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Transformation Related Defects in Vacuolar Acidification and Mannose 6-Phosphate Receptor Function: Effects on Lysosomal Targeting

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TRANSFORMATION RELATED DEFECTS IN VACUOLAR ACIDIFICATION AND MANNOSE 6-PHOSPHATE RECEPTOR FUNCTION; EFFECTS ON LYSOSOMAL TARGETING

by

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LIST OF ABBREVIATIONS

BCA	bicinchoninic acid
BCECF/AM	2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester
BSA	bovine serum albumin
cAMP	cyclic adenosime 5′-3′-monophosphate
CD-MPR	cation dependent mannose 6-phosphate receptor
CI-MPR	cation independent mannose 6-phosphate receptor
DMEM	Dulbecco's Modified Eagle Media
DSS	disuccinimidyl suberate
EDTA	ethylene dinitrilo tetraacetic acid, disodium salt
FITC	fluorescein isothiocyanate
GTP	guanosine 5′-triphosphate
HEPES	2 (N-morpholino) ethanesulfonic acid
IGF	insulin-like growth factor (I or II)
M6P/IGF-II	275 kDa (cation indendent) mannose 6-phosphate receptor
MMSV	Moloney murine sarcoma virus-transformed BALB/3T3 fibroblasts
CO	core oligosaccharide
PBS	phosphate buffered saline
PMP	pentamannosyl 6-phosphate

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
z-Arg-Arg-AMC	N α-benzyloxycarbonyl-L-arginyl-L-arginine 7-amido 4-methylcoumarin
z-Phe-Arg-AMC	N α–benzyloxycarbonyl-L-phenylalanyl-L-arginine 7-amido 4-methylcoumarin

CHAPTER I

GENERAL INTRODUCTION

The lysosomes are responsible for the degradation and turnover of endogenous cell components, as well as, foreign matter taken up by the cell through endocytosis and phagocytosis. Most long and short-lived proteins are eventually delivered to lysosomes where they are degraded within minutes by a battery of potent proteolytic and peptidylytic enzymes (Mortimore *et al.*, 1988). The number and types of proteases found in lysosomes often vary depending on the particular cell type and species, with as many as 15-20 proteases and a variety of other hydrolytic enzymes being present (reviewed in, Barrett, 1977; Ahlberg *et al.*, 1985; Bohley, 1987; and Agarwal, 1990). These proteases are synthesized as inactive precursors on the rough endoplasmic reticulum and are eventually sorted from newly synthesized secretory proteins and other proteins associated with the endolasmic reticulum/Golgi axis. Features which promote the delivery of proteins to the lysosome and inhibit their entry into the secretory pathway are the presence of the mannose 6-phosphate recognition marker, the existence of mannose 6-phosphate receptors, and the increasing acidity of the compartments on the pathway from the trans Golgi to the lysosome (reviewed in, Kornfeld and Mellman, 1989; Kornfeld, 1992).

Two receptors have been identified which are able bind the mannose 6-phosphate recognition signal found on these newly synthesized lysosomal enzymes; a 275 kDa receptor and a 46 kDa receptor. The 275 kDa receptor can also bind insulin-like growth factor-II, therefore it is referred to as the M6P/IGF-II receptor (Morgan *et al.*, 1987). Both receptors reside primarily in the Golgi apparatus and endosomes and to a lesser

degree on the plasmamembrane. The acidic nature of the lysosomal compartment provides optimal conditions for the activation and stabilization of lysosomal proteases (Henson, 1988; Young and Zygas,1987). Some portion of lysosomal enzymes may be secreted into the extracellular space, from where some can be recaptured and delivered by receptor-mediated endocytosis to lysosomes. However, it is presently unclear why some newly synthesized enzymes escape direct targeting to the lysosome and what purpose is served by their delivery to an extracellular space with a near neutral pH. At these pHs, most of the secreted enzymes are either inactive or unstable (Braulke *et al.*, 1988). The majority of these secreted enzymes are in the proenzyme form indicating that secretion occurs prior to delivery to the lysosome where proteolytic processing to the mature forms take place. Agents which increase the pH of the acidic compartments enroute from the site of synthesis to their final destination will result in an increased secretion of lysosomal enzymes (Gonzalez-Noriega, 1980; Hasilik and Neufeld, 1980; Braulke *et al.*, 1987). Cells lacking the 275 kDa M6P/IGF-II receptor have also been shown to secrete high levels of newly synthesized lysosomal enzymes (Nolan and Sly, 1987; Stein, 1987b). Several cancer cell lines secrete high levels of lysosomal enzymes, such as cathepsin L (Yamaguchi *et al*, 1990), cathepsin B (Sloane *et al.*, 1989), and cathepsin D (Capony *et al.*, 1984). It has been speculated that these enzymes may play a role in the destruction of the basal lamina, and as a result, promote the ability of cancer cells to invade tissues and ultimately to metastasize to distant sites (Yamaguchi *et al.*, 1990).

It has been observed that Moloney murine sarcoma virus-transformed BALB/3T3 (MMSV) fibroblasts secrete elevated levels of both procathepsins B and L when compared to nontransformed BALB/3T3 fibroblasts (Achkar *et al*, 1990). It was the intention of this research project to identify some of the factors which may contribute to the mistargeting of these lysosomal enzymes in the MMSV-transformed fibroblasts.

Endosomal and lysosomal pHs were determined using the pH sensitive probe, fluorescein isothiocyanate-dextran (FITC-dextran) (Ohkuma and Poole, 1978). These compartments were found to be 0.4 to 0.5 pH units higher in MMSV cells than in nontransformed BALB/3T3 fibroblasts. The transformed fibroblasts but not the BALB/3T3 cells, also lacked the ability to perform mannose 6-phosphate receptor (MPR)-mediated endocytosis, and they lacked appreciable levels of the 275 kDa M6P/IGF-II receptor as observed by Western blot analysis. When the MMSV cells were treated with potassium propionate, pH 7.4, acidification of endosomal and lysosomal compartments were enhanced, lowering their pH to values found in the nontransformed fibroblasts. This treatment reduced the levels of secreted procathepsin B and procathepsin L by 95-99%. Potassium propionate also activated MPR-dependent endocytosis in the BALB/3T3 cells by 160% and restored this activity in MMSV cells. However, the levels of the 275 kDa M6P/IGF-II receptor remained unchanged in the treated cells. These results suggest that a defect in vacuolar acidification and the absence of the 275 kDa M6P/IGF-II receptor contribute to the secretion of procathepsins B and procathepsin L by MMSV cells. They also suggest the possible participation of the 46 kDa mannose 6-phosphate receptor in lysosomal enzyme trafficking in potassium propionate treated cells.

CHAPTER II

REVIEW OF RELATED LITERATURE

Lysosomal Targeting-

Lysosomal, secretory, and membrane proteins all share the same early steps in a common biosynthetic transport pathway, but are eventually sorted from each other and delivered to different destinations (reviewed in, Pfeffer, S.R. ,1988; Hasilik, 1992). Each of these type of proteins contain a hydrophobic amino-terminal signal sequence which can interact with an 11 S ribonucleoprotein signal recognition factor, SRP. The binding to SRP of the newly made signal peptide interrupts translation and leads to the binding of the ribosome with its nascent polypeptide to the surface of the rough endoplasmic reticulum (RER). Translation resumes and the elongating protein is transferred into the RER lumen. Many of these proteins contain asparagine residues which undergo cotranslational glycosylation in the RER. This glycosylation involves the transfer of a large oligosaccharide group (3 glucoses, 9 mannoses, and 2 N-acetylglucosamines) from a lipid-linked intermediate to the new polypeptide in one step. Before the newly synthesized polypeptide leaves the ER, the signal peptide is cleaved off, the protein is folded, and some of the terminal sugars are removed. Most of these proteins are then delivered to the Golgi apparatus by vesicular transport where further post-translational modifications can occur.

Almost all proteins destined for the lysosomes undergo two important enzymatic reactions while present in the Golgi network. The first involves the enzyme, UDP-N-acetylglucosamine-lysosomal enzyme/ N-acetylglucosamine-1-phosphotransferase

(phosphotransferase) (Reitman and Kornfeld (1981); Lang *et al.*, 1984). This enzyme selectively transfers N-acetyl-glucosamine 1-phosphate from the nucleotide sugar, UDP-GlcNAc to the high mannose oligosaccharides on the newly synthesized lysosomal protein, resulting in a phosphodiester intermediate. The second enzyme, N-acetylglucosaminidase-1-phosphodiester-α-N-acetylglucosaminidase, removes the N-acetylglucosamine group, leaving an exposed mannose 6-phosphate residue on the protein. The resultant mannose 6-phosphate monoester serves as the recognition signal necessary for binding of lysosomal enzymes to mannose 6-phosphate receptors located in the trans Golgi network and cell surface. A basic overview of lysosomal enzyme synthesis and transport is represented in Figure 1. The importance in generating this mannose 6-phosphate recognition signal was discovered from studies of patients with I-Cell Disease (Hickman and Neufeld (1972). These patients lack N-acetylglucosamine-1-phosphotransferase and as a result secrete most of their lysosomal enzymes into the extracellular space due to their inability to generate the protein-linked mannose 6-phosphate. These secreted enzymes are also unable to bind with high affinity to cell surface mannose 6-phosphate receptors. NH_4Cl, which promotes lysosomal enzyme secretion, has recently been shown to prevent the exposure of the mannose 6-phosphate recognition signal by inhibiting the second enzyme, N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase (Isidoro *et al.*, 1990). As a result treated fibroblasts secrete high levels of cathepsin D. The site on lysosomal enzymes recognized by the phosphotransferase is more complex than a simple primary sequence or secondary structure found in some of the other intracellular targeting signals. Studies using site directed mutagenesis, the production of chimeric proteins, and computer modeling on cathepsin D, have revealed that a single lysine residue at position 203 and two noncontinuous sequences, each approximately 55 amino acids long are instrumental in the recognition event (Baranski *et al.*, 1990; Baranski *et al.*, 1991).

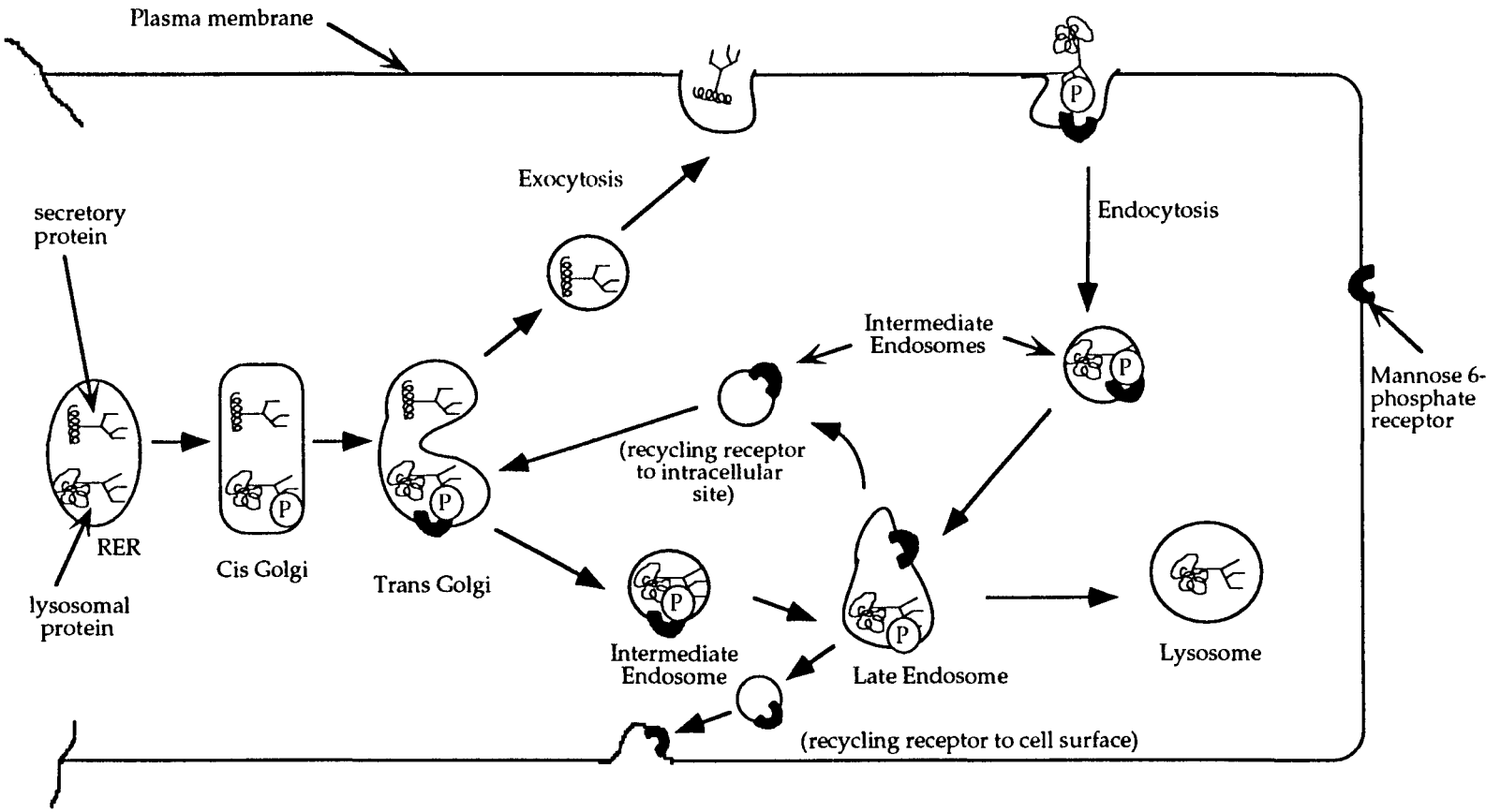

Fig. 1. INTRACELLULAR PROTEIN TRANSPORT PATHWAYS. General scheme showing selective routing between lysosomal and secretory proteins. Note: (P) represents phosphorylated lysosomal protein essential for ligand interaction with mannose 6-phosphate receptors. Adapted from Rothman *et al.* (1983) and Lehninger *et al.* (1993).

It is in the trans Golgi network (TGN) that proteins targeted to the lysosomes are separated from secretory and membrane proteins. A series of events involving membrane sorting, vesiculartransport and fusion of vesicular compartments occur as lysosomal enzymes are transported through the endocytic pathway. Lysosomal enzymes are packaged into clathrin coated vesicles which also house mannose 6-phosphate receptors. These transport vesicles bud off from the TGN and fuse to intermediate endosome. Intermediate endomes evolve into late endosomes, also referred to as prelysosomes or CURL (compartment for uncoupling receptors and ligands). Eventually the contents of late endosomes are delivered to the lysosomes. It is in the prelysosomal compartment that mannose 6-phosphate receptors dissociate from their ligands due to the acidic nature of this compartment (Hoflack and Kornfeld, 1985). The lysosomal enzymes proceed on to the lysosomes and the mannose 6-phosphate receptors recycle back to the TGN or to the plasma membrane. This intracellular 'endocytic pathway' is also believed to be connected to an incoming endocytic pathway at the level of the intermediate endosomes.

Little is known about the events and mechanisms involved in the transfer of molecules through the ER and Golgi network, to endosomes, lysosomes, and the plasma membrane. Recently the discovery of a fungal metabolite, Brefeldin A (BFA) has aided in the investigation of some of these events. BFA disrupts the Golgi structure by causing the microtubule-dependent retrograde transfer of Golgi associated components back to the ER, thereby blocking early events in exocytosis (Misumi *et al.*, 1986; Lippincott-Schwartz, 1990; Hendricks *et al.*, 1992). BFA may act by promoting the dissociation of a nonclathrin-coated protein called beta-COP from Golgi membranes (Orci *et al.*, 1991). BFA also causes changes in the distributions of both the 275 kDa M6P/IGF-II receptor and the 46 KDa M6P receptor (Damke *et al.*, 1991). It produces an increase in the number of both types of M6P receptors on the cell surface as well as an increase in the

internalization rate of the corresponding receptor/ligand complexes.

Regulation of Cytosolic pH-

Many cellular processes are affected by pH, such as protein synthesis, cell proliferation,metabolite transport, and enzyme activity. Most cells have a resting cytosolic pH of 7.0-7.2 (Moolenaar *et al.*, 1983; Moolenaar *et al.*, 1984; Paris and Pouyssegur, 1984; Grinstein *et al.*, 1985a), versus an average extracellular pH of 7.5 under physiological conditions (Tannock and Rotin, 1989). Cells utilize several mechanisms to maintain their intracellular pH (see Figure 2.) which might otherwise become too acidic because of the production of metabolically generated acids and a passive diffusion of protons into the cell due to a negative plasma membrane potential. Two ways by which cells achieve a homeostatic pH are by metabolically transferring acids from the cytosol into intracellular organelles and by eliminating acids or bases via several membrane- associated ion transport systems (reviewed in, Roos and Boron, 1981).

Three major ion translocators contribute to the regulation of pH at the plasma membrane. They are a Na^+/H^+ antiporter, a Na^+-dependent HCO_3^-/Cl^- exchanger, and a cation-independent HCO_3^-/Cl^- exchanger (Madshus, 1988). The Na^+/H^+ antiporter and the Na^+-dependent HCO_3^-/Cl^- exchanger act to prevent excessive cytosolic acidification, and the cation-independent HCO_3^-/Cl^- exchanger prevents alkalinization. In addition, some cell types also utilize H^+(ATPase) pumps and a lactate/proton symporter to maintain their cytosolic pH (Anwer and Nolan, 1988).

The Na^+/H^+ antiporter is found on the plasma membrane of almost all animal cells (Aronson, 1985). It is involved in the maintenance of intracellular pH, the regulation of intracellular Na^+, and the control of cell volume. It may also be involved in the early

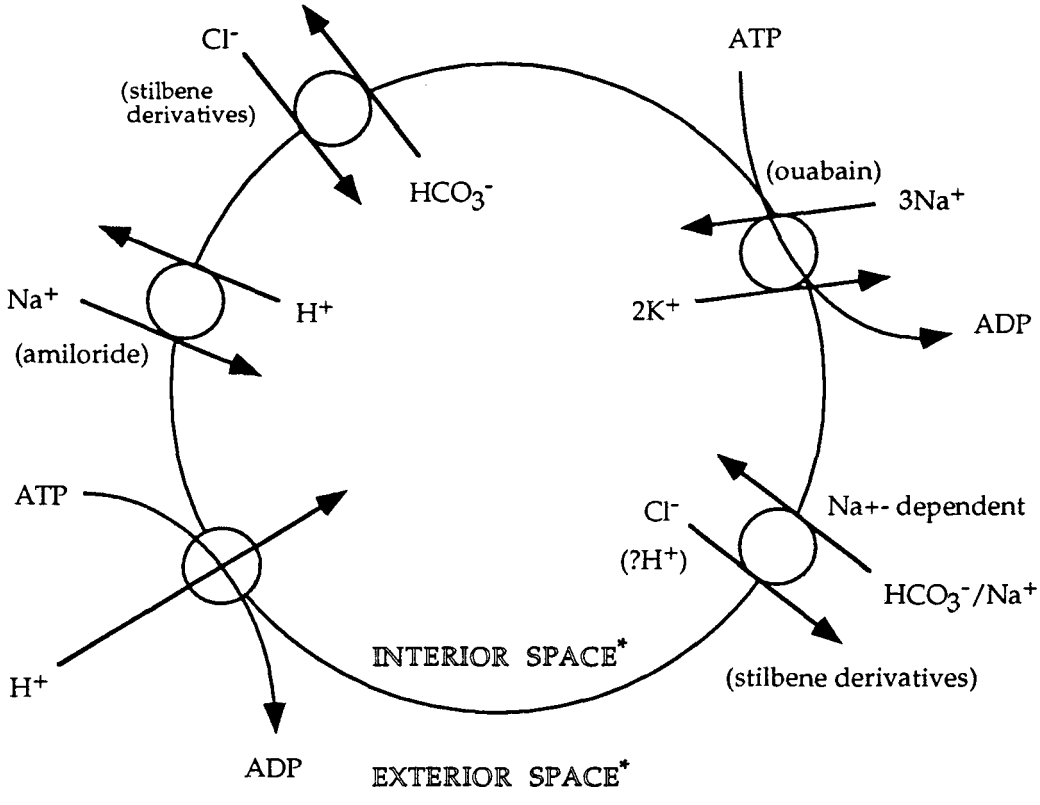

Fig. 2. pH REGULATION MECHANISMS. General summary of the various regulators which facilitate in the generation and maintenance of pH gradients across membrane barriers and their inhibitors. *Interior and exterior are general terms which refer to either the cytosol versus the extracellular space, or interior of lysosomes or endosomes versus the cytosolic space.

events of mitogenesis (Tannock and Rotin, 1989)). Under physiological conditions the Na^+/H^+ antiporter is almost quiescent. Normally it operates to couple the extrusion of H^+ from the cell to Na^+ uptake, although the reverse is possible. It is electroneutral and does not require metabolic energy directly. The driving force are the concentrations of Na^+ and H^+. Amiloride is a weak base which inhibits Na^+/H^+ exchange by competing for the extracellular Na^+ site (Grinstein and Smith, 1987).

Although most cells rely on the Na^+/H^+ antiporter as their major cytosolic pH regulator, some cell types also employ the Na^+-dependent and the cation independent HCO_3^-/Cl^- exchangers in cytosolic pH homeostasis (Reinertsen *et al.*, 1988). The Na^+-dependent HCO_3^-/Cl^- exchanger is electroneutral and acts by transporting one Na^+ and one HCO_3^- into the cell in exchange for one Cl^- and one H^+ (Boron and Russell, 1983). Under resting conditions, the Na^+-dependent HCO_3^-/Cl^- is inactive (L'Alleman *et al.*, 1985). The high concentration of extracellular Na^+ is sufficient to drive the exchanger. The cation-independent HCO_3^-/Cl^- is electroneutral and allows for one Cl^- to enter the cell and one HCO_3^- to leave. This exchanger only operates when the cytosolic pH becomes too alkaline which occurs very infrequently (Tonnessen *et al.*, 1987). Both types of HCO_3^-/Cl^- exchangers are inhibited by stilbene derivatives and are insensitive to amiloride (Tannock and Rotin, 1989). Other transporters may also participate in pH regulation. These include the H^+(ATPase) pump, which is very important in regulating intracellular pH in yeast and in some types of epithelial cells (Al-Alwqati, 1986), and to a lesser extent, the lactate/H^+ transporter which serves to remove protons from the cytosol of some cells by an electroneutral, symport mechanism (Balkovetz *et al.*, 1988).

The most commonly used technique for measuring cytosolic pH employs the fluorescent probe, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester, (BCECF-AM). It is nonfluorescent and lipid soluble in its ester form. Once it diffuses

into the cell, cellular esterases can convert it into its H_2O soluble, fluorescent form. This dye becomes trapped in the cytosol and has not been visualized in any other compartment (Grinstein *et al.*, 1989a). The fluorescent intensity of BCECF is extremely pH sensitive, especially in the pH range of 6.0 to 8.2 (Rink *et al.*, 1982).

