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Lysosomal Enzyme Secretion in Tumor Cells and the Purification and Partial Characterization of a 67 Kda Cathepsin B-Like Enzyme Secreted by B16 Melanoma Cells

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LYSOSOMAL ENZYME SECRETION IN TUMOR CELLS AND THE PURIFICATION AND PARTIAL CHARACTERIZATION OF A 67 kDA CATHEPSIN B-LIKE ENZYME SECRETED BY B16 MELANOMA CELLS.

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CHAPTER I

INTRODUCTION

The major characteristic of malignant tumor cells is their ability to invade foreign tissues and form metastatic foci at sites in the body distant from the primary tumor. At an early stage of invasion cells detach from the primary tumor and migrate into the adjacent tissue, possibly guided by chemotactic factors. Tumor cells which penetrate lymph or blood vessels can spread throughout the body. Tumor cells which escape destruction in the lymphatic or circulatory system can form new colonies by extravasating through the wall of the vessel and initiating cellular replication. Thus, the formation of metastases is the end result of a continuous invasive process that is associated with the breach of host protein barriers and the dissemination of viable tumor cells by the circulatory system throughout the entire body of the host (Thorgeirsson et al, 1985; Tryggvason, 1986).

Cancer metastasis is the major cause of treatment failure, morbidity, and death for patients with solid malignant tumors. Whereas the treatment modalities of surgery, chemotherapy, and radiotherapy can cure approximately 50% of patients who develop malignant tumors, the majority of patients who fail to respond to these modalities succumb to the direct effect of the metastases or to complications

associated with the treatment of metastases (Liotta, 1986). At the time of diagnosis of primary tumors, approximately 50% of patients already have occult micrometastic tumor colonies that ultimately expand and lead to direct anatomic compromise. A large clinically detectable metastic focus in a particular organ may indeed indicate the presence of numerous subliminal micrometastases of more recent initiation. The scattered anatomic deposits of metastases, the heterogeneity of their cellular subpopulations, and their size and age variations often prevent surgical removal and limit the response to systemic anticancer agents (Chabner et al., 1986; Liotta, 1986). In addition, whereas conventional therapy, such as chemotherapy or radiotherapy, can in some cases retard metastatic growth, conventional therapy can often indirectly lead to patient morbidity and death by compromising the immune response and causing complications of infectious disease (Liotta, 1986).

Metastatic migration is a complex phenomenon that involves a series of sequential steps which include: detachment of cells from the primary tumor; penetration of the extracellular matrix and basement membrane; invasion of the vasculature to enter the blood circulation (intravasation); arrest in the capillaries at the secondary sites (attachment); invasion through the capillary wall (extravasation) and finally, colonization and proliferation in the distant tissues.

Considerable experimental evidence generated by many research groups has supported the proposal that invasive tumor cells produce matrix-degrading proteolytic enzymes (Mullins et al., 1983; Thorgeirsson et al., 1985; Jones and De Clerk, 1982; Liotta, 1986). Since the extracellular matrices of different tissues vary qualitatively and quantitatively with respect to their levels of collagens, glycoproteins, proteoglycans, and other matrix constituents (Alitalo and Vaheri, 1982; Jones and De Clerk, 1982; Liotta, 1986), it is likely that multiple proteolytic enzymes are responsible for degradation of various extracellular matrices (Jones and De Clerk, 1982).

Invasive tumors with the potential to metastasize produce several proteolytic enzymes that may contribute to matrix degradation, including collagenases (Liotta, 1986; Liotta et al., 1982), plasminogen activators (Dano et al., 1985; Goldfarb, 1983; Henry et al., 1983), cathepsin B (Sloane and Honn, 1984; Qian et al., 1989), endoglucuronidases (Nakajima et. al., 1983; Nakajima et. al., 1984; Vlodasky et. al., 1983), additional neutral serine proteases (Distefano et al., 1983; Kramer et al., 1985; Zucker et al., 1985), and procoagulants (Badenoch-Jones and Ramshaw, 1985). Tumor cells can also secrete lysosomal proteases, as examples, transformed murine fibroblasts synthesize and secrete increased amounts of procathepsin B (Achkar et al., 1990) and procathepsin L (Gottesman et al., 1980), Morris hepatoma 7777 cells secrete

early or precursor forms of cathepsin C (Mainferme et al., 1985), and a mammary cell carcinoma MCF7 secretes procathepsin D in response to estrogen stimulation (Mainferme et al., 1985; Copony et al., 1989). Among the lysosomal proteinases which may play a role in invasion and metastasis, cathepsin B has been the one most extensively investigated.

