase in the phosphorylated levels of pp69 in the media, which could be due to a number of factors including increased kinase activity inside the cell, increased secretion, or a decrease in phosphatase levels outside the cell, EGF treated NRK-49F cells were analyzed relative to their secreted phosphatase activity (Fig. 2.1.B).

A 12 hr EGF-treatment causes a 70% reduction in the ALP activity and a 30% reduction in prot/ser specific phosphatase activity (prot/ser-P) which corresponds to the 12 hr peak in phosphorylated pp69 levels (Fig. 2.1.B,C). The 70% decrease in ALP levels after 12 hr of EGF treatment was not dependent on the ALP buffer system used since these results could also be achieved by using a different buffer system (Fig. 2.1.C; Fig. 2.2.B) as described in Materials and Methods. The reason for an elevation in phosphatases seen to take place approximately 8 hr prior to this 12 hr drop in activity and its possible effect on pp69 is unclear. However, it can be suggested that this increase in ALP activity is to maintain a constant pp69/pp69 ratio until EGF has been present for approximately 12 hr.

Overall, it appears that this decreased ALP activity and to a lesser degree prot/ser-P activity could affect pp69 protein in response to EGF-induced mitogenesis. Acid phosphatase (AcP) activity was low in comparison to both ALP and prot/ser-P
Figure 2.2 Time-course study of the effects of 5ng/ml EGF treatment on intracellular phosphatase activity

A) Time-course study of the effects of EGF treatment on the intracellular protein (dark-hatched bars) and ALP (lighter-hatched bars) activity of NRK-49F cells.

{Insert} Immunoprecipitated intracellular pp69 levels after EGF treatment.

B) Further comparison of untreated (white) and 12hr (grey-hatched) EGF treated intracellular ALP activity: n=9, P<0.02.

C) Secreted (sec)(dark-hatched lines) and intracellular (lys) (grey-hatched)AcP activity; comparison of untreated and 12hr EGF treated cells. Numerical observations of ALP and AcP activity profiles were similar to previously reported activity profiles of lysate derived ALP and AcP for kidney tissue (Farley, et.al., 1987).
which is in agreement with Farley, et al., (1987) observation of ALP and AcP activity levels in kidney tissue. Although AcP activity decreased somewhat in the media of EGF-treated NRK-49F cells, this phosphatase did not appear to follow the EGF-induced phosphorylation peaks as is seen with prot/ser-P and ALP (Fig. 2.3C). However AcP activity did demonstrate an approximately 40% increase in the lysate, which suggests it may be regulated by EGF in order to dephosphorylate certain intracellular peptides but does not appear to have any significant relationship to pp69. Similarly, AcP (lysate-derived) activity has been shown to be increased after bovine bone extract treatment of chick bone cells (Werdegal, et al., 1988).

**Effect of EGF on the Secretion Profile of pp69**

Detectable levels of pp69 appear in the secreted medium in approximately one hour and this protein has a half life of approximately 40-50 min (Chackalaparampil, unpublished results). In the presence of EGF the phosphorylated level of pp69 is enhanced and may be due to an alteration of the secretion profile. Cells were labeled for two hr in the presence or absence of 5ng/ml EGF and chased with cold media. The results demonstrate that although EGF increased the overall incorporation of label into pp69, no alteration in the rate of pp69 secretion (Fig. 2.3) could be detected. As a control and to alter the pattern of pp69 secretion, pepstatin (10ug/ml), a carboxyl protease inhibitor which can inhibit the degradation of certain proteins, was added in the presence of EGF. The results of this treatment suggest that certain proteases may regulate directly or indirectly the secretion of pp69. Although no alteration the the rate of secretion
Figure 2.3 Secretion Profile of EGF treated NRK-49F cells

- open squares, NRK-49F cells; • closed circles, +EGF; □ dark squares, +EGF +10ug/ml pepstatin.

(Insert), autoradiogram representation of pulse-chase analysis. Cells were labeled for 2hr prior to chase to ensure that the specific activity of intracellular pp69 would be sufficiently high. Pepstatin was added to alter the secretion of pp69 as a control.
A

![Graph showing cpm recovered vs. time of chase (min)]

NRK

<table>
<thead>
<tr>
<th>min.</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
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<td></td>
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<td></td>
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</tbody>
</table>
takes place in the presence of EGF, it is clear that EGF treatment increases the overall levels of $[^{35}S]$-labeled pp69 (Laverdure, et al., 1987).