Factors which alter cytosolic pH-

Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), have been shown to induce DNA synthesis, cell division, and metabolic changes (reviewed in, Moolenaar, 1986; Grinstein *et al.*, 1989b). These growth factors have also been shown to stimulate the Na^+/H^+ antiporter system and, as a result, cause an increase in the cytosolic pH (Rothenberg *et al.*, 1983; Moolenaar, 1986). The tumor promotor, tetradecanoyl-phorbol-acetate (TPA), can also activate the Na^+/H^+ exchanger (Moolenaar *et al.*, 1984). There is strong evidence that TPA acts through protein kinase C to activate the Na^+/H^+ exchanger (Nishizuka, 1984). Inhibitors of the Na^+/H^+ exchanger block cell growth, which emphasizes the important role this system has on the development of the proliferative response (reviewed in Grinstein *et al.*, 1989b). Studies have shown that when the yeast H^+(ATPase) pump is transfected into fibroblasts, two responses occur; an increase in cytosolic pH, and the transformation of the cells. These results indicate that it is the elevated pH that may be the primary mechanism which initiates cell proliferation, regardless of whether or not the Na^+/H^+ exchanger is activated (Perona and Serrano, 1988).

Cells are able to manipulate the pH of their extracellular environment by acid extrusion. The extracellular pH of the microenvironment of human and animal cells has been estimated using microelectrodes. The calculated pHs, which vary for different species and tissue types, had average values of 7.5 for humans, 7.32 for dogs, and 7.43 for rats (Wike-Hooley *et al.*, 1984) in normal tissue. However, when these measurements

were performed in solid tumors, an average pH of 7.0 was observed. The lower pH found in the extracellular space adjacent to tumor cells is not fully understood but increased glycolysis and lactic acid production by tumor cells is thought to contribute to some of the excess proton production (Hochachka and Mommsen, 1983). In murine B16F10 melanoma cell cultures the pH of the extracellular space located in close proximity to adherent cells was measured (Young and Spevacek, 1992). The average observed pH around individual cells was found to be 6.4 versus a pH of 7.4 for the bulk of the culture media. Some cells showed a pericellular pH as low as 5.5. The low pH environment created by these highly metastatic cells may serve to activate latent precursor forms of enzymes secreted by these tumor cells (Rozhin *et al.*, 1990).

Vacuolar pH-

Cells contain a number of intracellular compartments with acidic environments, including coated vesicles (Lemansky *et al.*, 1987), lysosomes (Ohkuma and Poole, 1978), endosomes (Ohkuma and Poole, 1978; Maxfield, 1982), condensing vacuoles (Orci *et al.*, 1987), and the trans Golgi network (Anderson and Pathan, 1985). Molecules which enter cells by endocytosis encounter an acidic environment within the first five minutes after entry (Maxfield, 1982). As these molecules transverse the intracellular endocytic pathway to lysosomes they encounter a gradual decreasing pH. Endosomes and lysosomes maintain their low pH with an electrogenic H^+(ATPase) pump (reviewed in Nelson, 1992). The vacuolar H^+(ATPase) differs from the two other classes of known H^+(ATPase), mitochondrial and gastric intestinal. It can be inhibited by alkalating agents such as N-ethylmaleimide (NEM) and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (Galloway *et al.*, 1983). Recently it was reported that the trans Golgi network may also use a pyrophosphate-driven proton transporter, H^+-PP_iase, to establish a transmembrane pH gradient (Brightman *et al.*, 1992). In addition, lysosomes may rely

on a Donnan-type equilibrium to help maintain the high concentration of protons transported into the lysosomal lumen (Moriyama *et al.*, 1992). In contrast, early endosomes appear to contain a plasma membrane derived electrogenic Na^+,K^+-ATPase pump which may reduce their capacity to be acidified (Fuchs *et al.*, 1989; Casciola-Rosen and Hubbard, 1992). The Na^+,K^+-ATPase pump is oriented in the endosomal membranes such that for every 3 Na^+ entering the endosomal lumen 2 K^+ would leave, thereby generating a positive interior membrane potential. This would limit the extent to which protons could accumulate within the endosomal lumen (Cain *et al.*, 1989) and might contribute to their slightly more alkaline interior when compared to late endosomes and lysosomes. This pump can be inhibited by ouabain.

In fibroblasts and macrophages, lysosomes maintain an acidic pH of 4.5 to 4.8 (Ohkuma and Poole, 1978; Dean *et al.*, 1984; Mellman *et al.*, 1986). Endosomes have a slightly higher pH of about 5.5 (Maxfield, 1982). The importance of the acidic nature of the endosomal pathway which includes the trans Golgi network, endosomes, and lysosomes, has been well established by the effects of agents which disrupt the proton gradient. Weak bases, such as NH_4Cl (Gonzalez-Noriega *et al.*, 1980; Hasilik and Neufeld, 1980; Braulke *et al.*, 1987b) and the carboxylic ionophore, monensin (Wileman *et al.*, 1984), inhibit the processing and activation of newly synthesized lysosomal enzymes, the recycling of receptors, the entry of viruses and toxins, and promote the missorting of proteins (Dean *et al.*, 1984).

Several methods have been developed for estimating the pH in vacuolar compartments. One method measures the partition of a weak base between the cytosol and various acidic vacuoles. Weak bases are membrane permeable and uncharged at neutral pH. They diffuse into acidic compartments where they accumulate as a result of aquiring a positive charge. Acridine orange is the most widely used fluorescent weak base (Van Dyke *et al.*, 1985). Its fluorescence intensity is concentration dependent and

not pH dependent, decreasing with increasing concentration. Estimations of vacuolar pH are based on the amount of dye which accumulates in these compartments. This method is not accurate because the volume of the acidic vauoles can not often be determined directly, and the vacuolar dye concentration is therefore uncertain. In addition, a variety of acidic compartments of varying intraluminal pH contribute to dye accumulation so that the pH of any one compartment is difficult to ascertain. A second method involves using a fluorescent probe which is taken up by the cell through the endocytic pathway. Fluorescein isothiocyanate (FITC) attached to a large dextran molecule enters the cell by fluid-phase endocytosis (Ohkuma and Poole, 1978). Because the fluorescent intensity of fluorescein is extremely pH sensitive around physiological pH ranges and is not otherwise strongly affected by environment, it is widely used for measuring the pH of vacuolar compartments on the endocytic pathway.

Receptor-mediated transport-

It is well established that newly synthesized lysosomal enzymes are sorted from secretory proteins and targeted to their final destination by way of the mannose 6-phosphate recognition system (reviewed in, Kornfeld and Mellman, 1989; Kornfeld, 1992). Two distinct mannose 6-phosphate receptors (MPR) have been identified; a 275-300 kDa receptor, also referred to as the cation-independent (CI)-MPR or mannose 6-phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor, and a 41-46 kDa receptor, often referred to as the cation-dependent (CD) receptor. Both receptors are involved in the intracellular transport of newly synthesized lysosomal enzymes (von Figura and Hasilik, 1986, Kornfeld, 1987). Both receptors continuously recycle between the Golgi apparatus, where they are able to bind ligands at near neutral pH values, and acidic prelysosomal compartments, where ligands uncouple from these receptors (Stein *et al.*, 1987a; Duncan and Kornfeld, 1988; Dahms *et al.*, 1989). Both receptors also

recycle to the cell surface. The cell surface M6P/IGF-II receptor can participate in receptor-mediated endocytosis of extracellular ligands under physiological conditions, whereas the 46 kDa MPR can not for reasons which are unknown (Griffiths *et al.*, 1988; Braulke *et al.*, 1990). Only when the 46 kDa receptor is overexpressed and the extracellular pH is lowered to 6.5 has it been shown to mediate low levels of receptor-mediated uptake of mannose 6-phosphate-containing ligands (Watanabe *et al.*, 1990). The two receptors can bind the same ligands but with different affinities and pH dependencies (Hoflack *et al.*, 1987; Tong and Kornfeld, 1989).

Cloning and sequencing of the bovine, human, and mouse 46 kDa MPR (Dahms *et al.*, 1987; Pohlmann *et al.*, 1987; Ma *et al.*, 1991; Koster *et al.*, 1991) and the bovine and human M6P/IGF-II receptor (Lobel *et al.*, 1988; Oshima *et al.*, 1988) has revealed that the receptors are unique but related proteins, which are encoded by different genes, but which may have developed from a common ancestor. The entire extracellular domain of the 46 kDa MPR is similar to each of the repeating units found in the M6P/IGF-II (discussed below) (Dahms *et al*, 1987). There is no homology in the signal, transmembrane and cytosolic domains between the two receptors (Kornfeld, 1992).

It takes approximately 15 min or less for receptors to recycle back to the trans Golgi network from prelysosomal compartments and as little as a few minutes for receptors to recycle back to the plasma membrane (Schwartz *et al.*, 1982; Harford *et al.*, 1983). Some receptors can recycle even in the absence of ligands (Oka and Czech, 1986; Braulke *et al.*, 1987a). However, when intracellular uncoupling of the receptor/ligand complexes is inhibited, as when vacuolar pH is increased, the receptor pool can become limiting and often the cell surface receptor pool is depleted (Gonzalez-Noriega *et al.*, 1980; Tietze *et al.*, 1982). Under these conditions, not only are the occupied receptors blocked from the recycling pathway but so are the unoccupied receptors. Two explanations have been proposed to explain this phenomenon : the first is based on the observation that when

receptor recycling is inhibited, a great deal of the cell membranes become internalized, causing swelling of intracellular vacuoles which in turn could limit movement within the cell (Stein *et al.*, 1984); the second possibility is that a change in vacuolar pH causes alterations in receptor conformation, state of aggregation, or in the charge of ionizable groups on the receptors which would inhibit its normal functions (Mellman *et al.*, 1986). Mutant cells with defects in the acidification of endosomes but not lysosomes exhibit reduced cell surface uptake by M6P/IGF-II receptors and fail to deliver newly synthesized lysosomal enzymes to the lysosomes (Robbins *et al.*, 1984; Park *et al.*, 1991). Instead, these cells secrete a majority of their newly synthesized lysosomal enzymes. Addition of the proton ionophore monensin to wild type cells mimicks the behavior of these mutant phenotypes (Robbins *et al.*, 1984).

M6P/IGF-IIreceptor-

The M6P/IGF-II receptor was first isolated (Sahagian *et al.*, 1981) by affinity chromatography using immobilized ß-galactosidase-Sepharose 4B affinity chromatography after it was discovered that receptor binding and endocytosis of newly synthesized lysosomal enzymes bearing a mannose 6-phosphate group was competively inhibited by mannose 6-phosphate (Kaplan *et al.*, 1977a; Kaplan *et al.*, 1977b). The M6P/IGF-II shows an optimal binding affinity toward ligands between pH 6.0-6.3 but still shows a 70 % binding capacity at pH 7.4 (Hoflack *et al.*, 1987). This receptor is a transmembrane glycoprotein (Sahagian and Steer, 1985) with has an average molecular mass of approximately 275 kDa with an additional 20-30 kDa of carbohydrate (reviewed in, Pfeffer, 1988). It also contains a number of intramolecular disulfide bridges (Sahagian and Neufeld, 1983). The M6P/IGF-II is composed of four structural domains, a single 44 amino acid N-terminal signal sequence, a 2269 amino acid extracellular domain, a 23 amino acid transmembrane region, and a 163 amino acid

carboxyl terminal cytoplasmic domain. The extracellular domain is made up of 15 repeating units, each approximately 147 amino acids long. The cytoplasmic domain contains four regions which contain sequences known to serve as substrates for different protein kinases, such as protein kinase C, cAMP-dependent protein kinase, and casein kinase I and II (MacDonald *et al.*, 1988). There is only one pool of M6P/IGF-II receptor which is located mostly in the late endosomal/prelysosomal compartment, with the remaining population distributed in lesser quantities throughout the plasma membrane, early endosomes and trans Golgi network (Griffiths *et al.*, 1990). The M6P/IGF-II receptor functions to sort and direct newly synthesized lysosomal enzymes as well as to recapture extracellular mannose 6-phosphate bearing ligands.

When cultured cells lack the M6P/IGF-II receptor (Gabel *et al.*, 1983; Nolan and Sly, 1987) or this receptor is blocked with antibodies (Stein *et al.*, 1987), the cells secrete a majority of their newly synthesized lysosomal enzymes and are unable to carry out receptor-mediated endocytosis of mannose 6-phosphate ligands. These functions are restored when M6P/IGF-II receptor deficient cells are transfected with M6P/IGF-II receptor cDNA (Kyle *et al.*, 1988; Lobel *et al.*, 1989). Similar studies with truncated receptors have shown that the cytoplasmic domain contains the signal needed for receptor internalization at the cell surface, and for proper lysosomal enzyme sorting and delivery (Lobel *et al.*, 1989; Johnson *et al.*, 1990; Canfield *et al.*, 1991). Studies using chimeric proteins have also indicated the importance of the extracellular and transmembrane domains in retaining the receptor in the endocytic pathway (Dintzis and Pfeffer, 1990).

It was discovered that the M6P/IGF-II receptor was able to bind to insulin-like growth factor II as well as mannose 6-phosphate (Morgan *et al.*, 1987). IGF-II is a nonglycosylated protein. The M6P/IGF-II receptor has unique binding sites for IGF-II and mannose 6-phosphate (MacDonald *et al.*, 1988), with both able to simultaneously

bind to the receptor (Kiess *et al.*, 1990). However, IGF-II and a lysosomal enzyme bearing a mannose 6-phosphate appear to bind in a mutually exclusive manner. Strangely, when IGF-II is overexpressed in NIH 3T3 cells, M6P/IGF-II dependent receptor-mediated uptake and the intracellular trafficking of newly synthesized lysosomal enzymes are not impaired (Braulke *et al.*, 1991). There are 19 potential asparagine-linked oligosaccharide sites located on the extracellular domain of the M6P/IGF-II receptor (Lobel *et al.*, 1988). The receptor does not need to be glycosylated for IGF-II binding but glycosylation is necessary for mannose 6-phosphate binding (Kiess *et al.*, 1991). Each M6P/IGF-II receptor can bind one molecule of IGF-II (Tong *et al.*, 1988), and two molecules of mannose 6-phosphate or one molecule of a diphosphorylated oligosaccharide (Tong *et al.*, 1989a; Distler *et al.*, 1991). IGF-II can also bind to the IGF-I receptor and the insulin receptor.

The M6P/IGF-II receptor differs from the IGF I receptor and the insulin receptor in the fact that it does not have intrinsic tyrosine kinase activity (Hari *et al.*, 1987). Several different responses have been observed when IGF-II binds to the M6P/IGF-II receptor; increased glycogen synthesis in rat hepatoma cells (Hari *et al.*, 1987), increased amino acid uptake in human myoblasts (Shimizu *et al.*, 1986), increased Na^+/H^+ exchange in canine kidney proximal tubular cells (Rogers *et al.*, 1990), and increased Ca^+ influx and DNA synthesis in BALB/c cells (Kojima *et al.*, 1988). IGF-II exerts its signaling effect by a direct coupling between $G_{i\text{-}2\alpha}$ and the M6P/IGF-II receptor (Okamoto *et al.*, 1990). Mannose 6-phosphate-containing ligands do not elicit this response. The M6P/IGF-II receptor is also capable of endocytosing IGF-II and delivering it to the lysosomes for degradation (Kiess et al., 1988). The M6P/IGF-II receptor has been implicated in the binding and activation of latent TGF-ß1 (Dennis and Rifkin, 1991). Extracellular acid hydrolases which are capable of degrading extracellular proteoglycans can bind and be retained by cell surface M6P/IGF-II receptors (Roff *et al.*, 1983; Brauker *et al.*, 1986).

A soluble form of the M6P/IGF-II receptor has been detected in the media from human colon HT-29 carcinoma cell cultures (Garrouste *et al.*, 1991). This truncated 250 kDa receptor was found to be missing the carboxy-terminal and transmembrane domains. These cells had previously been shown to secrete abnormally high levels of IGF-II (Culouscou *et al.*, 1990). The soluble M6P/IGF-II receptor retains a high affinity to both IGF-II and mannose containing ligands, however, most of the secreted IGF-II was found to be bound to a group of hetereogeneous IGF-binding proteins. Earlier studies have suggested that the synthesis of the truncated receptor may be developmentally regulated. High concentrations of soluble M6P/IGF-II receptors have been detected in fetal and neonatal sera which gradually decrease to undetectable levels in adult sera (Gelato *et al.*, 1989; Kiess *et al.*, 1987). Several suggestions have been made for possible roles for a soluble M6P/IGF-II receptor: inactivation of lysosomal enzymes, lysosomal enzyme transport and delivery to other locations, inhibition of lysosomal enzyme re-uptake into the cells, or a possible extension of the half life of these enzymes in the extracellular space (Garrouste *et al.*, 1991). Any of these functions could aid in tissue repair, remodeling, or in tumor invasion.

46 kDa mannose 6-phosphate receptor-

The 46 kDa receptor was first reported in cell lines that were deficient in the M6P/IGF-II receptor but were able to properly sort and deliver newly synthesized lysosomal enzymes (Hoflack and Kornfeld, 1985; Distler and Jourdian, 1987). Presently, no cell has been found which lacks this receptor. The 46 kDa receptor is also referred to as the cation-dependent MPR because it requires divalent cations (Mn^{2+} or Mg^{2+}) for optimum ligand binding (Watanabe *et al.*, 1990), although divalent cations are not essential (Junghans *et al.*, 1988). It can bind mannose 6-phosphate optimally in the pH range from 6.0 to 6.3, and at pH 7.4 shows very little binding affinity (Tong and

Kornfeld, 1989b). This receptor is an integral membrane glycoprotein with four structural domains; a 28 amino acid terminal signal sequence, a 159 amino acid extracellular domain, a 25 amino acid membrane spanning domain, and a 67 amino acid cytosolic domain (Dahms *et al.*, 1987). The newly synthesized receptor must obtain N-linked high mannose oligosaccharides and form intramolecular disulfide bonds (Hille *et al.*, 1990) before it can bind to mannose 6-phosphate containing ligands. When these alterations occur, the conformation of the receptor changes, resulting in trypsin resistance, an increased affinity toward antibodies directed against the mature protein, and the ability to form noncovalently linked homodimers. The detergent solubilized 46 kDa receptor exists as a mixture of noncovalently linked dimeric and tetrameric forms in solution (Waheed *et al.*, 1990). In baby hamster kidney cells which overexpress the 46 kDa MPR, crosslinking experiments have shown that the receptor exists in the cell membranes as monomers, dimers, and tetramers (Waheed *et al.*, 1990). The tetrameric form binds ligands with a greater affinity due to its higher valency. Both dimeric and tetrameric forms bind ligands with the same pH optimum. Tetramer formation is favored when the receptor number is high, at neutral pH, and in the presence of mannose 6-phosphate or mannose 6-phosphate containing ligands. From equilibrium dialysis data it is estimated that the monomeric form of this receptor is able to bind one mole of monovalent mannose 6-phosphate ligand and 0.5 moles of a diphosphorylated high mannose oligosaccharide per monomeric unit (Tong and Kornfeld, 1989a; Distler *et al.*, 1991). A dimer and tetramer would be expected to have twice and four times as many binding sites, respectively. The role of the 46 kDa MPR in the cell is still rather obscure. When this receptor is overexpressed in different cell systems, divergent results are obtained; it either promotes the secretion or the intracellular retention of newly synthesized lysosomal enzymes. In M6P/IGF-II receptor deficient BHK cells, overexpression of the human 46 kDa MPR results in an increased secretion of newly

synthesized lysosomal enzymes (Chao *et al.*, 1990). In M6P/IGF-II deficient mouse L cells, overexpression of the 46 kDa MPR results in a higher intracellular retention of newly synthesized lysosomal enzymes (Watanabe *et al.*, 1990).

Mannose 6-phosphate independent lysosomal targeting-

Most but not all newly synthesized lysosomal enzymes are targeted to the lysosomes by way of the mannose 6-phosphate signaling recognition mechanism. Acid phosphatase (AP) is properly sorted and delivered to the lysosomes independent of the mannose 6-phosphate system (Waheed and van Etten, 1985). Interestingly, when factors which normally increase lysosomal enzyme secretion such as chloroquine and tunicamycin were added to primary rat hepatocyte cultures, no increase in the secretion of AP was seen and maturation of the enzyme occurred normally (Tanaka *et al.*, 1990). Additionally, AP does not contain the same type of phosphorylated high mannose groups found in many of the other lysosomal enzymes. AP is synthesized as an integral membrane protein which is proteolytically processed to a soluble form once it reaches the lysosomes. ß-Glucocerebrosidase is also targeted to the lysosomes by a mannose 6-phosphate independent pathway (Aerts *et al.*, 1988).

In patients with I-Cell disease, their inability to generate mannose 6-phosphate recognition markers does not impede normal lysosomal delivery in hepatocytes, Kupffer cells, and leukocytes (Owada and Neufeld, 1982). This implies the existence of a M6P/MPR-independent lysosomal targeting pathway in these cells. Several investigators have observed that procathepsin D and procathepsin L can bind to microsomal membranes in a mannose 6-phosphate receptor-independent manner (Diment *et al.*, 1988; Rijnboutt *et al.*, 1991; McIntyre and Erickson, 1991). Only the precursor forms of these enzymes possess this ability to bind to microsomal membranes, and their association is pH-dependent (McIntyre and Erickson, 1991). The proenzymes

bind optimally to the membranes at pH 5.0. These authors suggest that this mechanism is responsible for the delivery of lysosomal enzymes from acidic prelysosomal compartments to the lysosomes since the proenzymes are no longer coupled to the mannose 6-phosphate receptor in these intermediate compartments. Additionally they propose that when agents which raise intravesicular pH are added to cell cultures, the increased secretion of latent lysosomal enzymes may be due to the failure of the proenzymes to bind to the prelysosomal membranes. However, the propeptide region alone has been shown to be insufficient as a lysosomal mannose 6-phosphate independent targeting signal when chimerically linked to secretory proteins (Connor, 1992). Conversely, the basis of M6P/MPR-independent targeting of lysosomal enzymes in hepatocytes, Kupffer cells, and leukocytes remains unknown.

Mistargeting of Lysosomal enzymes-

There have been many reports documenting elevated lysosomal enzyme secretion by tumor cells in both tissue and organ cultures, and by normal cells in response to transforming and growth-stimulating agents. High levels of latent cathepsin B have been measured in the ascitic fluid of patients with ovarian carcinoma (Mort *et al.*, 1983; Dufet *et al.*, 1984). Cathepsin D is overexpressed and abnormally high levels are secreted by human breast cancer MCF7 cells (Garcia *et al.*, 1984). Murine Kirsten sarcoma virus-transformed NIH fibroblasts, secrete much higher amounts of procathepsin L than non-transformed NIH 3T3 fibroblasts (Gal and Gottesman, 1986). Moloney murine sarcoma virus transformed BALB/3T3 fibroblasts show an increased secretion of procathepsin B and ß-glucuronidase, in addition to procathepsin L, when compared to non-transformed BALB/3T3 cells. This secretion could not be further stimulated by the addition of monensin (Achkar *et al.*, 1990). When growth factors are added to quiescent NIH 3T3 fibroblasts there is an elevation in the amount of procathepsin L secreted.

Growth factors can also enhance the already high levels of procathepsin L secretion by transformed KNIH fibroblasts (Stearns *et al.*, 1990).