Many studies have reported a positive correlation between cathepsin B activity and malignant tumors (Pool et al., 1978; Recklies et al., 1980; Sloane et al., 1984; Koppel et al., 1984; Keppler et al., 1988; Krepela et al. 1989, 1990; Qian et al., 1989). Increased secretion of cathepsin B-like enzymes has been observed in ascites fluid from patients with various kinds of malignant tumors (Pietras and Roberts, 1981; Mort et al., 1981; Dufek et al., 1984) and in conditioned media from cultured human and murine mammary carcinoma (Recklies et al., 1982; Petrova-Skalkova et al., 1987). The molecular basis for the increased levels of cathepsin B activity in the tumors remains unknown. Possible factors include increased transcription, translation, post-translational processing or stability of cathepsin B in tumors. Furthermore, the distribution of cathepsin B is often altered in tumor cells, being found associated with the plasma membrane (Pietras and Roberts, 1981; Keren and Le Crue, 1988), and secreted into the extracellular medium. It is not known whether this is a result of a cellular defect in the biosynthetic transport pathway in tumors, a defect in the post-translational

processing of cathepsin B, or from the synthesis in tumor cells of a form of cathepsin B which lacks amino acid sequences necessary to target this enzyme to lysosomes.

Although a selective elevation in the level of mRNA for cathepsin B has been demonstrated in B16 melanoma cells and other malignant tumors (Qian et al., 1989), the secreted cathepsin B-like activity has never been purified and its identity to lysosomal cathepsin B has not been proven. Furthermore, it is not known whether lysosomal enzyme secretion by tumor cells is selective or whether more than one kind of enzyme may be secreted by a given cell type. Finally, for specific lysosomal proteinases, explanations at the cellular and molecular levels for their secretion by tumor cells have not been achieved.

The goals of this study were: First to examine the secretion of latent and active forms of cathepsin B, cathepsin L and ß-glucuronidase by murine fibroblasts, virally transformed murine fibroblasts, and several murine melanoma cell variants which express elevated levels of cathepsin B mRNA. The purpose was to determine whether these cells show a general decrease in the efficiency of enzyme targeting to lysosomes on transformation, and if secretion of lysosomal enzymes is specific for cathepsin B. During the course of these studies we observed that the cathepsin B-like activity secreted by B16 melanoma cells did not resemble normal cathepsin B in size and immunoreactivity. Consequently, we

undertook to purify and characterize the cathepsin B-like enzyme secreted by murine B16 cells in order to determine whether these cells secrete the authentic lysosomal procathepsin B.

CHAPTER II

REVIEW OF THE RELATED LITTERATURE

The Cellular Proteases

Proteases are composed of two groups: exopeptidases (peptidases) which cleave either amino- or carboxy-terminal amino acids or dipeptides from proteins, and endopeptidases (proteinases) which cleave peptide bonds in the interior of the protein molecule. However, this distinction is not always clear cut as some proteases, can have both activities.

Proteases may be further characterized as belonging to one of five classes depending on the mechanism of peptide bond cleavage catalyzed by their active centers (Barrett, 1980a). These five classes are: serine proteinases, cysteine proteinases, aspartic proteinases, metallo-proteinases and a fifth category which includes enzymes with different or undertermined catalytic mechanisms (Barrett, 1980a). Each of the first four classes will be briefly discussed before introducing the lysosomal proteinases and more specifically cathepsin B.