Growth Analysis of Treated NRK-49F Cells

If indeed EGF's mitogenic effects include a decrease in the secreted phosphatase activity, implying that these phosphatases play some role either in altering cell morphology and/or growth, then it would be of interest to determine whether cells treated with phosphatase inhibitors have growth rates similar to those seen with cells grown in the presence of EGF. Levamisole, an antihelmintic drug (Van Belle, 1972), is a potent and specific ALP inhibitor, while PPA is a potent protein phosphatase inhibitor. These inhibitors have previously been used at concentrations of 10mM in vitro (Chan & Stinson, 1986), which can be seen to inhibit cell growth (Fig. 2.4.A). At lower concentrations, both these inhibitors increased cell growth to levels similar to that seen with EGF-treated cells (Fig. 2.4 A). Furthermore, spermine, which is known to activate certain protein phosphatases (Auberger, et al., 1984; Ballou & Fisher, 1986), at 5uM is able to inhibit cell growth by 50%.

The phosphorylated levels of pp69 from cells treated with the phosphatase inhibitors PPA, and levamisole, as well as two activators of phosphatase activity, insulin and spermine were analyzed. While EGF increased the phosphorylation of pp69 by approximately 2-fold both levamisole and PPA increased the phosphorylated levels of pp69 by 2.2 and 2.5-fold respectively (Fig. 2.4.B). It has been demonstrated that an increase in cell growth of NRK-49F cells and an increase in AlG occurs in the presence of 10uM vanadate with a concomitant increase in phosphorylated pp69 present in the media (Nemir & Mukherjee, 1988). Interestingly, at low concentrations vanadate in the presence of zinc has been
Figure 2.4

A) Growth curve analysis of NRK-49F cells treated with phosphatase activators and inhibitors.

symbols = dark hatched bars, Levamisole (mM); light hatched bars, PPA (mM); grey colored, Spermine (uM); open triangles and solid line, 5ng/ml EGF. average of three experiments, n=9.

B) Effect of phosphatase inhibitors and activators on the phosphorylation of pp69 with the corresponding LKB densitometric and CPM ratios.

NRK-49F, NRK-49F +5ng/ml EGF, NRK-49F +0.1mM levamisole, NRK-49F +0.1mM PPA, NRK-49F +10ug/ml Insulin, 2) NRK-49F, NRK-49F +5uM spermine.

C) Comparison of Levamisole and PPA on both ALP and protein phosphatases.

symbols = grey colored bar graph, NRK-49F; hatched bar graph, +0.1mM PPA; open bar graph, +0.1mM Levamisole. Average of three experiments, n=9.

*% cell number = cell number as % of control
shown to inhibit ALP (Sparks & Brautigan, 1986) Although EGF and insulin share a number of metabolic actions (Blackshear, et al., 1984; LeCam, 1982), both spermine (5uM) and insulin (10ug/ml) decreased the phosphorylated levels of pp69 by 50% (Fig. 2.4.B), while spermine increased the levels of unphosphorylated pp69 (np69). The results obtained with Insulin treatment can be used as an argument to rule out any possible cytotoxic effects of spermine. Whether pp69 phosphorylation is affected specifically by ALP or prot/ser-P cannot be determined since both levamisole and PPA appear to have overlapping effects (Fig 2.4.C).

ALP Sensitivity of pp69 Secreted from EGF, Insulin and Levamisole Treated Cells

It has been shown that phosphatase treatment of neurofilament proteins affect their mobilities in SDS-gels indicating that a conformational change has most likely taken place (George, et al., 1986). If pp69 is being modified by certain phosphorylation and/or dephosphorylation events, it is probable that EGF or levamisole treatment would alter and/or change the number of sites of phosphorylation of pp69. One way to ascertain this is by analyzing the susceptibility of this protein to ALP activity after different treatment regimes. Increased or decreased susceptibility of pp69 to ALP implies that some modification of the protein (addition or removal of phosphate bonds) has occurred to make other phosphate sites more or less accessible to ALP.

Untreated NRK-49F cells secrete pp69 containing phosphorylated serine residues which appears to be relatively insensitive to ALP treatment (Fig 2.5). One unit of ALP treatment for 30 min. decreased the amount of phosphorylated
**Figure 2.5** ALP Sensitivity of pp69 from NRK-49F Cells after EGF, Insulin and Levamisole Treatment.