Many mitogenic agents, such as growth factors, mitogenic lectins, the *ras* oncogene product, and tumor promoting phorbol esters are thought to activate the Na^+/H^+ exchange pump, raising the intracellular pH by 0.1 to 0.4 units (Grinstein *et al.*, 1985a). Jiang *et al.* (1990) reported a substantial increase in intralysosomal pH following the malignant transformation of mouse 3T3 fibroblasts with Kirsten murine sarcoma virus, and the transfection of human MSU-1.1 fibroblasts with v-K_i*ras* or T_{24} H-*ras*. The increased cellular pH may contribute to lysosomal enzyme mistargeting. Human breast cancer MCF7 cells are mitogenically stimulated by immunopurified cathepsin D (Vignon *et al.*, 1986). The mitogen, insulin-like growth factor-II (IGF-II), has also been found to be synthesized and secreted by these cells (Yee *et al.*, 1988; Osborne *et al.*, 1989) and stimulates growth in these cells in both an autocrine and paracrine manner (Karey and Sirbasku, 1988). Mathieu *et al.*, (1990) found that cathepsin D was able to modulate the activity of IGF-II on MCF7 cells. At low concentrations, extracellular IGF-II is able to bind to the M6P/IGF-II receptor. However, when there are competing high concentrations of cathepsin D present, IGF-II binding to the M6P/IGF-II receptor was barely detectable and showed substantially high binding to the IGF-I receptor.

Preliminary evidence suggests that secreted lysosomal proteases may be capable of activating other proteases and may augment the responses of growth factors. Secreted rat procathepsin L has been shown to be activated by the addition of mature cathepsin D (Wiederanders and Kirschke, 1989). Others have shown that procathepsin B can be proteolytically cleaved to its active, mature form by cathepsins D and L, as well as by autocatalysis (Rowan *et al.*, 1992). Low pH conditions are required for the maturation and activation of prolysosomal enzymes in these studies. Recently it has been observed that the autocatalytic activation of procathepsin L to its mature enzyme form could

occur at pH 5.5 and as high as pH 6.0, when a negatively charged group is present nearby (Mason and Massey, 1992).

CHAPTER III

MATERIALS AND METHODS

Materials-

BALB/3T3 Clone A31 (BALB/c, embryo; mouse) and MMSV [Murine sarcoma virus (Moloney) transformed BALB/3T3, embryo; mouse] fibroblasts were obtained from the American Type Culture Collection (Rockville, Maryland). FITC-dextran (M_r 71,200), monensin, pepsin (3900 units/mg protein), trypsin, and mannose 6-phosphate (sodium salt) were obtained from Sigma. Potassium propionate and sodium [^{125}I] iodide were obtained from ICN Biochemicals. Z-Arg-Arg-AMC and Z-Phe-Arg-AMC were obtained from BACHEM Bioscience Inc. (Philadelphia, PA). All other reagents not mentioned were obtained from Sigma.

Preparation of Pentamannosyl 6-Phosphate (PMP)-

Yeast phosphomannan from *Hansenula holstii* NRRL Y-2448 was the generous gift of Dr. M.E. Slodki, Northern Regional Research Laboratories, USDA (Peoria, IL). Pentamannosyl 6-phosphate (PMP) was prepared from o-phosphomannan by acid treatment as described (Slodki *et al.*,1973; Murray *et al.*,1980). This proceeded as follows; 1 gm of o-phosphomannan was rehydrated in 100 ml of distilled H_2O overnight at 4°C. Bio-Rad AG 50W-X5 cation exchange resin (H^+ form) was added to the swollen oligomer until the pH was below 2.5. The mixture was boiled at 100°C for 1 hr. After cooling to room temperature, the cation exchange resin was filtered off and discarded. The supernatant was adjusted to pH 11 with saturated $Ba(OH)_2$. To precipitate out the unhydrolyzable core oligosaccharide, an equal volume of 95 % ethanol was added and

the mixture was left overnight at 4°C. The supernatant was collectedafter centrifugation at 1,000 rpms at 4°C for 10 min. The unhydrolyzable core oligosaccharidewas retained for further use as an inhibitor of mannose 6-phosphate receptor-mediatedendocytosis. The supernatant was adjusted to pH 2.5 as above with Bio-Rad AG 50W-X5 resin and filtered. The sample was dried down on a rotary evaporator. Isolation of the pentasaccharide phosphomonester was performed by resuspending the sample in 3 ml of 0.1 N acetic acid and applying it to a 85 cm X 1.5 cm Sephadex G-25 (fine) (Pharmacia) column. The sample was eluted with 0.1 N acetic acid and 2 ml fractions were collected. Fractions were assayed for the appearance of carbohydrate using the method of Dubois *et al.*, (1956). This involved the addition of 10 µl of 80% phenol to 400 µl of sample followed by the addition of 1 ml of conc H_2SO_4. The absorbance of the samples were determined with a Gilford Response UV/Vis spectrophotometer at 490 nm. A standard curve was generated using 0.05-0.30 mM (D+)-mannose. Fractions were combined and dried down on a rotary evaporator.

The pentamannose phosphomonoester (PMP) was coupled to bovine serum albumin (BSA) by reductive alkylation according to the method of Schwartz and Gray, (1977). This involved the addition of 68 mg of BSA (fraction V) to 200 mg of PMP. To this was added 9.9 mg of sodium cyanoborohydride in 5 ml of 0.2 M $KHPO_4$ buffer, pH 8.0. The reaction was allowed to proceed for 3 days at 37°C. The extent of coupling was estimated by measuring the amounts of phosphate (Fiske and Subbarow, 1925) and mannose (Dubois *et al.*, 1956) that were incorporated into the protein product. Total protein was determined by the Bio-Rad Protein Assay Kit according to the manufacturer's protocol.

The unhydrolyzable core oligosaccharide which remained after acid treatment (described earlier) was converted into a sodium salt by redissolving the 95% ethanol

precipitate in distilled H_2O and adjusting the pH to below 2.5 with Bio-Rad AG 50W-X5 resin. The resin was filtered off and the solution was neutralized to pH 7.4 with 1 N NaOH. The product was lyophilized and stored at 0°C prior to use.

Cell Culture-

Cells were maintained in DMEM (BALB/3T3 and MMSV media contained 1 g/L and 4.5 g/L glucose, respectively) supplemented with 10% calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin , and 3.7 g/L sodium bicarbonate in a 5% CO_2, 95% air incubator at 37°C. Every 3 to 4 days cells were detached with 0.25% trypsin, 1 mM EDTA in PBS, and passaged at a dilution of 1 to 6. Prior to the collection of media and cells for enzyme assay, near confluent monolayers were exposed to serum-free DMEM for 5.5 h, or to one of the following buffers: 110 mM potassium propionate, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 44 mM $KHCO_3$ (buffer A); 111 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 44 mM $KHCO_3$ (buffer B); and 110 mM N-methyl glucosamine chloride, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 44 mM $KHCO_3$ (buffer C).

Fluorescent *Microscopy*-

The method used was adapted from that previously described (Schwartz *et al.*, 1988). BALB and MMSV cells were grown on glass slides previously sterilized with 70% ethanol. Cells were exposed to 1 mg/ml fluorescein dextran (FITC-dextran) for either 15 min, 2 h, or 24 h in complete DMEM. After incubation, cells were washed extensively with 130 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose, and 20 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 7.4. Fluorescent microscopy was performed on a Nikon Microphot system which included an inverted

epifluorecence microscope equipped with a 75-W xenon lamp and a Farrand microspectrofluorometer. A 50% neutral density filter was used to reduce the intensity of the fluorescent beam. The green fluorescence of fluorescein was observed using a Nikon B filter cassette (excitation was set at 420-485 nm; dichroic mirror at 510 nm; emission > 520 nm). Photographs were taken with a Nikon FG 35 mm camera attached to the camera port using Kodak Ektachrome film.

Measurements of Vacuolar pH-

The following methods are adapted from those previously described (Okhuma and Poole, 1978; Okhuma *et al.*, 1982). Excitation spectra were collected for FITC-dextran (1 µg/ml) in 10 mM citrate, 173 mM sodium phosphate buffer at various pHs between 3.0 and 8.1, with a SLM/Aminco SPF 500C spectrofluorometer at an emission wavelength of 519 nm, and with both excitation and emission monochromator bandwidths set at 5 nm. Using the ratio of relative fluorescent intensities at 495 and 450 nm, a pH titration curve was constructed for FITC-dextran. Estimates of vacuolar pH were made after exposing the cells to FITC-dextran at the following concentrations; 10 mg/ml for 15 min, 5 mg/ml for 2 h, or 1 mg/ml for 24 h in complete medium at 37°C. Different concentrations of FITC-dextran were used in order to optimize the fluorescence at shorter uptake periods. At these concentrations, the amount of fluorescent dye internalized by cells would not affect the pH dependency of FITC-dextran fluorescence (Ohkuma and Poole, 1978). Cell monolayers were washed extensively with ice-cold 130 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose, and 20 mM MES, pH 7.40 (buffer D). Cells were collected by scraping and centrifugation at 1,000 rpms at 4°C. The pellet was washed briefly, resuspended in buffer D at 37°C and placed in a stirred quartz cuvette. After obtaining an initial excitation spectrum, monensin was added (final concentration 60 µM) to equilibrate the intracellular pH to the extracellular

pH of 7.4, and additional excitation spectra were collected. Average vacuolar pH was estimated in two ways: by the F_{495}/F_{450} ratio method originally described (Okhuma and Poole, 1978; Okhuma *et al.*, 1982) or from the change in fluorescence at 485 nm after the addition of monensin. In the latter method, the average vacuolar pH is given by the relationship:

$$pH = 6.407 - \log \left[\frac{0.983 - R}{-0.132 - R} \right]$$

In this equation, R is $\Delta F485$ / (F485)monensin, where 6.41 is the midpoint of a titration curve obtained by plotting [(F485) pH 7.4 - (F485) pH] / (F485) pH 7.4 against pH, and 0.983 and -0.132 are respectively the upper and lower limits of the titration curve. This latter procedure was more accurate than the F495/F450 ratio method when the FITC-dextran signal at 450 nm was low relative to the background fluorescence at 450 nm.

Cytosolic pH determination using BCECF/AM-

Cytosolic pH was measured as described previously (Kiang *et al.*, 1990). BALB and MMSV cells were lifted from T 75 cm^2 flasks with 0.25 % trypsin, 1 mM EDTA. After centrifugation, cell pellets were resuspended in Hank's buffer (135 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.2 mM $CaSO_4$, 0.8 mM Na_2HPO_4, and 10 mM HEPES) containing 5 mM glucose and 0.2 % BSA. The cells were allowed to recover from trypsinization by returning the cell suspension to an incubator for 1 h at 37 ^{o}C. Cells were pelleted by centrifugation, and resuspended at a concentration of 2 X 10^6 cells/ml in Hank's buffer containing 5 mM glucose, 0.2 % BSA, and 2 µM 2',7'-

bis(carboxyethyl) carboxyfluorescein acetoxymethylester (BCECF/AM) (Molecular Probes, Eugene, OR). BCECF/AM was prepared as a 1 mM stock solution in DMSO. Cell suspensions were incubated for 15 min at 37°C. Cells were pelleted by centrifugation, buffer was aspirated off, and pellet was washed three times with Hank's buffer (glucose and BSA free). The cell pellet was resuspended in Hank's buffer at a concentration of 1 X 10^6 cells/ml and transferred to a stirred quartz cuvette. Fluorescence excitation spectra were collected on a SLM Amico Fluorescence Spectrometer at an emission wavelength of 530 mn and slit widths of 4 nm for both excitation and emission, at 37°C. Excitation scans were collected from 400 to 510 nm. The cytosolic pH was calculated from the ratio of relative fluorescence at the excitation wavelengths, 497 nm/437 nm. A standard pH curve was generated for BCECF using BALB cell suspensions which had internalized BCECF/AM. Basically, this involved placing BCECF/AM treated BALB cells (see above) in high potassium buffer at various pHs and adding nigericin, a potassium proton ionophore. The high potassium buffer contained 145 mM KCl, 5 mM NaCl, 1.2 mM $MgCl_2$, 1.6 mM $CaCl_2$, and 10 mM HEPES. The calibration buffer was adjusted to the appropriate pH by the addition of either HCl or KOH. A stock solution of 10 mM nigericin was prepared in absolute ethanol and was added to cell suspensions to give a final concentration of 10 μM in the cuvette. After recording an excitation spectrum, the cell suspension was removed and centrifuged. An excitation spectrum was recorded for the supernatant and this was subtracted from the excitation spectrum of the cell suspension to correct for leakage of the fluorescent probe. The pH of the supernatant was remeasured with a combination pH electrode and this value was plotted against the corrected cellular Ex497/437 ratio to create the standard curve. The effect of potassium propionate on the cytosolic pH

was determined as described previously (Grinstein *et al.*, 1984; Grinstein *et al.*, 1989). BALB and MMSV cells were placed in 140 mM potassium propionate, 1 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES, pH 7.4, for 90 min prior to exposure to BCECF/AM , during the 15 min incubation with 2 μM BCECF/AM, and during the collection of excitation spectra.

Radiolabeling of PMP-BSA and IGF-II-

Both PMP-BSA and IGF-II were radiolabeled with [^{125}I]-sodium iodide by the Chloramine-T Method (Hunter and Greenwood, 1962). All of the following reagents were added in rapid succession to a microfuge tube; 0.5 mCi of [^{125}I]-NaI, 50 μl of 0.5 M sodium phosphate buffer (pH 7.4), 1 μg of protein (in 0.05 M sodium phosphate, pH 7.4), and 25 μl of chloramine T (2 mg/ml in 0.05 M sodium phosphate buffer, pH 7.4). The reaction was allowed to proceed for 30 seconds at room temperature and quenched by the addition of 100 μl of cysteine-HCl (0.17 mg/ml in 0.05 M sodium phosphate buffer, pH 7.4). Free iodide was separated from labeled substrate by applying the reaction mixture to a 12 cm X 0.9 cm BioGel P-6 column which had been pretreated with 0.1% BSA in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and then washed extensively with this same buffer absent of BSA prior to the application of the samples. Fraction sizes of 0.5 ml were collected and counted on a TM Analytic 1191 Gamma Counter. Percent radiolabel incorporation was determined by precipitating the labeled PMP-BSA with ice-cold 10% trichloroacetic acid (TCA) /2% phosphotungstic acid (PTA). To remove the remaining unbound iodine, the [^{125}I]-PMP-BSA was extensively dialyzed against three buffer changes of PBS at 4^{o}C. After dialysis, [^{125}I]-PMP-BSA was concentrated down to a minimal volume using an Amicon

microconcentrator (Centricon 10). Protein levels were determined with the Bio-Rad Protein Assay Kit. Fractions from the Bio Gel P-6 column containing [^{125}I]-IGF-II were analyzed as above, pooled, lyophilized, and resuspended in a minimal volume. Both substrates were stored at 4^{o}C prior to their use.

Receptor-mediated Endocytosis-

BALB or MMSV cells, cultured in 35 mm multiwelled tissue culture plates, were preincubated for 90 min at 37^{o}C in either serum-free DMEM, pH 7.4, or in potassium propionate (buffer A), pH 7.4, after several washes to remove any traces of sodium. [^{125}I]-labeled-PMP-BSA (specific activity, 1.78 X 10^{3} cpms/ng) was added to each well at a concentration of 1 µg/ml. Uptake was allowed to proceed for 90 min at 37^{o}C. Cells were then washed five times with ice-cold Hank's balanced saline solution, pH 7.4, and lysed with 500 µl of 1 M NaOH. Radioactivity in the lysate was measured with a TM Analytic model 1191 Gamma Counter. To determine nonspecific uptake, either 60 mM mannose 6-phosphate or 10 mM core oligosaccharide was also added to some of the wells at the beginning of the 90 min uptake period. Protein was determined by BCA Protein Assay (Pierce). To distinguish between cell surface binding and uptake, some plates were incubated with [^{125}I] labeled PMP-BSA for 90 min at 4^{o}.

Crosslinking [^{125}I]-IGF-II to M6P/IGF-II receptor-

[^{125}I]-IGF-II was radiolabeled as described above to a specific activity of 3.2 X 10^{5}

cpms/ng. BALB and MMSV cells were grown in 75 cm^2 tissue culture flasks to ~80% confluency. Monolayers were washed two times with binding buffer containing, 118 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 8.8 mM glucose, 0.5% (w/v) BSA, 20 mM HEPES, and 10 mM NaHCO3, pH 7.4. To look at surface binding, cells were incubated with 5 ng/ml of [^{125}I]-IGF-II, either with or without cold IGF-II (500 ng/ml) in binding buffer, pH 7.4, for 4 h at room temperature. To look at total cellular binding, cells were incubated with 5 ng/ml of [^{125}I]-IGF-II in binding buffer, pH 7.4 containing 2 mg/ml saponin either with or without cold IGF-II (500 ng/ml) for 4 h at 4^0C. Immediately following the incubation, cells were washed four times in binding buffer, pH 7.4 (the last two washes BSA free). To initiate crosslinking, 1 ml of 0.1 mM of disuccinimidyl suberate (DSS) in binding buffer, pH 7.4 (BSA free) was added to each flask for 15 min at 15^oC. The reaction was quenched by the addition of 10 mM Tris, 1 mM EDTA, pH 7.4 for 20 min at room temperature. Cells were collected by scraping and centrifugation at 2,000 rpms for 10 min at 0^oC. The cell pellets were washed three times with ice-cold PBS. Pellets were resuspended in 1 ml of PBS and aliquots were removed for protein determinations. Cells were repelleted and resuspended in 100 µl of electrophoresis sample buffer at a final concentration of 4 µg/µl. Samples were loaded and electrophoresed on a 6% SDS-PAGE gel under reducing conditions according to Laemmli (1970). After drying the gel, the labeled receptor was quantified by autoradiography with Kodak X-AR film. Scanning densitometry of the autoradiographs was performed on a MicroScan 1000 Gel Analyzer (Technology Resources, Inc.).

Enzyme Activity Measurements-

Cells were grown to ~80% confluency in 75 cm^2 tissue culture flasks. To measure secreted enzymes, cells were incubated for 5.5 h in one of the following buffers: serum-free DMEM; buffer A, pH 7.4; buffer B, pH 7.4; or buffer C, pH 7.4 (see Cell Culture METHODS)) at 37^{o}C. After incubation, media was collected and centrifuged at 1,000 rpms for 5 min at 4^{o} to remove any particulate matter. Cell monolayers were washed three times with ice-cold PBS and collected by scraping into ice-cold PBS. Cells were pelleted, resuspended in 1 ml of ice-cold PBS, and aliquots removed for counting with a Coulter model ZM Counter equipped with a model 256 Channelyzer. Cells were repelleted and lysed by sonication in 50 mM sodium acetate, 1 mM EDTA, 0.1 M NaCl, 0.2% Triton X-100, pH 5.2. Samples of media and cell lysates were kept at 0^{o}C just prior to assay or were stored at-20^{o}C until activity could be determined. Latent cathepsin B and cathepsin L activities were determined by adding 100 µl of activator (30 mM dithiothreitol, 15 mM EDTA, pH 5.2), and 100 µl of pepsin (three concentrations of pepsin were prepared; 10 mg/ml, 5 mg/ml, and 2.5 mg/ml in 0.2 M sodium acetate, pH 4.2), to 100 µl of media or cell lysate. The mixture was incubated for 30 min at 37^{o}C and stopped by the addition of 800 µl of 0.2 M citrate/phosphate buffer, pH 6.2. . The activated enzyme was measured by the addition of 100 µl of substrate. The final substrate concentrations were either 94 µM Z-Arg-Arg-AMC for cathepsin B or 5 µM Z-Phe-Arg-AMC for cathepsin L (Qian *et al.* 1989; Achkar *et al.*, 1990). Hydrolysis rates were measured at excitation and emission wavelengths of 370 nm and 460 nm, respectively. Bandwidths were set at 10 nm for emission and 7.5 nm for excitation. Prior to the enzyme assays, the spectrofluorometer was standardized

with a known concentration of the product, 7-amino-4-methyl coumarin. Active cathepsin B and cathepsin L was determined as above except pepsin was omitted, the initial incubation was done at room temperature, and 900 µl of 0.2 M citrate/phosphate buffer, pH 6.2 was added prior to substrate.

ß-Glucuronidase activity was measured according to the method of Robins, (1979). BALB and MMSV cells were pretreated as described above for cathepsins B and L. The reaction and assay was performed as follows; 330 µl of media or cell lysate was added to 660 µl of 4-methylumbelliferyl-ß-D-glucuronide (1.5 mM stock in 0.15 M sodium acetate, pH 4.4). The mixture was incubated at 37^{o}C and 300 µl aliquots were removed at 25 min, 55 min, and 85 min and added to 1 ml of 0.5 M glycine, 0.5 M Na_2CO_3, pH 10. Fluorescence intensity was measured for each of these time points at excitation and emission wavelengths of 365 nm and 448 nm, respectively. The activity of ß-glucuronidase was expressed as amount of fluorescent product produced over time and was determined from (Δ F/min) / FSTD , where delta F is the change of fluorescence over time for each sample assayed and FSTD is the slope of the standard curve. The standard curve was constructed by plotting the fluorescence intensity against known concentrations of the standard 4-methylumbeliforone (1 nM -10 µM).

[^{3}H]-Leucine Incorporation into cellular proteins-

BALB and MMSV cell were cultured to ~80% confluency in 100 mm tissue culture dishes. Cells were washed three times with either serum and leucine free DMEM or with 110 mM potassium propionate, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 44 mM $KHCO_3$, pH 7.4 and left for 1 h at 37^{o}C in the same media as used in the wash. Cells were metabolically labeled with 0.2 mCi of (4,5-^{3}H)leucine (35-50 Ci/mol,

ICN Biochemicals) for 90 min in either serum and leucine free DMEM, serum and leucine free DMEM containing 100 μM cycloheximide, or 110 mM potassium propionate buffer. The cells were then washed five times with phosphate buffered saline (PBS) and lysed with 1 ml of 1 N NaOH. Total cellular radioactivity was determined by adding the cell lysate to Ultima Gold scintillation cocktail and counting with a Beckman LS 7500 scintillation counter. Protein was determined by the BCA Protein Assay .

Preparation of microsomal membranes-

To eliminate cytosolic proteins in sample preparations used for Western blot analysis of the M6P/IGF-II receptor, microsomal membranes were isolated. BALB and MMSV cells were collected by scraping, counted and pelleted in PBS by centrifugation at 1,000 rpms for 10 min. The cell pellet was resuspended in 1 ml of hypotonic buffer [20 mM HEPES, 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT), pH 7.4], vortexed, and left to stand for 10 min at 4°C. To ensure all the cells were lysed, the suspension was treated to 20 strokes of a Dounce homogenizer, and centrifuged at 2,200 rpms for 15 min at 4°C. The supernatant was retained and the pellet was treated with hypotonic buffer and rehomogenized as above. The second supernatant was combined with the first and centrifuged at 55,000 rpm for 60 min at 4°C. The resultant pellet was resuspended at a concentration of 200 mg wet weight to 1 ml of sample electrophoresis buffer (0.5 M Tris-Hcl, 10% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-b-mercaptoethanol, and 0.05% (w/v) saturated bromophenol blue, pH 6.8) (Soutar and Wade, 1989).