Serine Proteinases:

Due to extensive homologies between serine proteinases, it has been hypothesized that they have all diverged from one primitive enzyme (Hartley, 1970). This class of proteinase includes trypsin, chymotrypsin, elastase, coagulation factors,

plasmin, tryptases, cathepsin G, chymases, phasminogen activators, and many others. They are all active at a neutral pH without the need of a cofactor and all share the presence of the amino acid serine in their active centers. Adjacent histidine and aspartate residues form a catalytic "triad" which is essential for the activity of these enzymes (Dufton, 1990). Serine proteinases are first synthesized as inactive precursors that require limited proteolysis for activation (Moehle et al., 1989). Serine proteinases which participate in coagulation are controlled by a cascade of activating enzymes and a system of protein proteinase inhibitors. Serine proteinases of particular interest in cancer metastases are plasmin and the plasminogen activators urokinase PA and tissue PA.

Considerable experimental evidence has indicated that the production of plasminogen activator by tumor cells and the generation of plasmin by proteolytic activation of plasminogen is associated with various aspects of malignant transformation, tumor invasion, and metastasis (Bettelheim et al., 1984; Fairbairn and Quigley, 1980; Saksela, 1985). Several studies have indicated that matrix glycoproteins are sensitive to plasmin-mediated proteolytic degradation (Jones and De Clerk, 1982). Studies with human metastatic tumor cell lines and normal human fibroblasts have shown that the former, but not the latter, digest matrix glycoproteins (Jones and De Clerk, 1980). It has been suggested that the production of

plasminogen activator by invasive tumor cells and the generation of plasmin are key events in the hydrolysis of all matrix components, since glycoprotein removal from the matrix is required for the maximal degradation of collagens and elastin.

Cysteine Proteinases:

Members of the cysteine proteinase family contain a cysteine residue in their active site. Also essential to catalysis is the presence of a neighboring histidine residue which functions as a general acid-general base catalyst (Topham et al., 1990). Included in this group among others are papain from the papaya plant (Mitchel et al., 1970), a developmentally regulated proteinase in Dictiostelium (Williams et al., 1985), and the mammalian lysosomal enzymes cathepsins B, H, and L (Barrett, 1980b). The precise function of the lysosomal cysteine proteinases is unclear, but they are generally thought to play a role in intracellular protein degradation. Consistent with their lysosomal location, the mammalian cysteine proteinases are optimally active at acidic pH (Barrett, 1980b). However, tumor cells have been shown to secrete a cysteine proteinase which retains activity at neutral pH and may play a role in tumor metastasis (Mullins and Rohlrich, 1983). The lysosomal cysteine proteinases are synthesized as large precursors which undergo proteolytic maturation after exiting the Golgi apparatus (Nishimura and Kato, 1987a). Limited proteolysis is associated with the

acquisition of proteolytic activity by these enzymes. The secretion of active and inactive forms have been demonstrated (Bajkowski et al., 1984). Inactive forms can be activated by limited proteolysis with cathepsin D and pepsin (Nishimura et al., 1989). The active cellular form of cathepsin B is unstable above pH 7.0, but the form secreted by tumor cells shows an increased tolerance to elevated pH (Mort et al., 1981). Cathepsins B and L will be reviewed in more detail later.

Calpains are Ca^{2+} activated cysteine proteinases (Barth and Elce, 1981; Dayton and Schollmeryer, 1981) which in the presence of Ca^{2+} may undergo translocation to the cell membrane followed by autolytic activation (Murachi, T., 1989). Two forms of calpain have been detected which differ in the concentrations of Ca^{2+} required for their maximal activities (Mellgren, 1980). In other respects the two forms are similar, both are active optimally at pH 7.5, both are inhibited by sulfhydryl modifying reagents and exogenous inhibitors of cysteine proteinases and both appear to be composed of two subunits. Calpains have very restricted proteolytic activities, and their protein substrates include neurofilament proteins, c-protein, demin, filamin, rimentin and troponins I and T (Mellgren, 1980).