Cells were treated with either (5 ng/ml)EGF, Insulin (10 ug/ml), or levamisole (0.1 mM). The conditioned media from each were treated with 1 unit of ALP for varying lengths of time and pp69 was quantitated from gel slices and compared to untreated controls. (See Materials and Methods)

(Insert) autoradiogram representation of pp69 after treatment.

symbols - open squares, untreated NRK-49F; closed diamonds, +EGF; open diamonds, +Insulin; closed squares, +Levamisole.
TREATMENTS

NRK  EGF  LEVA

0  1  2  1  2  0  1  2

HRS

% pp69 remaining

20  40  60  80  100  120

0  20  40  60

ALP treatment (min.)
pp69 present in the media by 30%, whereas 5 min. ALP treatment of 12 hr EGF-treated cells results in a 63% reduction in phosphorylated pp69. In other words these cells secrete a pp69 protein with a dramatically increased ALP sensitivity. In support of these findings levamisole treated NRK-49F cells also secrete a pp69 protein with a similar ALP sensitivity to that of EGF induced pp69. Insulin which decreased the phosphorylated levels of pp69, did not cause an alteration in ALP sensitivity of pp69. Endogenous ALP present in the media during high levels of exogenous ALP treatment has little effect on the results of this assay, since it can be shown that media from EGF-treated cells contains much less ALP, but this pp69 is still more susceptible to exogenous ALP treatment than is control pp69. Prior work reveals that the tryptic peptide map of pp69 secreted by 96 hr post EGF and TGF-β treated NRK-49F cells has been altered in comparison to control pp69 (Fig.1.8.A), but without an increase in the number of sites. Therefore it is clear that certain phosphate site alterations do take place in this molecule. Further analysis of the tryptic peptide maps of pp69 from EGF and levamisole treated cells would be interesting to verify to what extent modifications of pp69 take place under these conditions.

LA-23 and KNRK Variants Demonstrate Altered: Morphology, ALP Activity and Phosphorylated PP69 Levels

KNRK cells were separated into two sub-populations based on morphological criteria (Fig. 2.6.1) (Noda M et.al., 1983). KNRKf (Fig. 2.6.1b) cells were selected on the basis of being more normal in appearance, i.e., large, flattened, and more adherent to the substratum. When the ALP activity present in the secreted media of these cells are compared to that of KNRKr (Fig. 2.6.1a) cells, which are smaller,
Morphologically altered KNRK cells (flat vs. round) were obtained through isolation by repeated gentle versene (tyrodes 100ml, ddH2O 900ml, EDTA 0.02%) washes of cells which were rounded until two relatively stable populations were obtained in which the enlarged and flattened cells formed a large proportion of the cell population.

1) a) KNRKr, b) KNRKf, c) LA-23 [32°C], d) LA-23 [39°C]

2) Graphical representation of % ALP activity versus cell line and culture condition. n=6.

3) Phosphorylated levels of pp69. a) LA-23 [32°C], b) LA-23 [39°C], c) KNRK-f, d) KNRK-r.

The lower pp62 protein which is present in these transformed cells is immunologically related to pp69 (Laverdure, et.al, 1987)
The observation that rapidly growing transformed cells have lower levels of pp69 in the media (Fig. 2.6) in comparison to their normal EGF-induced counterparts, at first glance appears to contradict the phosphatase/pp69 results obtained with EGF (Fig. 2.2). However, a large amount of the transformation-specific pp62 and much less pp69 is present in the media of transformed cells, which suggests that an aberrant control of pp69 with respect to phosphatase activity exists. When transformed cells recover partial regulation i.e. a ts-mutant at non-permissive temperature or KNRKf, regulation of pp69 is also partly restored (i.e. more normal). This in effect resolves the apparent contradiction with the EGF-treated NRK results (Fig. 2.2) that can be seen in terms of pp69 levels in Fig. 2.6, because the level of pp69 secreted by partially-transformed cells is now similar to that seen with untreated NRK-49F, although pp62 is still present. It can be postulated that the increased pp69 synthesis as well as specific phosphorylation or dephosphorylation events account for the first of 2-steps involved in AIG. The first step relating to proliferation and the second, a key signal leading to the breakdown of a regulated step (induction of pp62 ?) which results in a more permanent type of malignancy.
Figure 2.7 PP69, Secreted Phosphatase Activity and Tumor Progression

A

- Pseudonormal
- Primary cell
- Immortalized cell
- Increase pp69
- Increase np69
- PP69 induction
- Partially transformed
  - ts-mutant at non-permissive temperature
- Increase pp69
  - Similar to normal
- Increase secreted phosphatase activity
- Decrease secreted phosphatase activity

B

- pp69 level
- Phosphatase level
- PP62 levels

(normal units)

- Normal
- +EGF
- Transformed
- Partially transformed
rounded and barely adherent to the substratum, KNRKr cells demonstrate 33% lower activity than their more flattened counterparts (Fig. 2.6.2). This is accompanied by an approximately 2-fold decrease in the phosphorylated levels of pp69 (Fig. 2.6.3d), whereas KNRKf demonstrate more pp69 in the media (Kf/Kr = 2.3/1.0) (Fig. 2.6.3c).