Western blot analysis-

For detection 1of membrane or soluble forms of the M6P/IGF-II receptor, either microsomal membrane preparations or media samples were loaded on an SDS 4-15% gradient gel and run under nonreducing standard SDS-PAGE conditions (Laemmli, 1970) using a Bio-Rad Mini Gel Apparatus and a running buffer of 25 mM Tris-base, 192 mM glycine, pH 8.3. The outer lanes contained prestained high molecular weight markers (Sigma). Proteins were transferred from an SDS-PAGE gel to a membrane support (PVDF-Plus membrane, Micron Separations Inc.) in a Bio-Rad Transfer Apparatus with transfer buffer (25 mM Tris-base, 192 mM glycine, and 15% (v/v) methanol, pH 8.2) at 190 mA (constant) for 1 h at 4°C. Before transfer, gels were first placed in transfer buffer for 15 min at room temperature. After transfer, PVDF membranes were placed on an orbital shaker with 1X TBST (10 mM Tris-base, 150 mM NaCl, 0.05% (v/v) TWEEN-20, 0.2% (v/v) nonidet P-40 (NP-40), 2% (w/v) SDS, pH 8.0) containing 10% BSA (fraction V) for 1 h at room temperature. The primary antibody used was raised in rabbits and directed against human M6P/IGF-II receptor, and was a gift of Dr. William Sly (St. Louis University, St. Louis, MO). A titer of 1:125 was used in 1X TBST containing 1% BSA. The membrane was incubated with the primary antibody overnight at 4°C. The membrane was then washed three times with a 1:10 dilution of 10X TBST (100 mM Tris-base, 1.5 M NaCl, 0.5% (v/v) TWEEN-20, 2% NP-40, 20% (w/v) SDS,and 60 mM sodium deoxycholate, pH 8.0) for 10 min per wash per wash at room temperature. The secondary antibody (anti-rabbit IgG tagged with alkaline phosphatase, BRL) was used at a titer of 1:10,000 in 1X TBST containing 0.1% BSA. The membrane was incubated with the secondary antibody for 1 h at room temperature. The membrane was then washed three times with diluted 10X TBST for 10 min per wash. Prior to developing the membrane with the alkaline phosphatase

substrate, the membrane was placed in alkaline phosphatase buffer (100 mM Tris-base, 100 mM NaCl, 5 mM MgCl2, pH 9.5) for 10 min at room temperature. Color development was initiated by placing the membrane in alkaline phosphatase buffer containing 66 µl of Nitroblue tetrazolium (NBT) (50 mg/ml in 70% dimethyl formamide (DMF)) and 35 µl of 5-Bromo-4-chloro-3-indolyphosphate (BCIP) (50 mg/ml in 100% DMF). The membrane was removed from this solution when the protein bands were clear or the background was becoming too intense and placed in stop buffer (20 mM Tris-HCl, 5 mM EDTA, pH 8.0). Molecular weights were determined from the position of the bands and those of the prestained molecular weight markers.

Immunoprecipitation of cathepsin D and cathepsin L-

The procedures followed were adapted from several sources (Stein *et al.*, 1987c; Waheed and von Figura, 1990; Ma *et al.*, 1992). BALB and MMSV cells were grown in 60 mm tissue culture dishes under standard conditions. At time 0 min all cells were placed in methionine and cysteine free DMEM supplemented with 5% heat inactivated calf serum previously dialyzed against PBS. At 30 min, half of the culture dishes (BALB and MMSV cells) were extensively washed with 110 mM potassium propionate (buffer A, see cell culture) and then incubated in buffer A. At 60 min all cells were metabolically labeled by the addition of 200 µCi of [^{35}S]- L-methionine (specific activity, 1000 Ci/mmol) per dish and the cells were incubated for 1 hours at 37^{o}C. After labeling, the cells were washed two times with ice-cold 0.9% NaCl, and harvested by scraping in 1 ml of ice-cold lysis buffer (0.1 M sodium acetate, 0.2 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM iodoacetamide, pH 6.0) and disrupted by sonication. The resulting lysate was centrifuged at 17,000 rpm for 30 min

at 4°C. The membrane pellets were discarded and the supernatants were treated with an equal volume of -20° acetone, and the samples were left at -20°C overnight. The acetone precipitated proteins were recovered by centrifugation at 55,000 rpm for 60 min at 4°C. The protein pellets were redissolved in 600 µl of 10 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.2% (w/v) SDS, 10% (w/v) BSA (Sigma #A7638, IgG free), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 5 mM iodoactamide, pH7.4. The sample was precleared by the addition of 2 mg of Immunoprecipitin (BRL) and 5 µl of rabbit preimmune serum and left for 30 min at 4°C, followed by centrifugation at 55,000 rpm for 60 min. The supernatants were incubated overnight at 4°C with 10 µl of either anti-rat cathepsin D (a gift of Dr. Chung Lee, Northwestern Univ., Evanston, IL), or affinity purified anti-murine cathepsin L (a gift of Dr. Gary Sahagian, Tufts Univ., Boston, MA) along with 4 mg of pretreated Immunoprecipitin (see below). The mixtures were centrifuged at 55,000 rpm for 60 min and the pellets washed with the following buffers: twice with 1% Triton X-100, pH 7.4 containing 0.5 % SDS and 20 mg/ml BSA (IgG free); once with the fore mentioned buffer containing 2 M KCl; once with 10 mM Tris buffer, pH 8.5 containing 0.6 M NaCl, 0.1 % SDS, and 0.05 % Nonidet P-40; and twice with 1 mM sodium phosphate, containing 15 mM NaCl, pH 7.4.. The precipitate was resuspended by heating at 95°C for 5 min in SDS electrophoresis buffer containing 10 mM dithiotreitol and electrophoresed on a 10% SDS PAGE-gel. The proteins were visualized by fluorography.

Pretreated Immunoprecipitin was prepared according to Stein *et al.* (1987c) by making a 10% suspension of the Immunoprecipitin in 50 mM Tris-HCl, 0.15 M NaCl, 5

mM EDTA, pH 7.4 and heating the mixture for 30 min at 85°C. The sample was centrifuged at 1,000 rpm for 5 min and the precipitant was resuspended in 9 volumes of 50 mM Tris buffer used above containing 1 % SDS. The mixture was heated for 5 min at 95°C and centrifuged as above. The pellet was washed three times with 50 mM Tris buffer, followed by two washes with 10 mM sodium phosphate, pH 7.4 containing 0.15 M NaCl. The pretreated Immunoprecipitin was stored at 4°C in the 10 mM sodium phosphate buffer to which 10 mg/ml BSA and 0.04% NaN_3 had been added.

CHAPTER IV

RESULTS

Measurements of Vacuolar pH in BALB and MMSV cells-

Excitation spectra (Em=519 nm) were obtained for FITC-dextran in buffers of different pH (Figure 3) in order to generate standard curves for estimating vacuolar pH. Figure 4 shows the effect of pH on the ratio of the fluorescence at Ex_{495nm} over the fluorescence at Ex_{450nm} (Panel A), and on the ratio $\Delta F_{485}/F_{485}$ (explained in greater detail in METHODS and in Figure 4 legend). As previously reported, maximum changes were observed between pH 4.5 and 7.5, with the curve for F_{495}/F_{450} being nearly linear between pH 4.5 and 7.0, a range which includes the expected pH values of lysosomes and endosomes (Okhuma and Poole, 1978; Okhuma, 1984).

Lysosomal pH was measured in BALB and MMSV cells preloaded with FITC-dextran for 24 h , as described in METHODS. In Figure 5, lysosomal pH, calculated by the 495/450 ratio method (Okhuma and Poole, 1978; Okhuma, 1984) is shown for BALB cells as a function of the pH of the external buffer in which the cells were suspended. In the absence of monensin, this calculated pH was about 5.6 at pH 7.5, and varied by less than 0.3 units between 4.5 and 7.5. This indicated that nearly all the cell associated FITC-dextran was intracellular with little or none bound to the cell surface. Photomicrographs of cells with a Nikon Microphot fluorescence microscope confirmed that virtually all the fluorescence was cell associated and as characteristic of a vacuolar localization its distribution was not uniform, (Figure 6). Figure 5 also shows that the subsequent addition of 60 μM monensin to the cell suspensions produced a

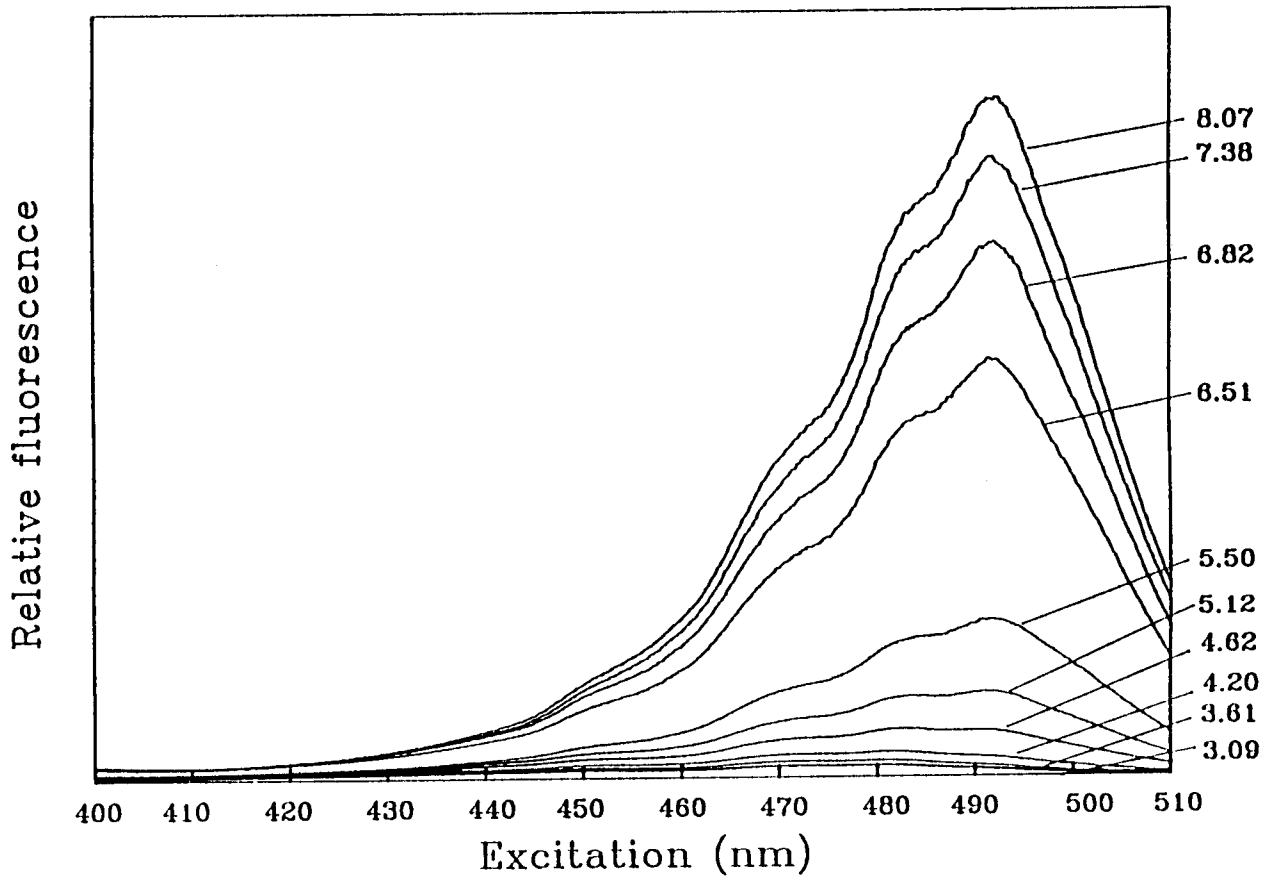

Fig. 3. FLUORESCENT EXCITATION SPECTRA OF FLUORESCEIN ISOTHIOCYANATE DEXTRAN AND ITS DEPENDENCE ON PH. Excitation spectra were collected for fluorescein isothiocyanate (FITC)-dextran (1 µg/ml) in 10 mM citrate, 173 mM sodium phosphate at twelve different pH values, ranging from 3.09 to 8.07. These spectra were used in the estimations of vacuolar pH by producing standard calibration curves (see Figure 4 and METHODS). Due to the storage limitations of the plotting program two out of the twelve pH spectra are not represented here (pHs 6.28 and 5.99). Each line represents 550 data points. Numbers on the right margin represent the pH of the buffer in which FITC-dextran spectrum was generated. These results are representative of at least three independent experiments.

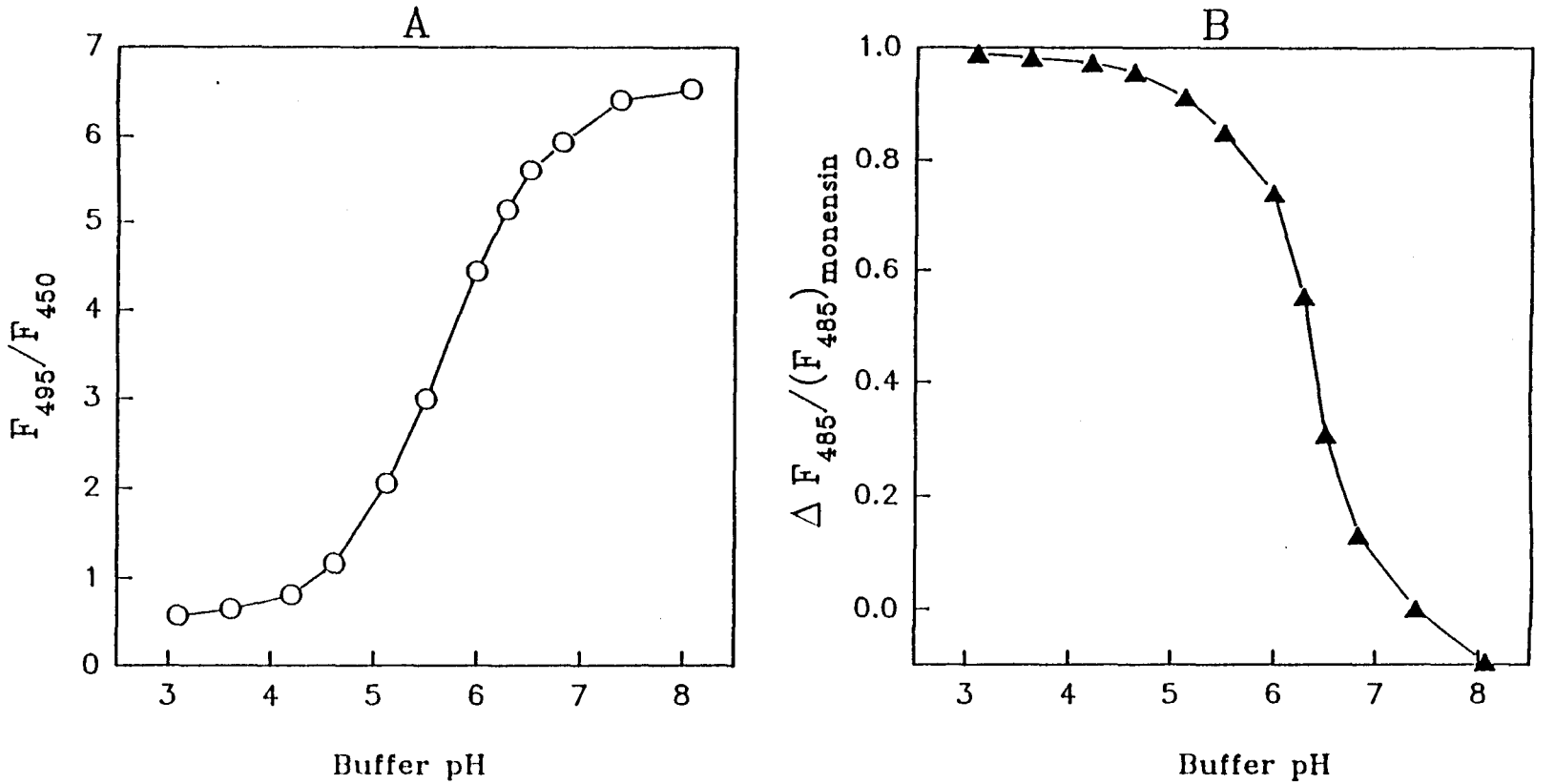

FIG. 4. TWO DIFFERENT TYPES OF STANDARD CALIBRATION CURVES USED FOR ESTIMATING VACUOLAR PH WITH FITC-DEXTRAN. A and B were generated from fluorescent excitation spectra presented in Fig. 3. A represents the ratio of the relative fluorescence at $Ex_{495nm/450nm}$ plotted against buffer pH, (method of Ohkuma and Poole,1978; Ohkuma *et al.*,1982). Vacuolar pH of unknowns could then be estimated from their spectra by calculating the F_{495}/F_{450} and extrapolating to corresponding pH. B represents the difference in relative fluorescence at Excitation 485nm between two different pH values. Where, ΔF_{485} = (F_{485} at pH 7.4) -(F_{485} at particular buffer pH) and F_{485} monensin = (F_{485} at pH 7.4). The pH of 7.4 was used because in the determination of vacuolar pH, cells were suspended in pH 7.4 buffer and the intracellular and extracellular pH were equilibrated by the addition of monensin. (See METHODS) Vacuolar pH could then be estimated from the change in fluorescence Ex485nm prior to and after the addition of monensin by comparison with the standard curve. In both methods the use of a fluorescence ratio corrects for differences in the loading of FITC-dextran between experiments.

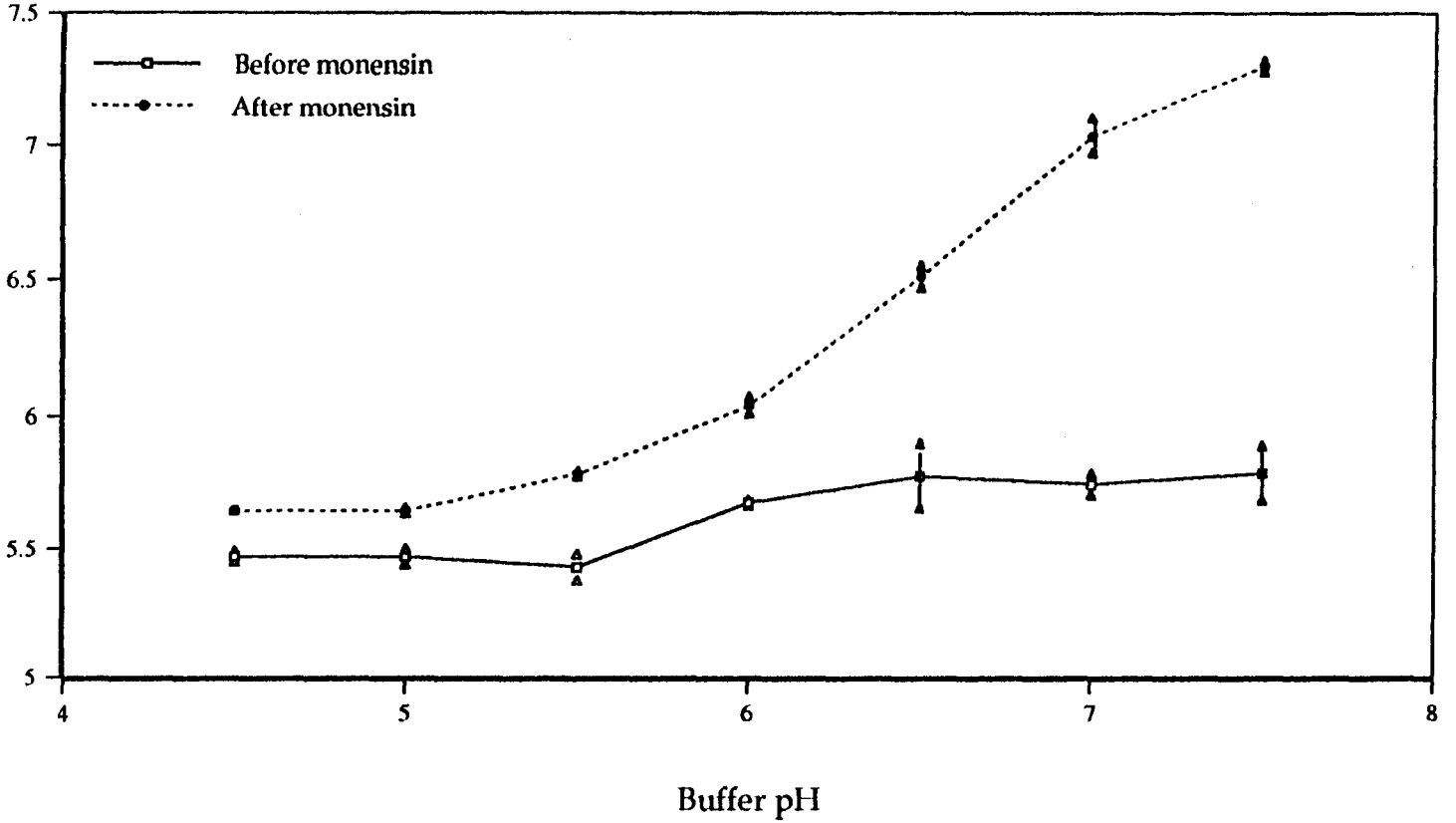

Fig. 5. FLUOROMETRIC TITRATION OF FITC-DEXTRAN IN LYSOSOMES OF BALB/3T3 FIBROBLASTS IN THE PRESENCE AND ABSENCE OF MONENSIN. Balb cells were preincubated with FITC-dextran, 1 mg/ml in DMEM, for 24 h. Cells were resuspended with continuous stirring in 20 mM MES buffer containing 130 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 5.5 mM glucose at the indicated pHs. Excitation spectra were collected before and after the addition of 60 μM monensin at emission wavelength of 519 nm. Vacuolar pH was calculated by Ex 495/450 fluorescence ratio method as described in METHODS and figure 4. The results are the mean and range of two independent sets of data.