Aspartic Proteinases

Members of this family of proteinases which all contain an active site aspartate include pepsin, renin, and

cathepsin D. Pepsin, which functions in the acidic milieu of the stomach, is responsible for the initial steps in protein digestion. Renin is thought to have been originated from the hypothetical cathepsin D-like archetype. However, unlike cathepsin D it is stable and active at neutral pH and is highly specific for the Leu-Leu bond that generates angiotensin I from angiotensinogen.

Cathepsin D is a major proteolytic enzyme of lysosomes and widely distributed in animal tissues. It is presumed that cathepsin D plays an important role in physiological protein degradation and pathological processes (Barrett, 1977; Schimke, 1975). However, the precise role of cathepsin D is still unclear, largely because the mechanism of protein degradation is presumably a complex and multistep process. Estrogen receptor positive human breast cancer cell lines (MCF7, ZR75-1) produce a M_r 52,000 protein, identified as a pro-cathepsin D (Capony et al., 1987), in response to estrogen treatment in culture. Procathepsin D is also produced constitutively at high levels in some hormone-independent cell lines (MDA-MB231, BT20). In addition, cathepsin D can be detected in primary breast cancer cells and assayed in the cytosol using specific monoclonal antibodies (Rochefort et al., 1987). In vitro, the secreted Mr 52,000 procathepsin D displays an autocrine mitogenic activity on MCF7 cells (Vignon et al., 1986) and interacts with the mannose 6-phosphate

receptor via mannose 6-phosphate residues on asparagine linked oligosaccharide side chains (Capony et al., 1987). At acidic pH in vitro, this protease can also degrade the extracellular matrix (Briozzo et al., 1988), suggesting that it may be involved in mammary carcinogenesis and metastasis (Rochefort et al., 1987b).

Metallo-Proteinases:

Metallo-proteinases are characterized by the presence of Zn^{2+} in their active sites. Examples include pancreatic carboxypeptidase, thermolysin and collagenases. This group of enzymes has a slightly alkaline pH optimum and is sensitive to inhibition by 1,10 orthophenanthroline, EDTA and thiols. They seem well suited to function extracellularly and in fact metallo-proteinases are secreted from cells rather than being active intracellularly.

Type IV collagenase, a metallo-proteinase, degrades the basement membrane type IV collagen (Liotta et al., 1979) whereas interstitial collagenases do not cleave this molecule (Babel and Alanville, 1984). Type IV collagenase has been found in malignant tumor tissues (Liotta et al., 1979; Salo et al., 1982) and cultured tumor cells (Salo et al., 1982), and also in proliferating human fibroblasts (Salo et al., 1985). Type IV collagense has been purified from a highly metastatic mouse tumor (Salo et al., 1982; Liotta et al., 1981) and cultured human mononuclear phagocytes (Garbisa et al., 1986). The enzyme is a glycoprotein with a molecular weight of either

72,000 or 92,000 when studied by SDS polyacrylamide gel electrophoresis (Salo et al., 1982; Liotta et al., 1981; Garbisa et al., 1986). Serine and sulfhydryl proteinase inhibitors such as phenylmethylsulfonyl fluoride, aprotinin and N-ethylmaleimide do not inhibit the enzyme whereas metal chelators do, indicating its identity as a metalloproteinase. The enzyme can be activated in vitro by pretreatment with trypsin, plasmin sodium dodecyl sulfate or organic mercurials and in this aspect it resembles interstitial collagenases. Type IV collagenase cleaves the native type IV collagen molecule at a single site in the helical domain at about one fourth the distance from the N-terminal end (Fessler et al., 1984). Accordingly, the enzyme may be able to decompose the collagen network of basement membranes and thus facilitates penetration by tumor cells.