Similarly, when tsLA-23 cells (Becker, *et al.*, 1977), which are NRK cells transformed by a ts-mutant of RSV, are shifted between permissive (Fig. 2.6.1c) and non-permissive (Fig. 2.6.1d) temperature (Chambers & Wilson, 1985), striking morphological changes take place (Chen, *et al.*, 1977; Kawai & Hanafusa, 1971) due to activation or inactivation of the viral oncogene product (Durkin, *et al.*, 1983). As well, many biochemical changes take place, such as: a change in total calmodulin content (Durkin & Whitfield, 1984), a 5-7 fold increase in PI turnover, a general alteration of the phospholipid profile of these cells (not shown). Furthermore, %ALP activity present in the secreted media of LA-23 cells grown at the permissive temperature of 32°C is dramatically reduced by 66% over those cells grown at the non-permissive temperature (Fig. 2.6.2). LA-23 cells shifted to the non-permissive temperature also demonstrate an approximately 50% increase in the phosphorylation of pp69 (Fig. 2.6.3b) similar to that seen with KNRKf cells. Both KNRKr cells and LA-23 cells at the permissive temperature do not demonstrate a change in the levels of expression of pp69 (Craig, *et al.*, 1988). In this context, it is interesting to note that rubella virus-infected embryonic palate mesenchymal cells also show a dramatic decrease in cellular ALP activity (Yoneda, *et al.*, 1986). These results suggest that: 1) ALP is involved in growth related changes (EGF-treated and transformed cell systems clearly demonstrate decreased secreted phosphatase activity) and 2) that this event may no longer be coupled in the same way as normal NRK-49F cells are to changes in the phosphorylation of
pp69. The relationship between pp69 and pp62 is unclear at present and may represent transformation events which could influence the phosphatase/pp69 interaction (see Fig. 2.7).

DISCUSSION

Temperature sensitive LA-23 cells, morphological variants of KNRK (r and f) cells, both NRK derived lines, and EGF treated NRK-49F cells clearly demonstrate a change in secreted ALP activity corresponding to their morphological and growth characteristics. Similarly, rubella virus-infected embryonic palate mesenchymal cells show a dramatic decrease in cellular ALP activity (Yoneda, et.al., 1986) and EGF-induced morphological changes of A-431 cells could be inhibited by Li⁺, PO₄⁻³, K⁺ and vanadate which inhibit phosphatase activity (Emura-Yamaguchi, et.al., 1987). As well, EGF-induced morphological changes in an osteoblastic cell line is accompanied by a decrease in cellular ALP activity after 24 hr EGF treatment (Kumegawa, et.al., 1983). Exposure of cultured human choriocarcinoma cells to methotrexate causes the formation of "giant cells" coupled to an increase in ALP activity (Burres & Cass, 1986).

Our conclusion that ALP and protein phosphatases are involved in cell proliferation (involving both cell morphology and cell multiplication) can be further supported by a number of observations. For example, retinoic acid which causes changes in growth and morphology of 9-1C cells also induces ALP activity (Reese, et.al., 1985). Transformed hamster embryo cells which are ALP⁺ showed decrease DNA synthesis, cell multiplication, reduced ability to form colonies in soft-agar and lower tumorigenicity in vivo (Sela & Sachs, 1974). Furthermore, 3-day bovine bone extract treatment of chick bone cells and skin fibroblasts
increase thymidine uptake and significantly decrease cellular ALP activity (Wergedal, et.al., 1988). Although no prior study has clearly linked EGF with alterations in secreted phosphatase levels, it is quite clear that cellular phosphatases are indeed controlled by various activators and inhibitors such as: insulin (Olsson & Belfrage, 1987; Londos, et.al., 1986), polyamines (Friedman, 1986), parathyroid hormone (Majeska & Rodan, 1982), corticosteroids (Ox, et.al., 1985), Vitamin D3 (Wong, et.al., 1977), isoproterenol and adrenalin (Ballou & Fisher, 1986). In NRK-49F cells, a normal rat kidney line, it appears that the secreted form of ALP as well as the secreted form of prot/ser-P are affected by EGF and have a functional role in the events that surround cell proliferation.