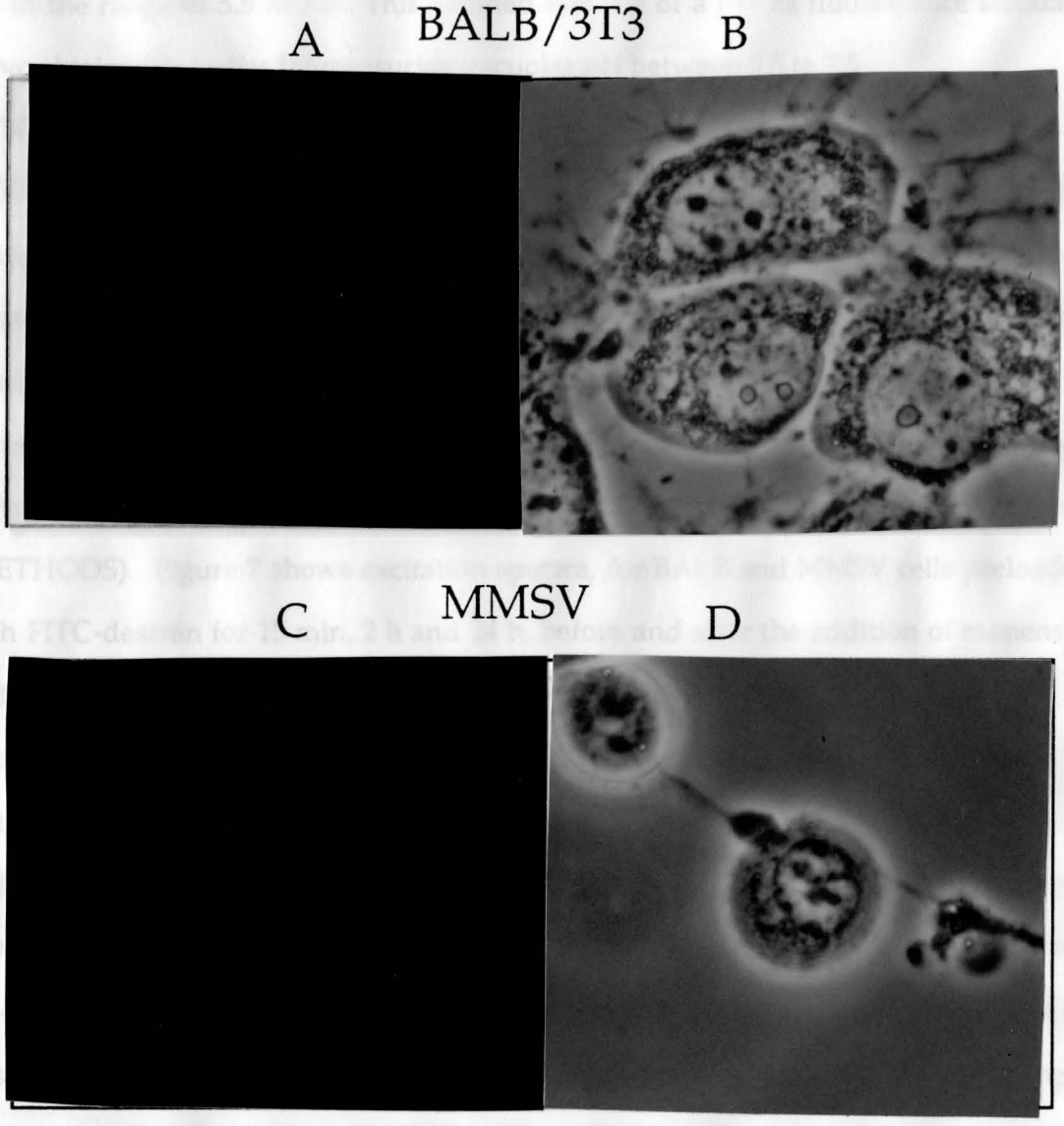

Fig. 6. FLUORESCENT AND PHASE-CONTRAST PHOTOMICROGRAPHS OF BALB/3T3 AND MMSV CELLS PREINCUBATED FOR 24 H WITH FITC-DEXTRAN. BALB and MMSV cells were grown on glass coverslips and exposed to FITC-dextran (1 mg/ml) in DMEM for 24 h. Cells were washed extensively and photographed with an 100X objective on a Nikon Microphot system. Fluorescence was observed using a filter set at $Ex_{420\text{-}485nm}$ and Em>510nm. Photographs **A** and **C** represent the fluorescence of internalized FITC-dextran in the BALB and MMSV cells, respectively. Photographs **B** and **D** are the corresponding phase contrast images.

nearly exact correspondence between the calculated lysosomal pH and the extracellular pH in the range of 5.5 to 7.5. This justified our use of a pH vs fluorescence standard curve obtained in buffer for measuring vacuolar pH between 5.5 to 7.5.

When the fluorescence signal at 450 nm was weak, as occurred after short periods of FITC-dextran uptake, vacuolar pH was more reproducibly estimated by measuring the change in fluorescence at EX 485 nm after addition of 60 μM monensin to the cell suspensions in buffer at pH 7.4. At this concentration of monensin, complete equilibrium between intracellular and extracellular pH was observed after < 1 min. To correct for differences in FITC-dextran loading between experiments, values of Δ F were divided by the fluorescence obtained in the presence of monensin at 485 nm (METHODS). Figure 7 shows excitation spectra, for BALB and MMSV cells preloaded with FITC-dextran for 15 min, 2 h and 24 h, before and after the addition of monensin. Different concentrations of FITC-dextran were used for each uptake period was to maximize the fluorescence signal at the shorter uptakes, since FITC-dextran is internalized by fluid-phase endocytosis. At any of these concentrations, the levels of internalized FITC-dextran will not excede the amounts which would alter pH dependence of its fluorescence (Ohkuma and Poole, 1978). All spectra were normalized for differences in FITC-dextran loading after the addition of monensin. In all cases it cn be seen that the fluorescence above 450 nm in the absence of monensin is more strongly quenched in BALB cells than in MMSV cells, indicative of a more acidic environment. Table 1 contains the calculated average vacuolar pH of the various compartments which were progressively filled during the proceeding period of uptake. The values in Table 1 are the average of 5 measurements. Cell viabilities, determined by the trypan blue exclusion method at the end of the measurements, were found to range from 80 to 90%. Additionally, excitation spectra were collected for cell cultures which were not cell scraped prior to collection (as described in METHODS) but rather lifted from culture

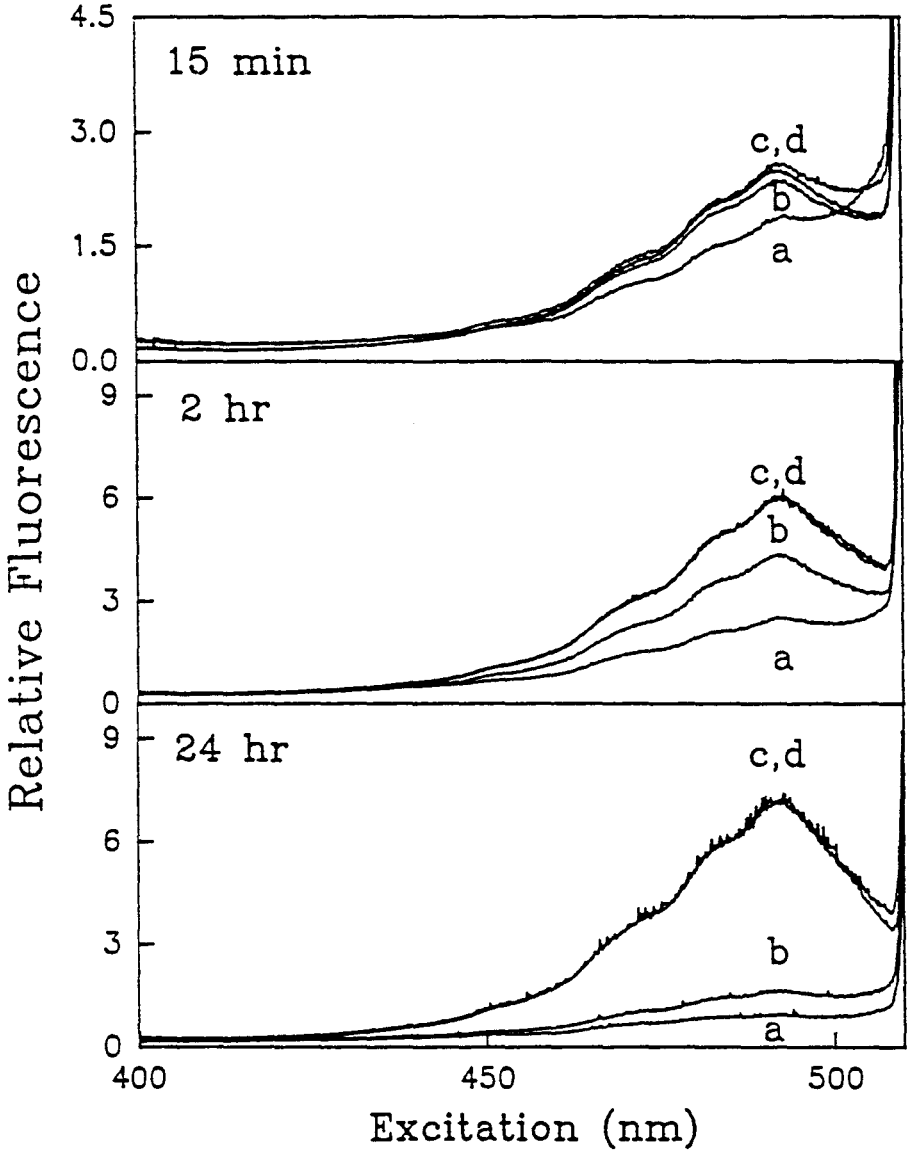

Fig. 7. MEASUREMENT OF VACUOLAR PH ALONG THE ENDOSOMAL/LYSOSOMAL PATHWAY IN BALB AND MMSV-TRANSFORMED BALB FIBROBLASTS. Cells were incubated with FITC-dextran: 10 mg/ml for 15 min; 5 mg/ml for 2 h; 1 mg/ml for 24 h. Cells were resuspended in buffer D (METHODS). The spectra in the presence and absence of 60 μM monensin were normalized to correct for differences in FITC-dextran between BALB and MMSV cells. Vacuolar pH was calculated from ΔF at Ex485 after addition of monensin. The spectra are identified as follows: a, BALB cells prior to monensin; b, MMSV cells prior to monensin; c, BALB cells after monensin; and d, MMSV cells after monensin. The results are representative of four independent experiments.

TABLE 1. Vacuolar pH along the endosomal/lysosomal pathway in BALB/3T3 fibroblasts and Moloney murine sarcoma virus-transformed BALB/3T3 fibroblasts[a]

	Incubation Time		
Cell line	15 min	2 h	24 h
BALB/3T3	6.50 ± 0.06	5.87 ± 0.15	5.27 ± 0.06
MMSV	6.97 ± 0.16 [d*]	6.37 ± 0.28 [d**]	5.68 ± 0.08 [d***]
ΔpH [c]	0.47	0.50	0.41

a
Cellular monolayers were incubated with FITC-dextran for 15 min (10 mg/ml), 2 h (5 mg/ml), and 24 h (1 mg/ml). Excitation spectra were collected from stirred cell suspensions as described in METHODS.

b
The average pH of vacuolar compartments which were filled with FITC-dextran during indicated times was estimated from the change in fluorescence at Ex 485nm after the addition of monensin as described in METHODS. The results are the mean and standard deviation of five independent set of data.

c
Represents the difference between the mean estimated pH values for BALB/3T3 cells and MMSV cells at a specific incubation time.

d
The statistical significance of the difference between the pH at a given incubation time for BALB and MMSV cells was determined with the Student T-test.
*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$

flasks with 0.25% trypsin/ 1 mM EDTA. This was performed to confirm the former pH measurements were not influenced by FITC-dextran linkage due to the possible damage the cells had to endure with scraping. These results confirmed that the pHs of vacuolar compartments in MMSV cells are consistently 0.4 to 0.5 units higher than in nontransformed BALB cells.

Acidification of BALB and MMSV cells with Potassium Propionate-

The cell cytoplasm can be acidified after a brief incubation with a short chain fatty acid in the absence of sodium or in the presence of an inhibitor of the plasma membrane sodium/proton antiporter (Grinstein *et al.*, 1984). The general mechanism for this is presented in Figure 8. Figure 9 shows the normalized excitation spectra of BALB and MMSV cells preloaded with FITC-dextran for 24 h. The results indicate that potassium propionate in a sodium free medium can lower the pH of vacuolar compartments in cultured cells. The calculated pH decreased from 5.7 to 5.0 in MMSV cells and from 5.3 to 5.1 in BALB cells. These lower pHs were maintained for the duration of the exposure of the cells to potassium propionate (> 1 h) in the spectrofluorometer cell.

Measurements of Cytosolic pH in BALB and MMSV cells-

The membrane permeate probe, BCECF/AM, was used to determine the effect of potassium propionate on cytosolic pH and has been well established as an indicator of cytosolic pH (reviewed in, Haugland, 1992). A standard curve used in estimating cytosolic pH for cultured cells was produced by preloading BALB cells with BCECF/AM and suspending the intact cells in high potassium containing buffers of different pH in the presence of nigericin. Because the BCECF/AM hydrolysis product, BCECF is also a fluorescein derivative, the excitation spectra have the same general shape as that of FITC-dextran. However, the standard curve for BCECF is produced in the same environment, the cytosol, from which the unknown pH'sare to be estimated. When preloaded BALB cells are placed in a high potassium buffer with the K^+/H^+

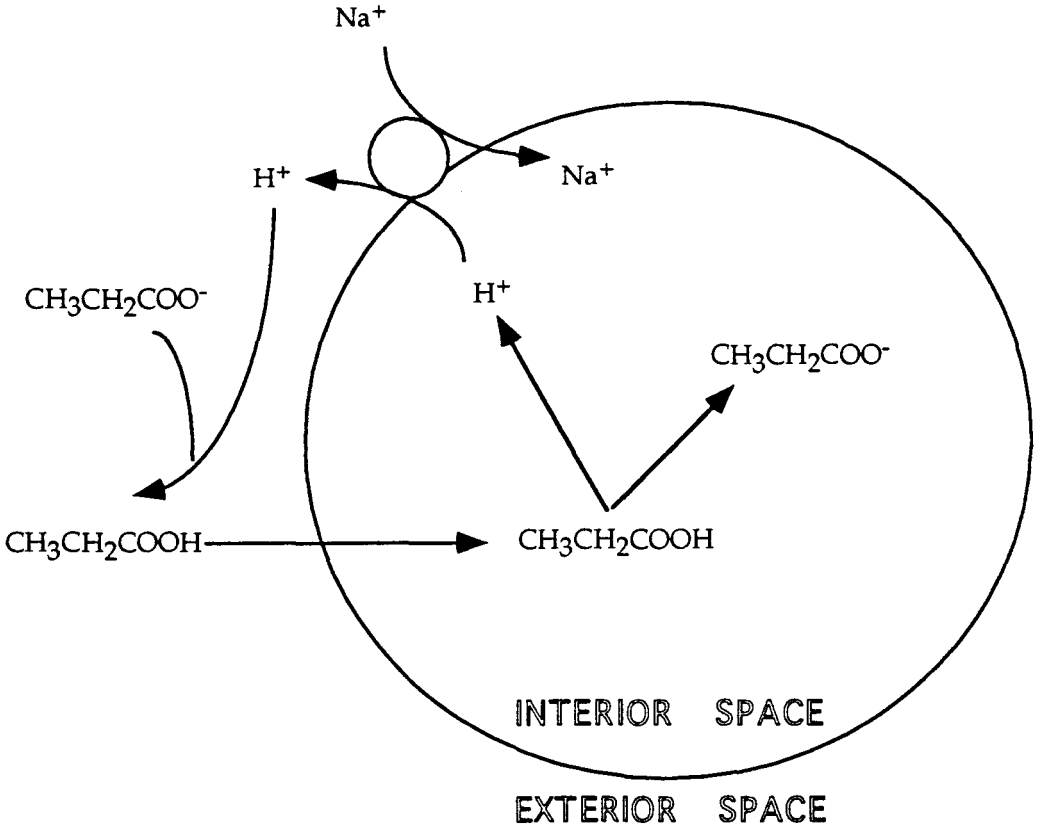

Fig. 8. MECHANISMS BY WHICH POTASSIUM AND SODIUM PROPIONATE MECHANISM DECREASE CELLULAR PH. Scheme for generating an acidic interior either by treating cells with potassium propionate in the absence of extracellular sodium or with sodium propionate in the presence of amiloride (an inhibitor of the Na^+/H^+ pump exchanger). $CH_3CH_2COO^-$ represents the propionate ion. (Adapted from Grinstein *et al.*, 1989)

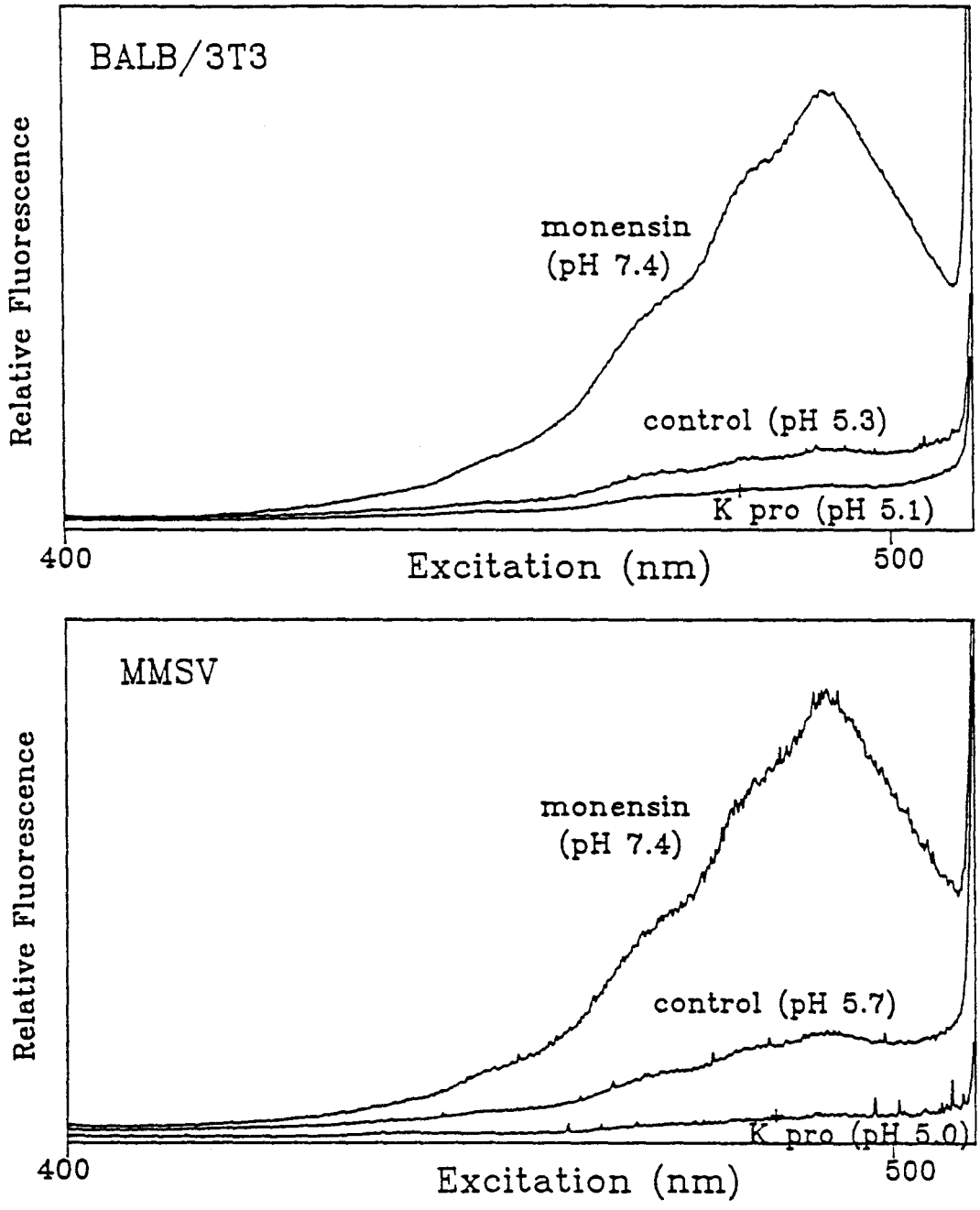

Fig. 9. EFFECT OF POTASSIUM PROPIONATE ON THE PH OF LYSOSOMES IN BALB AND MMSV FIBROBLASTS. Cells were preincubated for 24 h with FITC-dextran (METHODS). Cells were then resuspended in either buffer D (130 mM NaCl) or 20 mM HEPES buffer, pH 7.4, containing 140 mM potassium propionate, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose. Excitation spectra were recorded before and after the addition of 60 μM monensin. The spectra were normalized for differences in FITC-dextran uptake, and vacuolar pH was calculated from the ΔF at Ex485 after addition of monensin. The results are representative of at least three independent experiments.

ionophore, nigericin, intracellular pHs will equilibrate with the extracellular pH. Figure 10 represents the excitation spectra for BALB cells loaded with BCECF/AM under these conditions. Because BCECF/AM slowly leaks out of cells, correction for leakage was performed on the supernatant after initial data was collected. In Figure 10, the inset A, represents the pH calibration curve constructed from the ratio of the relative fluorescence at Ex 497 nm/437 nm. Figure 11 contains the fluorescent spectra for BALB and MMSV cell suspensions from which the values of cytosolic pH were calculated. These are summarized in Table 2.

Measurements of Cathepsin B and Cathepsin L Secretion -

MMSV cells, when incubated for 5.5 h in serum free DMEM, secreted 10-fold and 22-fold more total cathepsin B and total cathepsin L, respectively, than did nontransformed BALB cells (Table 3 and Figures 12 and 13) and about 2-fold more active ß-glucuronidase (Figure 14). Ninety two and 95 % of the secreted cathepsins B and L were in the latent, precursor forms. In addition, a very large proportion of the intracellular cathepsin L (80 %) in MMSV cells was in a latent form as compared with cathepsin B (12 %). When MMSV cells were incubated for 5.5 h with isotonic potassium propionate (buffer A), pH 7.4, in place of DMEM, there occurred dramatic, 93 % and 97 %, reductions in the secretion of total cathepsin B and total cathepsin L (Table 3). Sonme of the reduction in the levels of latent cathepsin B and cathepsin L may be due to a partial inhibition of protein synthesis caused by potassium propionate (see below). In Figure 12 and 13 levels of latent cathepsin B and cathepsin L were corrected for possible effects on protein synthesis. This reduced the the apparent effect of potassium propionate on cellular levels of latent cathepsin B and cathepsin L. However, the almost complete inhibition of secretion of the latent form of these enzymes is much too great to be explained in this way (see below). Potassium propionate produced little change in the already low levels of enzyme secretion by BALB cells, and potassium

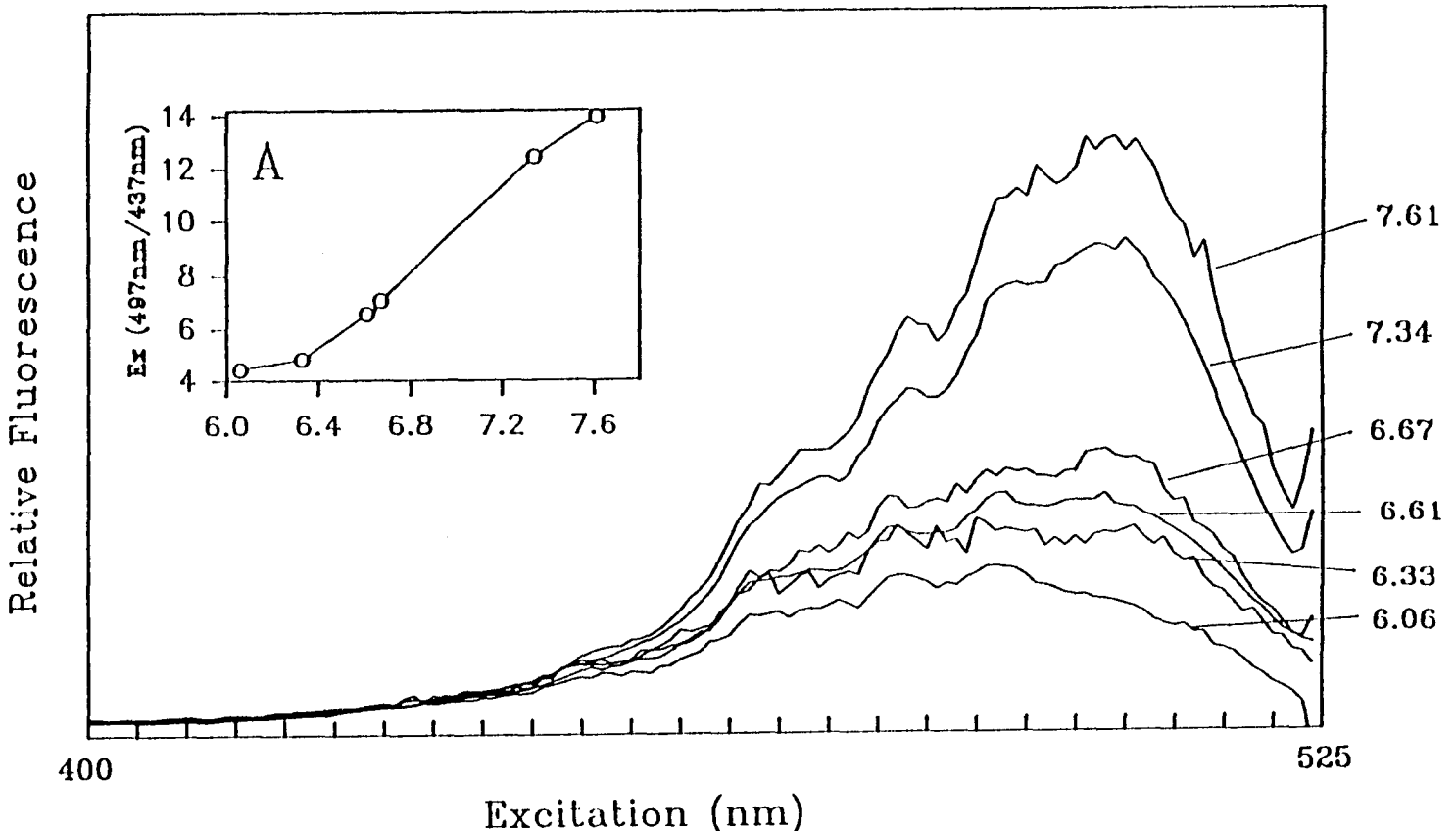

Fig. 10. *IN SITU* GENERATED FLUORESCENT EXCITATION SPECTRA AND pH STANDARD CURVE FOR BCECF IN BALB/3T3 CELLS USED IN THE DETERMINATION OF CYTOSOLIC PH. BALB cells were loaded with BCECF/AM and prepared as described by Thomas *et al.* (1979) and in METHODS. Each spectrum was generated when the preloaded cells were placed in high potassium buffer (145 mM KCl) containing 10 μM nigericin at different pHs. Nigericin will equilibrate the internal and the external pH in the presence of high concentrations of potassium. Excitation spectra were collected at Em_{530nm}. Values on the right represent the external buffer pH. Inset A, represents the standard prepared from curve these spectra used to estimate cytosolic pH. Essentially, the ratio of the relative fluorescence at Ex_{497}/Ex_{437nm} was plotted against external pH (similiar to that performed for FITC-dextran). These spectra and inset A are representative of one set of data collection.