Normal NRK-49F cells secrete predominantly a 69kDa phosphoprotein which accounts for as much as 30% of all [32P]-orthophosphate uptake into this class of proteins. Transformed cells, on the other hand, secrete either a significantly reduced amount of the phosphorylated form of this protein or its truncated form (Laverdure, et.al., 1987). PP69 appears to undergo at least two well known regulatory mechanisms: 1) at the level of protein secretion (Laverdure, et.al., 1987) and 2) at the level of phosphorylation at serine residues possibly altering this protein's tertiary structure (Sparks & Brautigan, 1986). Phosphate regulation of protein function has been shown to alter the relative strength of the interaction of a membrane-membrane skeleton-binding protein (Cianci, et.al., 1988). Phosphorylation of the human erythrocye protein 4.9 (Husain-Chishti, et.al., 1988), inhibits its actin bundling properties which returns upon dephosphorylation by ALP. It has been suggested that phosphorylation distorts protein geometry in such a way as to cause inhibition of filament bundling (Beckman, 1988). One can see that alteration of protein geometry could indeed alter ALP effectivity. In addition it has been determined that phosphorylation of
Synapsins stimulate microtubule binding activity while reducing its actin binding activity (Petrucci & Morrow, 1987). Interestingly, pp69 also appears to be a fibronectin-binding protein related to osteopontin (Craig, et al., 1988, Nemir & Mukherjee, 1988). It has been recently suggested that pp69 does in fact contain sialic residues (Nemir & Mukherjee, 1988). This would result in a molecule having an overall net negative charge. Tryptic peptide analysis on thin layer DEAE cellulose chromatography (Fig. 2.6) confirms that pp69 does appear to have a net negative charge. Sialation may aid in the binding of fibronectin and in the protection of pp69 from proteolytic degradation (Schauer, 1985). The phosphorylated form of the osteopontin-like pp69 protein exists in a steady state with the non-phosphorylated form of this protein which can be altered by EGF. This relationship may be necessary for the proper co-ordinate interaction between pp69, ALP, the cell surface and various extracellular matrix components. It appears likely then that pp69, on the basis of immunological (Craig, et al., 1988), and biochemical similarities, may be a member of a family of related secreted phosphoproteins possibly involved in cell growth, bone formation and developmental processes such as the mesenchyme-dependent folding seen with kidney tissue.

Therefore, we conclude, that pp69 is a key protein involved in the regulation of cell proliferation in NRK-49F cells. Furthermore, EGF induces growth and morphological alterations by affecting the activity of the secreted forms of ALP and protein phosphatases, which in turn regulates the phosphorylation and conformation of pp69.
GENERAL CONCLUSIONS

From the results presented in this thesis, three major conclusions emerge:

1) \[^{35}S\]methionine

The secreted levels of a major 69kDa phosphoprotein can be increased upon EGF treatment, and that this effect is modulated by TGF-\(\beta\) antagonistically or cooperatively dependent on TGF-\(\beta\) as well as EGF concentrations. Optimum levels appear to correspond to those required for AIG.

RA acts to non-cooperatively modulate the increase seen with EGF and TGF-\(\beta\) TPA acts synergistically with EGF and TGF-\(\beta\) to increase the levels of pp69. However, TGF-\(\beta\), RA, or TPA alone do not affect the secreted levels of this phosphoprotein.

2) \[^{32}P\]orthophosphate

The phosphorylation of pp69 is significantly altered by EGF, and RA to effect a large apparent increase in the phosphorylation of pp69, while TGF-\(\beta\) treatment results in lower phosphorylated levels of pp69.

The relative phosphorylation of pp69, when in the presence of TGF-\(\beta\) and EGF is dependent on concentration. At low TGF-\(\beta\) concentrations + EGF, phosphorylation of pp69 is increased, although the level is lower than that seen with EGF alone. This suggests once more that TGF-\(\beta\) modulates in some way the effect seen with EGF. At high TGF-\(\beta\) concentrations, phosphorylation of pp69 is dramatically reduced.
3) EGF, Phosphatases and PP69

Whereas intracellular kinases may affect phosphate incorporation of pp69, we show in the present study that secreted phosphatase activity may regulate the degree of phosphorylation of pp69 once it has been secreted. Secreted ALP is decreased in cell systems which are actively dividing such as: LA-23 cells grown at 32°C, KNRKr, and 12hr EGF-treated NRK-49F cells. The modulation of phosphorylation of pp69 described in (2) is a result of regulation at two levels, the increase in pp69 secreted levels and changes in phosphate incorporation. A strong correlation now exists between the effect of EGF, secreted phosphatase activity and pp69.

The above observations may provide some insight into the complex interactions that make up some of the numerous cellular changes taking place outside the cell during cell growth.
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