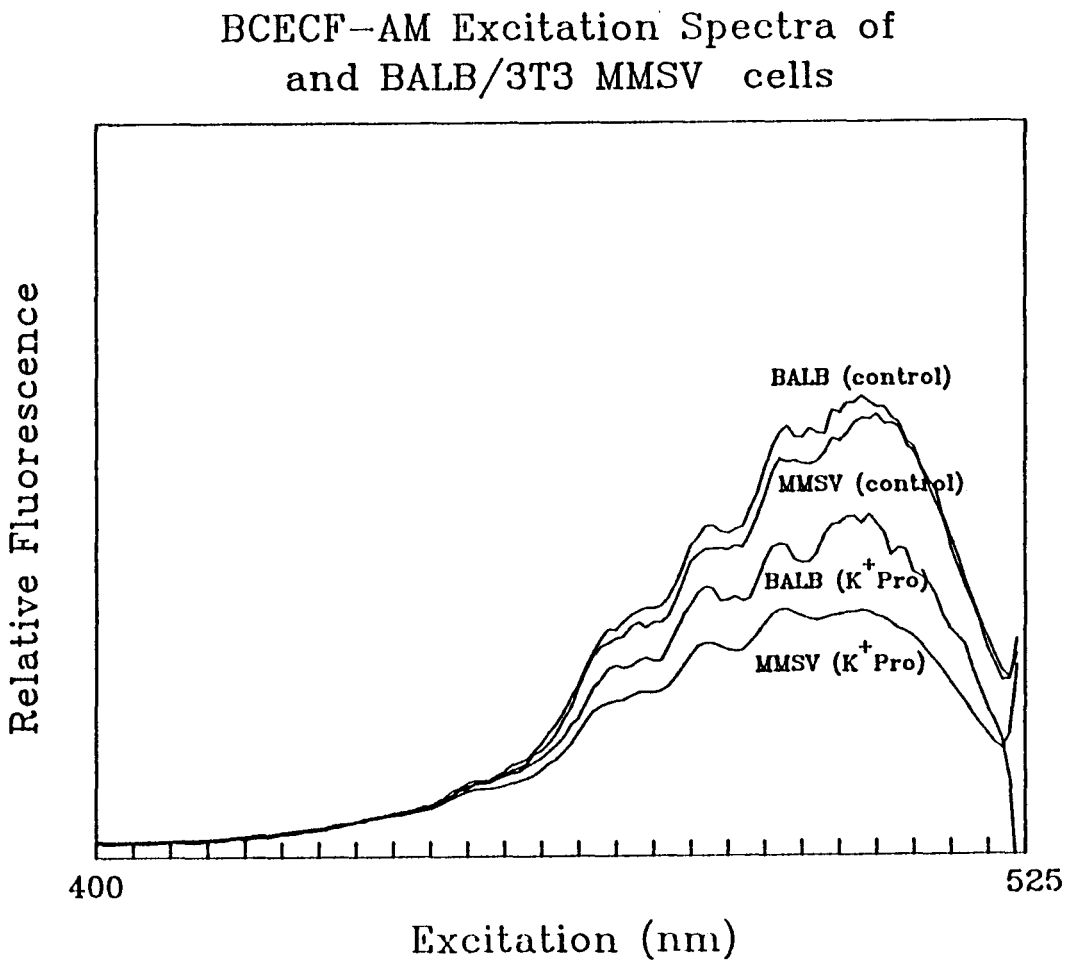

Fig. 11. EFFECT OF POTASSIUM PROPIONATE ON THE CYTOSOLIC PH FOR BALB/3T3 AND MMSV FIBROBLASTS. BALB and MMSV cells were loaded with 2 μM BCECF/AM in either HANK's buffer (control), pH 7.4 or 110 mM potassium propionate, pH 7.4, as described in METHODS. Cytosolic pH estimations were determined for each of the spectrum by calculating the ratio for the relative fluorescence at Ex_{497nm}/Ex_{437nm} and extrapolating to the corresponding pH using the standard calibration curve generated in Figure 10. These spectra are representative of one of the two independent experiments performed.

TABLE 2. Estimated cytosolic pH in BALB/3T3 fibroblasts and Moloney murine sarcoma virus-transformed BALB/3T3 fibroblasts [a].

	Estimated cytosolic pH	
Cell Type	BALB	MMSV
Control [b] (DMEM)	7.19 ± 0.01	7.140 ± 0.004
KPRO [c]	6.79 ± 0.05	6.570 ± 0.014

[a] BALB and MMSV cell suspensions were loaded with 2 μM BCECF/AM as described in METHODS. Cytosolic pH estimations were calculated from the $Ex_{497/437nm}$ ratios obtained from the fluorescent excitation spectra as presented in Figure 11 and extrapolating the pH from the standard curve presented in Figure 10. This is analogous to measuring vacuolar pH from the Ex_{495}/Ex_{450} ratio. However, in this case the standard curve was generated from spectra obtained with intracellular fluorophore and rhus corresponds to the actual cellular environment.

[b] For the determination of BALB and MMSV cytosolic pH under standard culture conditions, cells were loaded with BCECF/AM for 15 min in Hank's buffer (10 mM HEPES containing 135 mM NaCl, pH 7.4), as described in METHODS, washed and pelleted and resuspended in Hank's buffer. Excitation spectra were collected as before.

[c] BALB and MMSV cells were placed in 10 mM HEPES containing 110 mM potassium propionate, pH 7.4 for 75 min prior to the addition of BCECF/AM, during 15 min loading with BCECF/AM and during collection of spectra. Excitation spectra were collected as above.

[d] The results are the mean and range of two independent studies.

TABLE 3. Effect of culture medium on intracellular and secreted forms of cathepsin B and cathepsin L in BALB/3T3 and Moloney murine sarcoma virus-transformed BALB/3T3 fibroblasts.

		[a] CATHEPSIN B (pmol/min/10^6 cells)			[b] CATHEPSIN L (pmol/min/10^6 cells)		
	[c] medium	cell latent	cell active	total media	cell latent	cell active	total media
BALB/3T3	DMEM	28 ± 17	270 ± 28	22 ± 12	263 ± 50	255 ± 26	103 ± 36
	KPRO	ND	323 ± 11 *	15 ± 12	133 ± 76 *	337 ± 55 *	80 ± 18
	KCl	ND	251 ± 43	32 ± 3	113 ± 15 *	370 ± 25 *	91 ± 5
	NMG	5 ± 4	310 ± 61	21 ± 6	200 ± 27	219 ± 10	98 ± 2
MMSV	DMEM	81 ± 13	637 ± 13	209 ± 28	1563 ± 167	214 ± 29	2227 ± 373
	KPRO	27 ± 30 *	439 ± 44 **	15 ± 8 **	590 ± 112 **	324 ± 8 **	56 ± 39 **
	KCl	ND	1813 ± 274**	235 ± 3	1077 ± 210 *	591 ±150 *	2426 ± 94
	NMG	53 ± 7 *	590 ± 27	237 ± 16	1408 ± 14	251 ± 35	2423 ± 110

[a] Cathepsin B determined with 94 μM Z-Arg-Arg-AMC.
[b] Cathepsin L determined with 5 μM Z-Phe-Arg-AMC.
[c] DMEM refers to serum-free Dulbecco's modified Eagle's medium, pH 7.4;
KPRO refers 110 mM potassium propionate, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 44 mN $KHCO_3$, pH 7.4;
KCl refers to 111 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 44 mM $KHCO_3$, pH 7.4;
NMG refers to 110 mM N-methyl-D-glucamine chloride, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 44 mM $KHCO_3$, pH 7.4.
[d] ND indicates not detected
[e] All values are the mean and standard deviation of four determinations. The statisical significance of the differences between DMEM and KPRO,KCl, and NMG were determined with the Student T-test. *, $P < 0.05$; **, $P < 0.005$. NOTE: The the values represented in this Table have not been corrected for protein synthesis inhibition by potassium propionate.

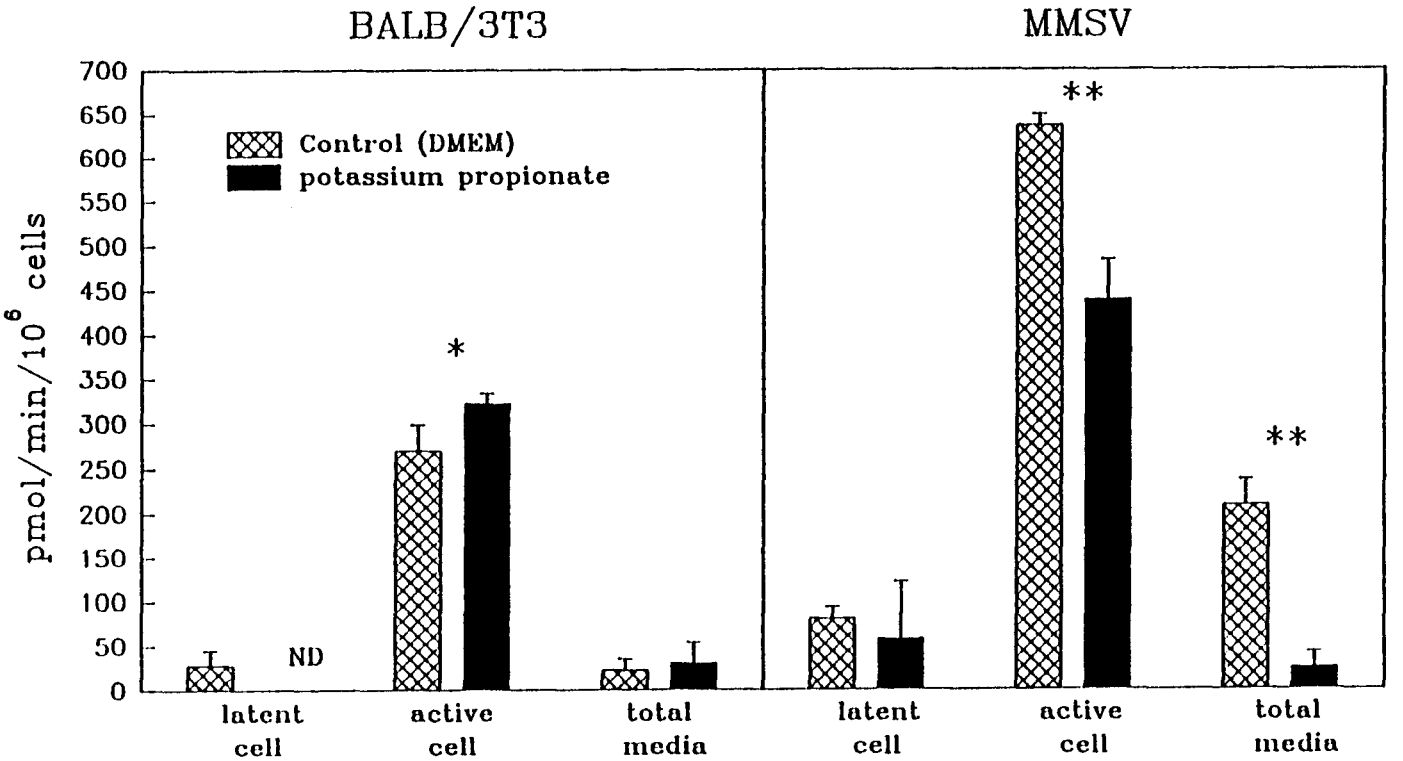

Fig. 12. EFFECT OF POTASSIUM PROPIONATE ON CELLULAR AND SECRETED CATHEPSIN B IN BALB/3T3 AND MMSV FIBROBLASTS. Cells were preincubated for 5.5 h in either serum-free DMEM (CONTROL) or 110 mM potassium propionate, pH 7.4 at 37°C. After collecting cells and media, cathepsin B was measured with 94 μM Arg-Arg-AMC, as described in METHODS. Latent enzyme represents the difference in activity before and after pretreatment for 30 min with pepsin. Media activity is expressed as total media since the latent enzyme contributes greater than 80 % to total activity. Note: All potassium propionate treated sample activities have been corrected for inhibition of protein synthesis using % inhibition calculated from [^{3}H]-leucine incorporation (see figure 15 and text). The results are the mean and standard deviation of four experiments. The statistical significance of differences between the control and potassium propionate treated samples was determined with the Student T-test. *, $P < 0.05$; **, $P < 0.005$. ND indicates not detected.

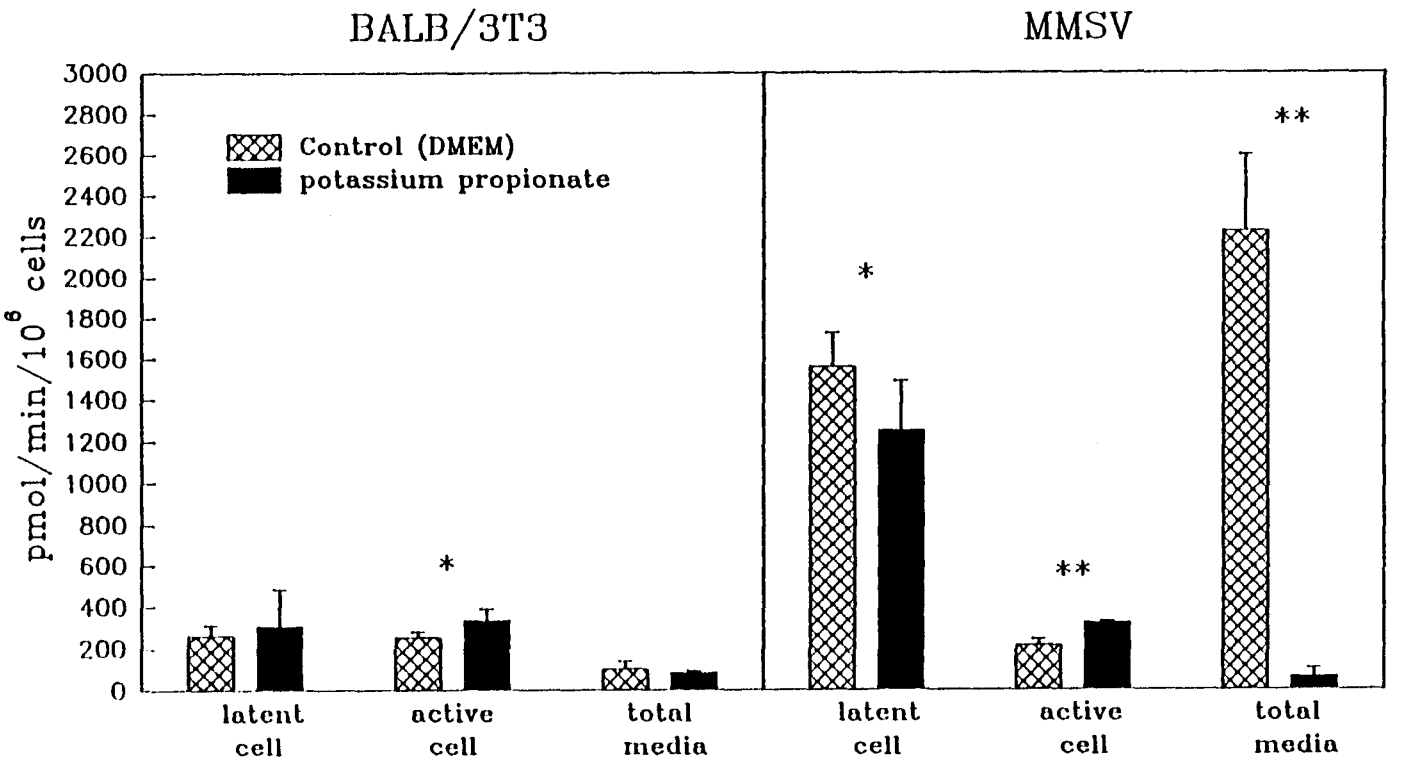

Fig. 13. EFFECT OF POTASSIUM PROPIONATE ON CELLULAR AND SECRETED CATHEPSIN L IN BALB/3T3 AND MMSV FIBROBLASTS. Cathepsin L was measured with 5 μM Z-Phe-Arg-AMC, as described in figure 12 and METHODS. Activities for potassium propionate treated samples have been corrected for inhibition of protein synthesis as done in figure 12. The results are the mean and standard deviation of four experiments. The statistical significance of differences between the control and potassium propionate treated samples was determined with the Student T-test. *, $P < 0.05$; **, $P < 0.005$. ND indicates not detected.

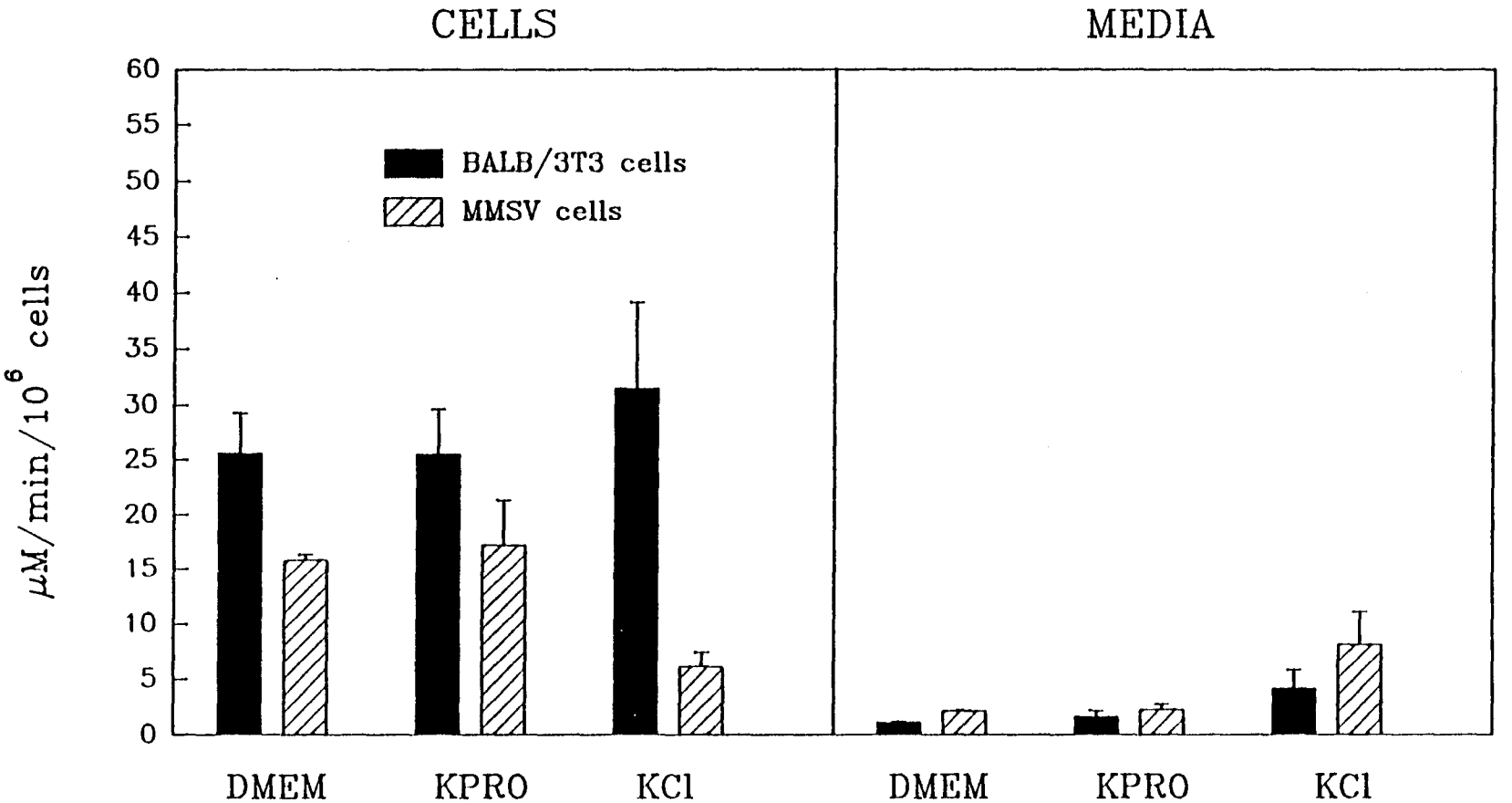

Fig. 14. EFFECT OF POTASSIUM PROPIONATE ON CELLULAR AND SECRETED β-GLUCURONIDASE IN BALB/3T3 AND MMSV FIBROBLASTS. Cells were preincubated for 5.5 h in either serum-free DMEM (CONTROL), 110 mM potassium propionate, (buffer A), or 110 mM KCl(buffer B), all at pH 7.4 and at 37°C. After collecting the cells and media, β-glucuronidase was assayed according to the method of Robbins, (1979). This involved a time point assay where samples were incubated with the substrate 4-methylumbelliferyl-β-D-glucuronide at 37°C. Fluorescent product was measured at 3 times over the course of 85 min. The results represent the mean and range of two independent experiments.

propionate did not strongly affect cellular levels of active cathepsin B and cathepsin L. Isotonic potassium chloride (buffer B), pH 7.4 and N-methyl-D-glucamine chloride (buffer C), pH 7.4, were unable to substitute for potassium propionate in reducing the secretion of procathepsin B and procathepsin L by MMSV cells (Table 3). However, potassium chloride did appear to increase cellular levels of active cathepsins B and L while decreasing cellular levels of the latent forms.

It has previously been shown that most of the latent cathepsin B and latent cathepsin L secreted by BALB and MMSV cells were newly synthesized (Achkar *et al*, 1990). To demonstrate that the inhibition of secretion was not due to an effect of potassium propionate on protein synthesis, we measured the incorporation of tritiated leucine into cellular proteins (Figure 15). Potassium propionate inhibited protein synthesis in BALB and MMSV cells by 57 % and 53 %, respectively. However, this inhibition was insufficient to account for the much more profound inhibition of procathepsin B and procathepsin L secretion caused by potassium propionate under these same conditions. When levels of latent forms were adjusted for changes in protein synthesis (Figures 12 and 13), potassium propionate could still be seen to inhibit MMSV cell secretion of cathepsin B and cathepsin L by 89 % and 96 %, respectively.

Effects of Potassium Propionate on the Synthesis of Cathepsins D and L-

Immunoprecipitation studies were performed in order to verify that the inhibition of lysosomal enzyme secretion caused by potassium propionate was not due to an inhibition of synthesis. BALB and MMSV cells were labeled for 60 min with [^{35}S]L-methionine in the presence or absence of potassium propionate buffer and the newly synthesized cathepsin D and cathepsin L were immunoprecipitated as described in METHODS. In Figure 16 the arrows indicate the position of both enzymes, with procathepsin at a Mr~42 kDa and procathepsin L at a M_r~36 kDa.

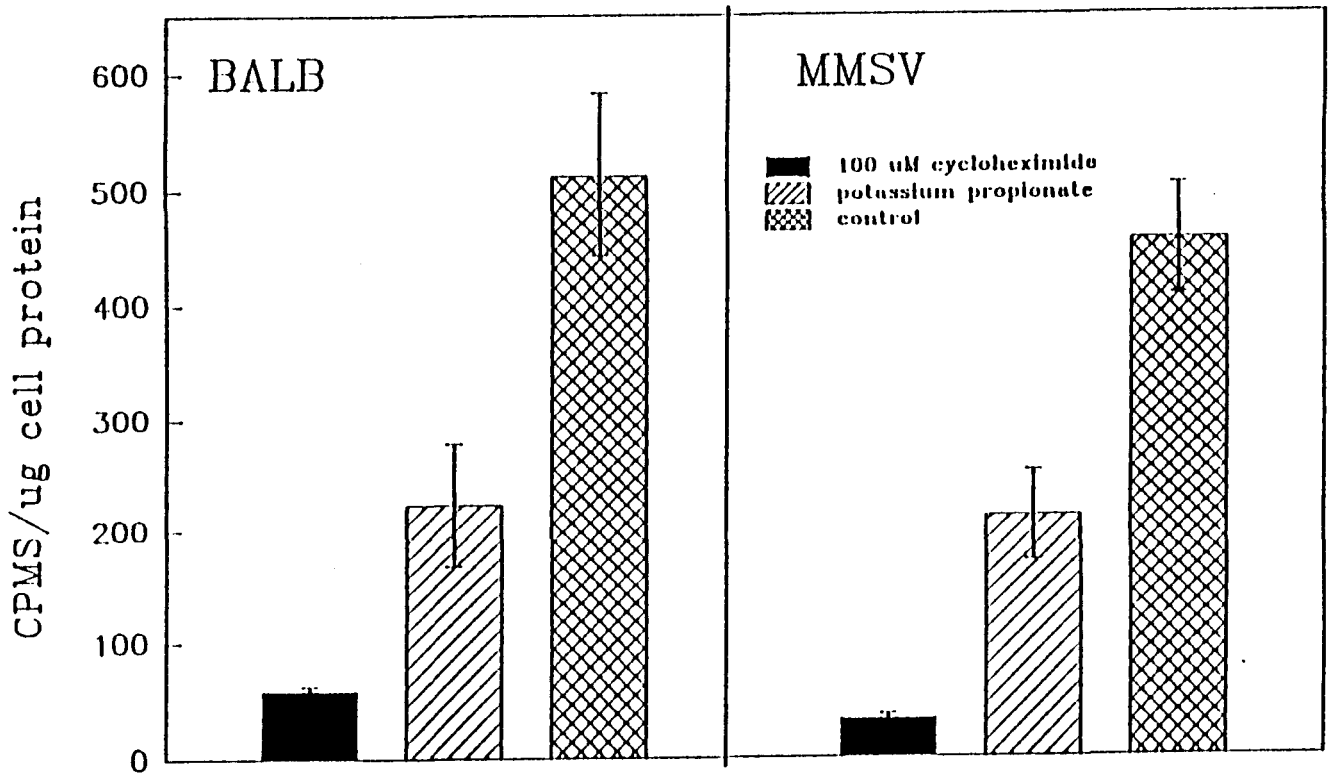

Fig. 15. EFFECT OF POTASSIUM PROPIONATE ON THE INCORPORATION OF [^{3}H]-LEUCINE INTO NEWLY SYNTHESIZED PROTEINS IN BALB/3T3 AND MMSV CELLS. Activity represents the amount of [^{3}H]-leucine incorporation in BALB and MMSV cells as described in METHODS. The total activity shown has been normalized to total cellular protein. The results are the mean and standard deviation of three independent experiments.

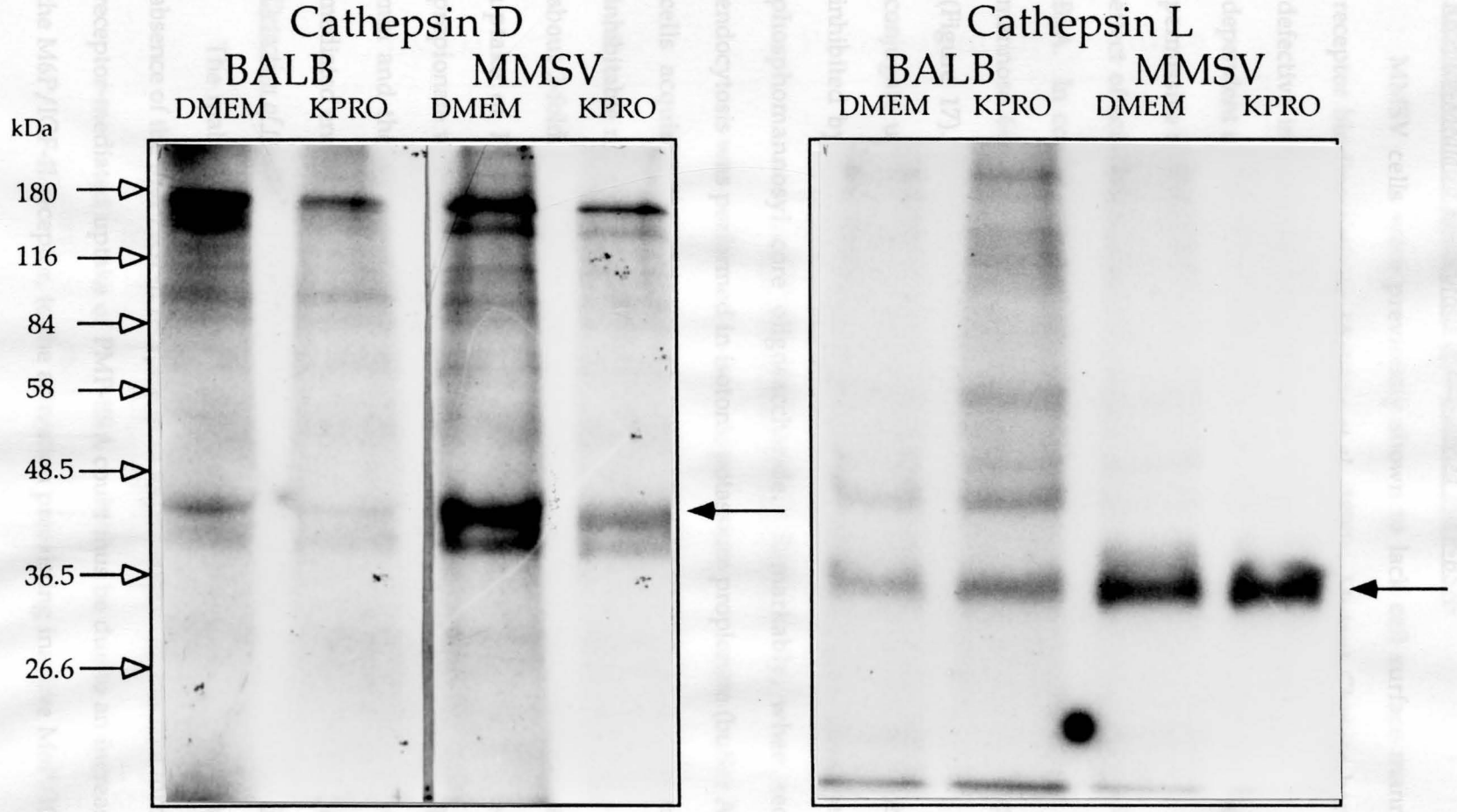

Fig. 16. EFFECT OF POTASSIUM PROPIONATE ON THE LEVELS OF NEWLY SYNTHESIZED CATHEPSIN D AND CATHEPSIN L IN BALB/3T3 AND MMSV-TRANSFORMED BALB/3T3 FIBROBLASTS. BALB and MMSV cells were either pretreated with potassium propionate or in DMEM (METHODS) and soluble proteins were immunoprecipitated with anti-cathepsins D or L. Immunoprecipitants loaded on 12% SDS-PAGE gels under reducing conditions. Detection [^{35}S]-radiolabeled proteins was done by fluorography.

Receptor-Mediated Endocytosis of ^{125}I-labeled PMP-BSA-

MMSV cells were previously shown to lack cell surface mannose 6-phosphate receptor binding activity (Achkar *et al.*, 1990). Mutant Chinese hamster ovary cells, defective in endosomal acidification, recover the capacity to carry out the receptor dependent uptake of mannose 6-phosphate containing ligands after being returned to a permissive temperature (Roff *et al.*, 1986). Consequently, we sought to investigate the effect of acidification with potassium propionate on the uptake of ^{125}I-labeled PMP-BSA. In confirmation of earlier studies, MMSV cells were unable to carry out the mannose 6-phosphate dependent uptake of pentamannosyl 6-phosphate linked BSA (Figure 17). In contrast, BALB cells took up appreciable amounts of the PMP-BSA conjugate under these same conditions. Internalization of radiolabeled ligand was inhibited by the competing ligands, mannose 6-phosphate and the unhydrolyzable phosphomannosyl core oligosaccharide. Remarkably, when receptor mediated endocytosis was performed in isotonic potassium propionate (buffer A), pH 7.4, MMSV cells acquired the capacity to internalize PMP-BSA in a mannose 6-phosphate inhibitable manner. This uptake exceeded that of BALB/3T3 cells in DMEM alone by about 2-fold. Curiously, potassium propionate also stimulated the receptor mediated uptake of PMP-BSA by BALB cells to similar levels. This effect of potassium propionate was reversible. When cells were pretreated in potassium propionate for 90 min and then allowed to take up PMP-BSA in DMEM, no stimulation of receptor mediated endocytosis was observed for either the BALB or the MMSV cells.

Detection of the M6P/IGF-II Receptor by Affinity crosslinking and Western Blot Analysis-

The inability of MMSV cells to bind and internalize PMP-BSA may be due to an absence of the M6P/IGF-II receptor or to its presence in an inactive form. Stimulation of receptor-mediated uptake of PMP-BSA could thus be due to an increase in the levels of the M6P/IGF-II receptor, to the activation preexisting inactive M6P/IGF-II receptor, or

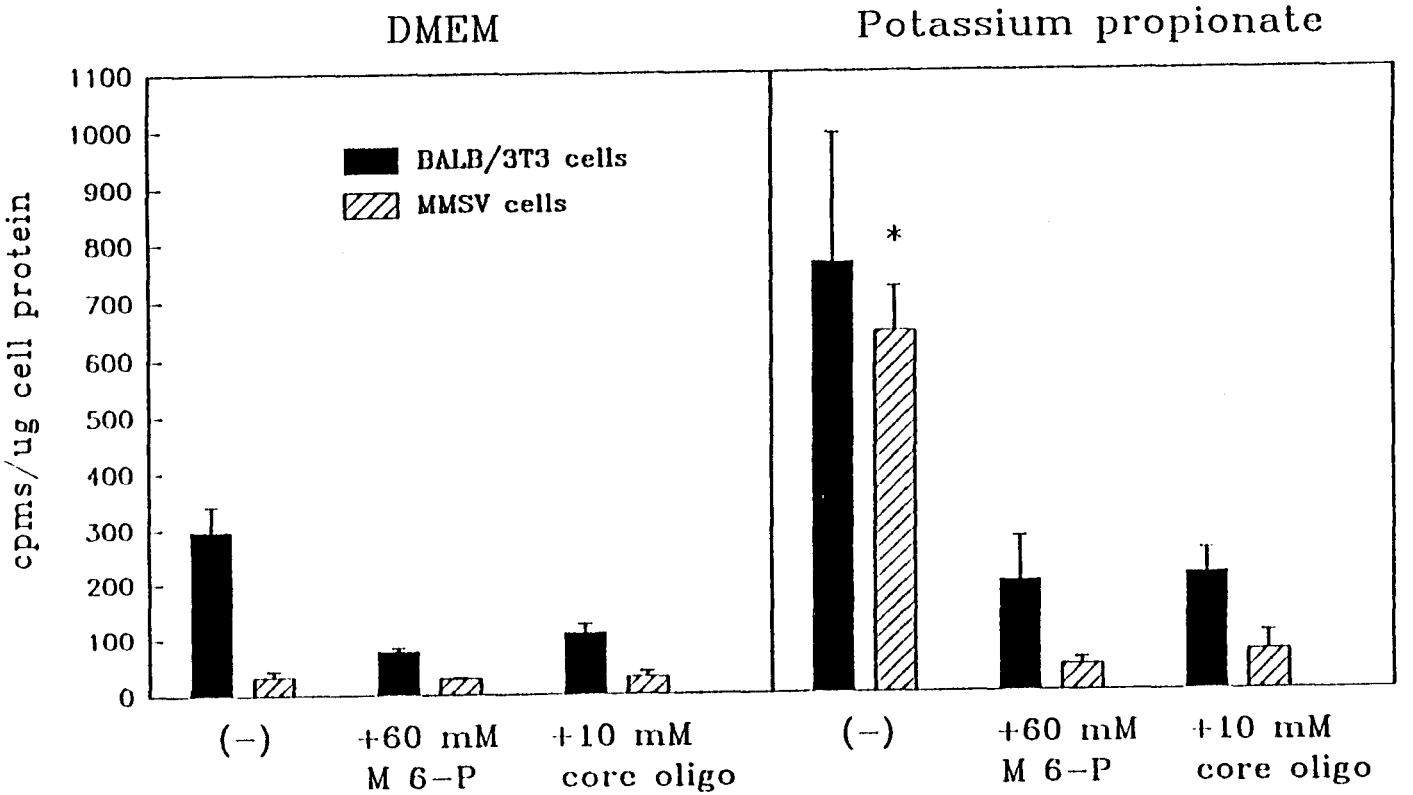

Fig. 17. EFFECT OF POTASSIUM PROPIONATE ON RECEPTOR-MEDIATED ENDOCYTOSIS OF [^{125}I]-RADIOLABELED PMP-BSA IN BALB/3T3 AND MMSV FIBROBLASTS. Cells were preincubated for 90 min in either serum-free DMEM or 110 mM potassium propionate, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 44 mM $KHCO_3$, pH 7.4 at 37°C. Uptake was initiated by the addition of [^{125}I]-PMP-BSA (1 μg/ml, specific activity=1.78 X 10^6 cpms/μg) in the same buffer as used in the preincubation step (METHODS). Nonspecific uptake was measured in the presence of either 60 mM mannose 6-phosphate or 10 mM unhydrolyzable core oligosaccharide (METHODS). The results are the mean and standard deviation of four independent experiments. The statistical significance of the differences between control (DMEM) and potassium propionate treated samples was determined with the Student T-test. *, $P < 0.01$.

to activation of the 46 M6P kDa receptor which does not normally participate in receptor-mediated endocytosis (Watanabe *et al.*, 1990). To distinguish between some of these possibilities, the status of the M6P/IGF-II receptor was evaluated by crosslinking to IGF-II and by Western blot analysis. Initially, [^{125}I] IGF-II was used to probe for M6P/IGF-II receptor on the cell surface and in the total population of cellular membranes of BALB and MMSV cells as described in METHODS. After autoradiography, BALB cell surface M6P/IGF-II receptor appeared as a very faint band at ~290 kDa, just barely visible to the eye. However, due to the limitations of the densitometer, no quantitation could be made. A much more pronounced band appeared for binding of [^{125}I]-IGF-II to total membranes from BALB cells permeabilized with saponin (Figure 18). Potassiumpropionate did not have any effect on the levels of either cell surface or total M6P/IGF-II receptor. MMSV cells showed the absence of any M6P/IGF-II receptor which could be crosslinked to [^{125}I] IGF-II. These results were confirmed by Western blot analysis. A band at 270 kDa was seen for BALB cell membrane preparations with equal intensity for control (DMEM) and potassium propionate treated cells. In contrast, the MMSV cells lacked any detectable M6P/IGF-II either before or after potassium propionate treatment (Figure 19). Additionally, conditioned media samples revealed no detectable soluble M6P/IGF-II receptor in either of the cell types (Figure 20).

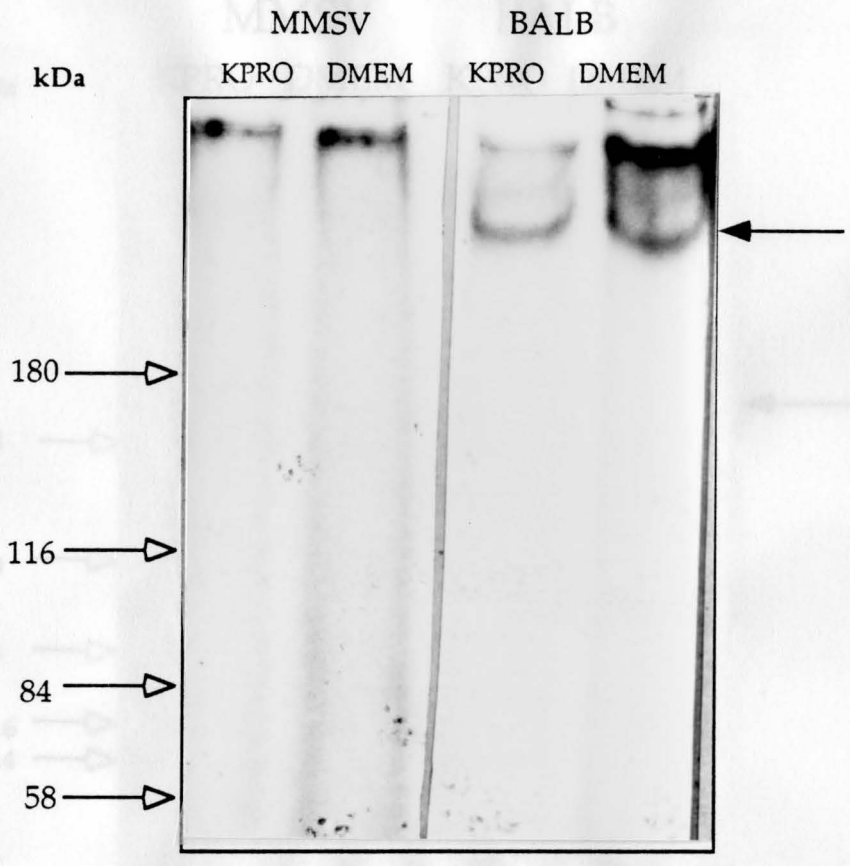

Fig. 18. [^{125}I] IGF-II CROSSLINKING TO THE 275 KDA M6P/IGF-II RECEPTOR IN MEMBRANES ISOLATED FROM BALB/3T3 AND MMSV-TRANSFORMED FIBROBLASTS. BALB and MMSV cells were pretreated either to 110 mM potassium propionate or DMEM (METHODS). Cells were permeablized in the presence of saponin and crosslinked to [^{125}I] IGF-II with DSS. Solubilized membranes were loaded on a 4% SDS-PAGE gel and run under reducing conditions. Bands were visualized by autoradiography. The positions of the prestained molecular weight markers are shown on the left (⟶▷). The position of [^{125}I] IGF-II crosslinked to M6P/IGF-II receptor is also indicated (⟶).

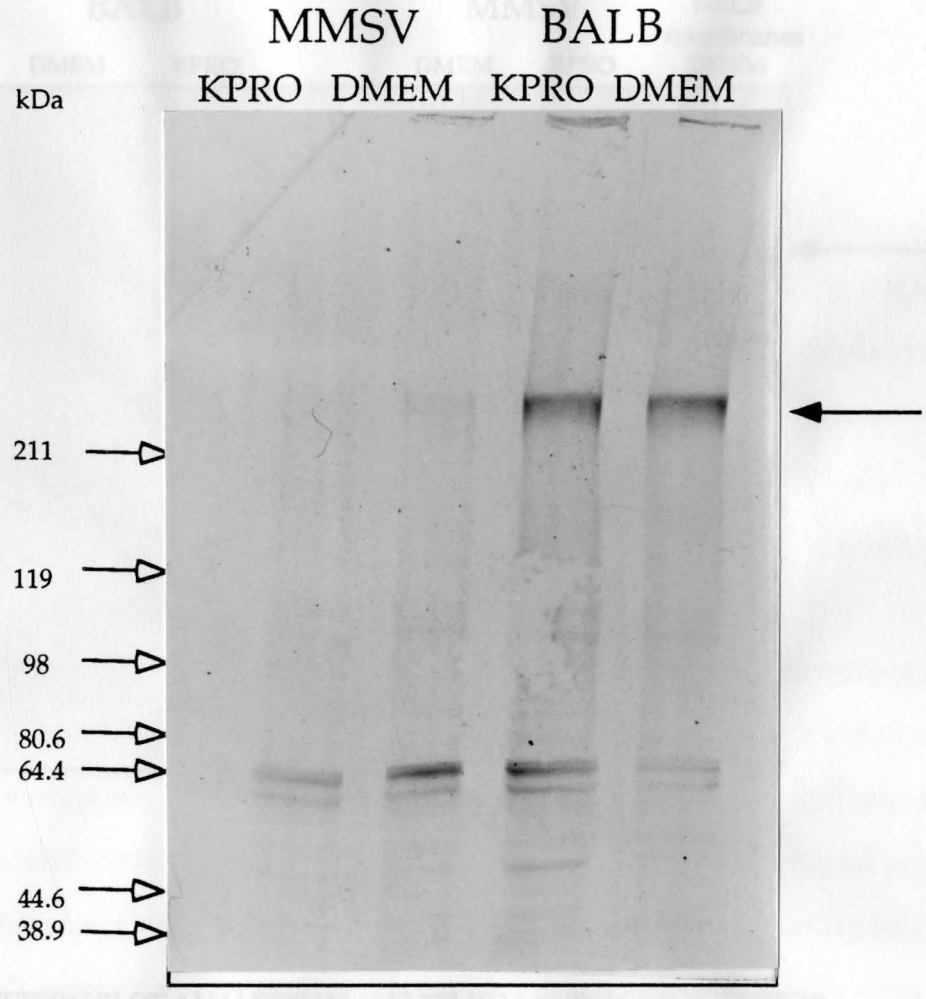

Fig. 19. WESTERN BLOT ANALYSIS OF THE 275 KDA M6P/IGF-II RECEPTOR FROM TOTAL MEMBRANE PREPARATIONS IN BALB/3T3 AND MMSV-TRANSFORMED FIBROBLASTS. Detergent solubilized membranes were electrophoresed on a 4-15 % SDS-PAGE gradient gel under non-reducing conditions, and transferred on to a membrane support and probed with anti-human M6P/IGF-II receptor. Positions of the for prestained molecular weight markers are shown on the left (⟶). The M6P/IGF-II receptor is shown on the right (⟵). MMSV and BALB cells were pretreated with either 110 mM potassium propionate (KPRO) or DMEM (METHODS).

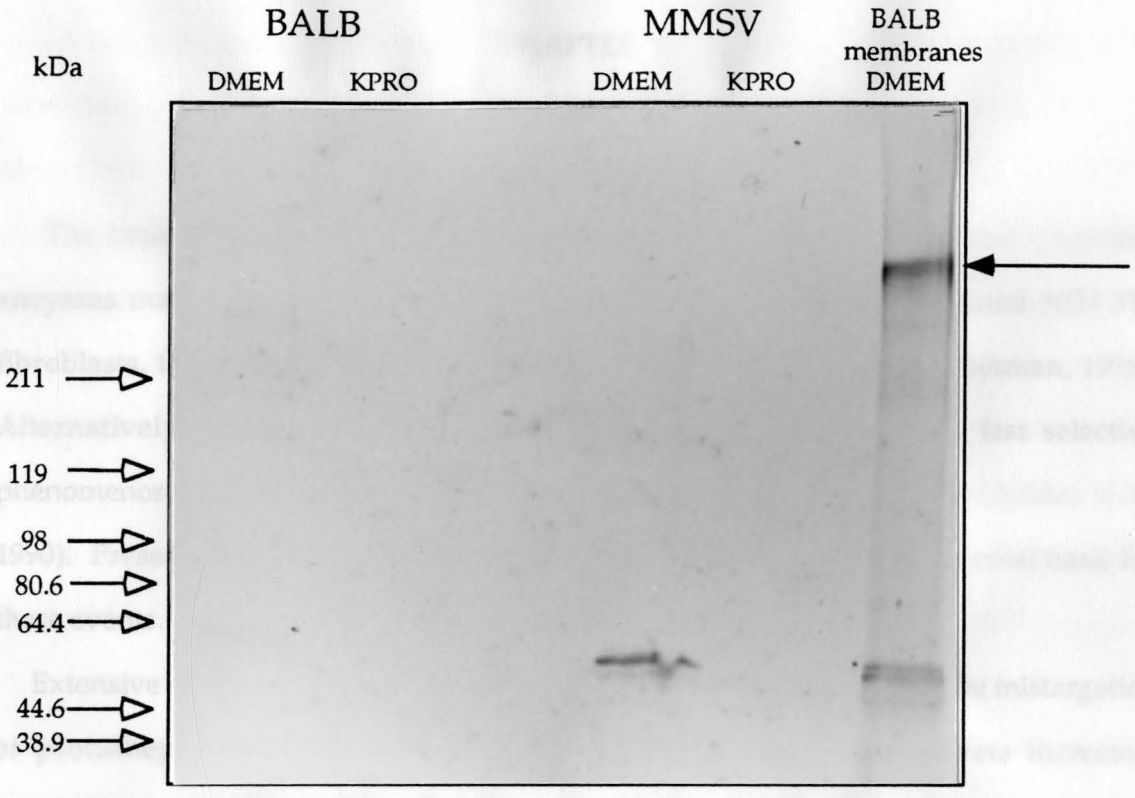

Fig. 20. WESTERN BLOT ANALYSIS OF THE 275 KDA M6P/IGF-II RECEPTOR FROM CONDITIONED MEDIA SAMPLES FROM BALB/3T3 AND MMSV-TRANSFORMED FIBROBLASTS. Cell culture media was concentrated down and loaded on a 4-15% SDS-PAGE gradient gel under nonreducing conditions and transferred on to a membrane support and probed with anti-human M6P/IGF-II receptor. The positions of the prestained molecular weight markers (⟶) and the (⟶) M6P/IGF-II receptor are identified with arrows. MMSV and BALB cells were treated with either 110 mM potassium propionate (KPRO) or DMEM (METHODS).

CHAPTER V

DISCUSSION

The basis for mistargeting and increased secretion of newly synthesized lysosomal enzymes may differ for different cell lines. For Kirsten virus transformed NIH 3T3 fibroblasts, the predominant enzyme secreted was procathepsin L (Gottesman, 1978). Alternatively, the mistargeting of lysosomal enzymes may involve a less selective phenomenon, resulting in a general secretion of several lysosomal enzymes (Achkar *et al.*, 1990). Presently very little information has been reported on the fundamental basis for these events.

Extensive studies have elucidated some of the factors which promote the mistargeting of procathepsin L. Cultured mouse fibroblasts synthesize and secrete increased amounts of procathepsin L in response to growth factors, tumor promoters, and viral transformation (Troen *et al.*, 1988). The increased secretion has been attributed to both a high level of procathepsin L synthesis and an intrinsic low affinity of procathepsin L for M6P/IGF-II receptor (Dong *et al.*, 1989). This reduced affinity results from the synthesis in murine fibroblasts of a glycosylated form of procathepsin L which contains one instead of two phosphorylated oligosaccharide side chains (Dong and Sahagian, 1990). Overproduction of cathepsin L would then result in a ligand which could compete only poorly for limited amounts of the M6P/IGF-II receptor. However, this explanation may be incomplete. In NIH 3T3 fibroblasts stimulated by platelet derived growth factor, procathepsin L synthesis and secretion are uncoupled; at early times a nearly quantitative secretion of procathepsin L occurs in the absence of increased synthesis and at late times, secretion ceases even after high levels of synthesis persist

(Prence *et al.*, 1990). In contrast to the behavior of stimulated NIH 3T3, MMSV-transformed BALB/3T3 fibroblastssecrete procathepsin B and procathepsin L in a manner suggestive of a more generalized defect in lysosomal enzyme targeting.

A cell type that secretes most of its newly synthesized lysosomal enzymes is the osteoclast, a polarized cell located where bone resorption takes place (Vaes, 1968). A characteristic of this cell is that it expresses high amounts of the M6P/IGF-II receptor (Baron *et al.*, 1988). These investigators observed the receptor to be colocalized with β-glucuronidase, cathepsin C, arylsulfatase and β-glycerophosphatase. The predominant site of residence was along the exocytic pathway. Although this study was incomplete, it was speculated that in the osteoclast, the MPR is involved in the vectorial transport of newly synthesized lysosomal enzymes to the apical membrane where they encounter an acidic environment that optimally favors ligand/receptor dissociation. What signals cause this alternative use of the MPR in this pathway remain to be determined.

Intracellular targeting to lysosomes depends on both a mannose 6-phosphate recognition marker on the ligand, as well as a mannose 6-phosphate receptor. This is supported by the observation that mutants lacking either of these will secrete most of the lysosomal enzymes they synthesize (Robbins and Myerowitz, 1981; Lemansky *et al.*, 1985; Kornfeld, 1986). Adding to the complexity of mistargeting are the observations that a number of cell lines deficient in the M6P/IGF-II receptor can still transport and correctly deliver their lysosomal enzymes (Gabel and Foster, 1986).

My observations from studies of MMSV-transformed fibroblasts have lead to the conclusion that these cells have several defective properties which may contribute to the secretion of newly synthesized enzymes. The first characteristic is the overall more alkaline nature of their endosomal and lysosomal compartments. The 0.4-0.5 unit increase in the pH found in the lumen of endosomes and lysosomes for MMSV cells when compared to the nontransformed BALB cells could be sufficient to disrupt proper

enzyme delivery. The nature of this acidification defect is unknown. Observations that growth factors and tumor promoters act to stimulate the plasma membrane Na^+/H^+ antiporter and increase cytosolic pH suggests the possibility that vacuolar alkalization is secondary to cytosolic alkalization caused by cellular transformation and activation. However, my observation that the cytosolic pH for MMSV and BALB cells are similar, 7.1 and 7.2, respectively, is directly counter to this explanation.

The maintenance of a gradient of decreasing luminal pH along the biosynthetic transport pathway from the trans-Golgi network to the lysosomes is essential for the sorting of newly synthesized enzymes to the lysosomes. When treated with agents which disrupt this proton gradient, cells will constitutively secrete high levels of lysosomal enzymes (Gonzalez-Noriega *et al.*, 1980). One factor which contributes to this secretion may be the failure of ligands to dissociate from the M6P/IGF-II receptor in an intermediate acidic compartment, the endosome, when its luminal pH is increased (Gonzalez-Noriega, 1980). Chinese hamster ovary (CHO) cells with a conditional defect in endosomal acidification secrete high levels of precursor forms of lysosomal enzymes at nonpermissive temperatures. These cells behave as if they lack functional mannose 6-phosphate receptors at nonpermissive temperatures even when the receptors are present (Park *et al.*, 1991). Substantial increases in intralysosomal pH have been reported for malignant transformed mouse 3T3 fibroblasts with Kirsten murine sarcoma virus and the transfection of human MSU-1.1 fibroblasts with v-Ki *ras* or T24 H-*ras* (Jiang *et al.*, 1990).

To test whether the observed increase in vacuolar pH for MMSV-transformed fibroblasts contributed to a disruption of normal lysosomal enzyme traffic, a method was applied to reacidify or return the pH of these compartments back to levels found in the nontransformed BALB fibroblasts. The small chain fatty acid, propionate, has previously been reported to lower the cytosolic pH in cultured cells (Grinstein *et al.*,

1984). Potassium propionate did lower the cytosolic pH by 0.4 to 0.5 units in both the MMSV and BALB fibroblasts (Figure 11). Fortuitously, potassium propionate also significantly lowered the lysosomal pH in the MMSV cells from 5.7 to 5.0 (Figure 9). Lysosomal pH in BALB cells decreased to a similar level from 5.3 to 5.1. When potassium propionate, pH 7.4, was replaced with a sodium containing buffer at the same pH, such as PBS or DMEM, lysosomal pH returned to initial levels almost immediately. Therefore, the effect potassium propionate has on vacuolar pH is reversible and transient.

In potassium propionate containing buffer, MMSV cells secrete greatly reduced levels of both cathepsin B and cathepsin L (Table 3 and Figures 12 and 13). In contrast, potassium propionate had only a small effect on the already low levels of enzyme secretion found with BALB cells. To rule out the possibility of that the potassium ion was contributing to these observations, enzyme levels were measured in the presence of potassium chloride. No significant change was observed in the secreted levels for both cell types. However, potassium chloride did lower the levels of latent forms of cathepsin B and cathepsin L in BALB and MMSV cells for unknown reasons. N-methyl-D-glucamine chloride, which contains a nontransportable cation, also failed to inhibit enzyme secretion. It is also possible that the decreased cytosolic pH resulting from potassium propionate treatment contributed to the decrease in secreted enzyme levels for the MMSV cells. To eliminate the possibility that cytosolic acidification by potassium propionate contributed to the inhibition of enzyme secretion in MMSV cells, one would need to find a method by which only endosomal and lysosomal pH would be altered. Currently, such a method has not been reported.

The dramatic inhibition of enzyme secretion caused by reacidification with potassium propionate in MMSV cells was not due to an inhibition of protein synthesis. [^{3}H]-Leucine incorporation under the same conditions as those used for the enzyme

assays, showed that potassium propionate does produce a general inhibition of protein synthesis in both the BALB and MMSV cells, by 57 % and 54 %, respectively (Figure 15). However, after adjusting for changes in protein synthesis, potassium propionate still inhibited cathepsin B and cathepsin L secretion by 89% and 96%, respectively in MMSV cells (Figures 12 and 13 have been corrected for this possible inhibition, Table 3 represents levels prior to correction). [^{3}H]-Leucine incorporation measured total protein synthesis. To selectively look at potassium propionate's effect on lysosomal protein synthesis, immunoprecipitation was performed to directly measure the rate of synthesis of cathepsin D and cathepsin L. After labeling with [^{35}S] methionine for 1 h, in the presence and absence of potassium propionate, newly synthesized cathepsin D and cathepsin L were immunoprecipitated by their corresponding antisera. Little or no difference was observed between control (DMEM) and potassium propionate treated BALB and MMSV cells with regard to cathepsin L synthesis (Figure 16). Cathepsin D synthesis appeared to be inhibited with regard to cathepsin L synthesis about 50 %. In neither case could the magnitude of these inhibition of lysosomal proteinase synthesis explain the much greater inhibition of secretion. The ability to inhibit lysosomal enzyme secretion by acidifying vacuolar compartments in MMSV cells suggests that a defect in vacuolar acidification may contribute to the secretory phenotype of MMSV cells.

The second deviant feature found in the MMSV cells was their inability to perform mannose 6-phosphate receptor-mediated endocytosis. The absence of functional M6P receptors on the cell surface of MMSV cells could be due to several possibilities: (1) these cells are unable to synthesize functional M6P receptor; (2) the synthesis of the receptor is normal but its degradation is enhanced; or (3) the vacuolar compartments responsible for ligand-receptor uncoupling are too alkaline to promote ligand dissociation and/or receptor recycling. The reappearance of functional mannose 6-phosphate receptor-mediated uptake after acidification with potassium propionate led

to the following possibilities: (1) there might be an increase in M6P/IGF-II receptor synthesis; (2) M6P/IGF-II receptor turnover is decreased; or (3) there is an increased recycling of the M6P/IGF-II receptor to and from the cell surface. The first two explanations do not appear likely as the effect of potassium propionate on receptor-mediated endocytosis is rapidly reversed on transfer to DMEM. This appears to resemble the effect of transferring CHO cells, conditionally defective in endosomal pH from a permissive to nonpermissive temperature (Park *et al.*, 1991).

IGF-II affinity crosslinking and Western blot analysis both confirmed the absence of the M6P/IGF-II receptor in the MMSV cells (Figures 18 and 19). This observation could explain the general mistargeting of lysosomal enzymes by MMSV cells, which is consistent with other reports on cell types deficient in the M6P/IGF-II receptor (Robbins and Myerowitz, 1981; Lemansky *et al.*, 1985; Kornfeld, 1986). It would be interesting to investigate whether other cells which lack the M6P/IGF-II receptor would also show an increase in the pH of their endosomes and lysosomes. If so, this might suggest that the absence of the M6P/IGF-II receptor is related to defects in the regulation of vacuolar pH.

The existence of a soluble, truncated M6P/IGF-II receptor was first reported in rat sera (Kiess *et al.*, 1987). Others have since shown that the soluble receptor in rat sera is about 240 kDa and lacks the cytosolic and transmembrane domains (MacDonald *et al.*, 1989). The soluble receptor was present in fetal, neonatal, and adult sera. The levels in fetal and neonatal sera were 3 to 4 times higher than those in adult sera. The soluble receptor still has the capability to bind M6P containing ligands. Speculations with respect to possible functions of a soluble M6P receptor have been proposed as follows: to retrieve extracellular lysosomal enzymes; to inactivate secreted lysosomal enzymes; or possibly to chaparone these enzymes during their transit to other extracellular locations (MacDonald *et al.*, 1989). Western blot analysis was performed on the conditioned media from BALB and MMSV cells, with the possibility that the M6P/IGF-II receptor

was absent in MMSV cells because it was proteolytically cleaved and released into the extracellular environment. Western blot analysis revealed no detectable M6P/IGF-II receptor for either cell type in the conditioned media, even when the cells were pretreated with potassium propionate (Figure 20). It therefore, remains uncertain whether or not MMSV cells have the necessary capability to produce the M6P/IGF-II receptor protein.

Potassium propionate treatment stimulated the uptake of [^{125}I] labeled PMP-BSA in both MMSV and BALB cells to about 200% over untreated BALB cells (Figure 17). Potassium propionate treated BALB cells failed to show any increase in the total level of M6P/IGF-II receptor determined by Western blot analysis under the same conditions. Additionally, the M6P/IGF-II receptor was still undetectable in potassium propionate treated MMSV cells. The fact that the M6P/IGF-II receptor did not reappear after treatment to potassium propionate, complicated the previous observation that MMSV cells regained the ability to internalize [^{125}I]-PMP-BSA after acidification. This suggests the possibility that potassium propionate stimulation involves the 46 kDa mannose 6-phosphate receptor which normally does not function in receptor-mediated endocytosis in cultured cells (Watanabe *et al.*, 1990). If the 46 kDa MPR is involved in the recovery of active mannose 6-phosphate uptake after potassium propionate at pH 7.4, this would be a unique finding. Presently, this receptor has been reported to bind extracellular mannose 6-phosphate bearing ligand only at pH 6.5 (Ma *et al.*, 1991). The reappearance of cell surface mannose 6-phosphate inhibitable receptor-mediated uptake in potassium propionate treated MMSV cells does not necessarily indicate, however, that intracellular M6P/IGF-II receptor activity has been enhanced. Activation of an intracellular M6P receptor would support my finding that potassium propionate was able to inhibit lysosomal enzyme secretion.

Alternatively, mannose 6-phosphate may be only one of several signals utilized by

cells in the proper sorting and delivery of newly synthesized enzymes to lysosomes. Diment *et al.* (1988) observed cathepsin D to be associated with membranes in macrophage endosomes. This membrane associated form was proteolytically processed while still bound to the membranes prior to its delivery to the lysosomes. This association was not found to involve any known glycoprotein receptor. Additionally, these investigators observed that an increase or decrease in vacuolar pH would disrupt this membrane binding. Therefore, an alternative explanation for the decrease in lysosomal enzyme secretion by MMSV cells after acidification with potassium propionate may involve a reassociation of these enzymes to transport vesicle membranes.

The results presented in this work have many important implications for future research. Since two deviant characteristics which may influence increased lysosomal enzyme secretion have been observed for the MMSV fibroblasts, they need to be addressed individually. The first observed defect, is the increased vacuolar pH. It has been reported (Moolenaar, 1990) that a shift in pH of 0.2 in any intracellular compartment would have substantial effects on a number of pH-sensitive processes in the cell. This supports the idea that the observed increase in endosomal and lysosomal pH of 0.4 - 0.5 for the MMSV cells would alter a number of intracellular processes. It is well established that lysosomal enzymes are generally active and stable under acidic conditions. Chloroquine has been shown to inhibit proteolytic processing of these enzymes by competing for the H^+, thereby raising vacuolar pH and increasing secretion. The effect of chloroquine appears to mimic the phenotype found for MMSV cells. In addition, the secretion of lysosomal enzymes by MMSV cells can not be further enhanced by monensin, another disrupter of vacuolar pH. (Achkar *et al.*, 1990). Procathepsin D undergoes activation and maturation at acidic pH (Samarel *et al.*, 1986; Samarel *et al.*, 1985). When rabbit cardiac cells were treated to chloroquine, the

proteolytic processing of cathepsin D was inhibited and these enzymes were mistargeted to the extracellular space. Evidence also suggests that cathepsin L undergoes autocatalytic activation at acidic pH (Salminen and Gottesman, 1990). The proteolytic maturation of other lysosomal enzymes should also be pH dependent. Therefore, an increase in vacuolar pH in MMSV cells would not only interrupt lysosomal enzyme targeting but it should also increase the relative abundance of precursor and partially processed forms of various lysosomal enzymes within the cells.

Interestingly, the secretion by fibroblasts of transforming growth factor-beta (TGF-β), which also contain phosphorylated mannose, is also stimulated by agents like chloroquine and monensin (Sha *et al.*, 1989). TGF-β is a potent modulator of cell growth and differentiation producing either growth stimulation or growth inhibiton depending on target cell type (Cohen *et al.*, 1990; Lipzonova *et al.*, 1990; Daniel and Sporn, 1990). Consequently, further studies comparing TGF-β secretion by BALB and MMSV cells would also be justified.

The second characteristic defect present in the MMSV cells is the absence or extremely low levels of the M6P/IGF-II receptor. The importance of this receptor in lysosomal enzyme targeting and recapture has been previously discussed. However, the absence of this receptor in potentially metastatic cells raises other interesting possibilities. This receptor also serves to bind IGF-II and it activates a signaling transduction pathway only in response to this ligand (Okamoto *et al.*, 1990). Little is currently known about the nature ot this secondary signaling effect except for that it involves a GTP-binding protein. IGF-II will bind to the IGF-I receptor in the absence of free M6P/IGF-II receptors. The IGF-I receptor differs from the M6P/IGF-II receptor in that it possesses an intrinsic tyrosine kinase activity (Hari *et al.*, 1987). Because of the absence of the M6P/IGF-II receptor in the MMSV cells the response induced by IGF-II would be mediated by the IGF-I receptor and be fundamentally different from that

evoked by the M6P/IGF-II receptor. Future studies on the nature of the secondary signaling pathways and responses for these receptors will help to determine the effect the absence of the M6P/IGF-II receptor has in MMSV cells.

In addition to binding mannose 6-phosphate containing ligands and IGF-II, the M6P/IGF-II receptor is responsible for the binding and activation of the latent form of TGF-β (Dennis and Rifkin, 1991). Most cells from a wide spectrum of organisms produce one or more isoforms of TGF-β (Pfeilschifter, 1990). It is involved with the synthesis and maintenance of the extracellular matrix and it can exert stimulatory or inhibitory effects on cells depending on their type (Pfeilschifter, 1990). TGF-β has been shown to be the most potent growth factor in the stimulation of collagen and fibronectin matrix protein synthesis (Ignotz and Massague, 1986). It has also been implicated in the maturation of procollagen (Rossi *et al.*, 1988). TGF-β not only promotes matrix synthesis, it also decreases matrix degradation. It has been shown to inhibit the synthesis of thiol proteases in fibroblasts and can inhibit other growth factors which promote collagenase activity (Edwards *et al.*, 1987). Since its discovery as a potential growth inhibitor (Roberts *et al.*, 1981), it has been speculated that its activity toward metastatic tumor cells is some how repressed by these cells. Because of the recent discovery that the M6P/IGF-II receptor serves in the activation of latent TGF-β, this research project suggests that the absence of the M6P/IGF-II receptor may have other implications besides lysosomal enzyme mistargeting in MMSV cells. It would be very interesting to see if the absence of the M6P/IGF-II receptor results in an inability of MMSVcells to activate latent TGF-β. If this were true, MMSV cells, should be unresponsive to latent TGF-β, have a decreased ability to manufacture the extracellular matrix, and should secrete increased amounts of other matrix degrading enzymes in addition to cathepsins B and L. Other cells which have been shown to possess high metastatic potential could be screened to see whether they lack or rapidly turnover the

M6P/IGF-II receptor. The absence or appearance of only low levels of the M6P/IGF-II receptor might enhance malignancy in several ways: by limiting TGF-β activation with its matrix deposition and growth inhibitory activities (Border *et al.*, 1990); and by increasing the secretion of collagenases and lysosomal acid hydrolases which degrade the constituents of the extracellular matrix.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 27, 1993
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