Cathepsin B

Cathepsin B, a cysteine proteinase with a M_r of approximately 26000-27000, is located in the lysosomes of mammalian cells (Barrett, 1977). The enzyme can function as an exopeptidase, an endopeptidase, and an esterase (Bajkowski and Frankfater, 1983a) and is optimally active below pH 7.0 (Bajkowski and Frankfater, 1983b; Snellman, 1971). It is very widely distributed (Kominami et al., 1985; Qian et al., 1989; Qian et al., 1990) and has been isolated from rat liver, bovine spleen and liver (Otto, 1971), and human spleen and liver (Barrett, 1973). Using immunochemical methods,

cathepsin B has been located within macrophages (Hourie et al., 1985) of various tissues. Important functions have been inferred for cathepsin B in a number of physiological and pathological processes including: degradation of endocytosed proteins (Bystryn and Perlstein, 1982); the turnover of intracellular proteins (Shaw and Dean, 1980); the processing of secreted proteins (Quinn and Yudah, 1978) and hormonal and neuromodulatory polypeptides (Docherty and Steiner, 1982; Steiner et al., 1984), tissue resorption and remodeling (Bayliss and Ali, 1978); inflammation (Daves et al., 1978); and mediation of hormonal response (Pietras et al., 1975). At present, the full spectrum of the biological role of cathepsin B is still not quite clear but its very broad tissue distribution suggests it has important functions.

The mature form of cathepsin B has a molecular weight of 26 KDa. Its pH optimum is about 6 with many substrates and it becomes unstable above pH 7.0 (Barrett, 1977). Several synthetic substrates are reported to be very specific to cathepsin B, such as carbobenzyloxy-L-alanine-L-arginine-4-methoxy-B-naphthylamide and carbobenzyloxy-L-arginine-L-arginine-2-naphthylamide. Fluorogenic and chromogenic synthetic substrates are the most common substrates used in enzyme assays for determination of the cathepsin B activity (Barrett, 1986; Bajkowski and Frankfater, 1975).

Numerous reports have associated cathepsin B-like activities with polypeptides of M_r greater than 26,000.

^{125}Ityrosyl-Arg-Arg-CH_2Cl, a cathepsin B substrate analog, has been reported to covalently label three proteins having the M_r of 39,000, 31,500 and 25,000 in rat islet cells (Docherty et. al., 1983) and in a transplantable rat insulinoma (Docherty et. al., 1984). The M_r 31,500 form cross-reacted with anti-rat liver cathepsin B antibodies. Pepsin treatment of the M_r 39,000 form converted it to a 31,500 protein which was also precipitated with anti-rat liver cathepsin B antibodies, suggesting that the 39,000 and 31,500 polypeptides are related as precursor to product. These workers have also found that cathepsin B in rat islets is synthesized initially as a precursor of an approximate M_r of 43,000. In the insulinoma the M_r 39,000 form was found in insulin secretory granules and lysosomes, whereas the M_r 25,000 species was only found in the lysosomal fraction. Consequently, these workers have suggested that the larger precursor forms of cathepsin B arise from incomplete proteolytic processing, possibly due to their not being efficiently targeted to lysosomes in the insulinoma.

The biosynthesis and processing of cathepsin B has also been studied in rat liver (Nishimura and Kato, 1987a, 1987b), and macrophages (Kominami et al., 1988) and in normal human and I-cell fibroblasts (Honewinkel et. al., 1987). In each case the enzyme is synthesized as a precursor which is N-glycosylated (M_r of 39,000 to 44,000) and which undergoes proteolytic processing to yield both an active single chain form (M_r of 29,000 to 33,000) and a two chain form (H chain,

Mr 25,000 to 27,000 and L-chain, M_r 4000 to 5000). In rat macrophages the conversion of precursor cathepsin B to the single chain form is blocked by inhibitors of metalloendopeptidases and formation of the two chain form is sensitive to a cysteine proteinase inhibitor, E-64 (Hara et al., 1988). Agents which decrease vacuolar acidity such as ammonium chloride, chloroquine and monensin also block the proteolytic maturation of cathepsin B (Hanewinkel et al., 1987; Nishimura et al., 1988).

The mRNA for rat liver cathepsin B has been cloned as the cDNA. The predicted translated product corresponds to a polypeptide having a M_r of approximately 41,000 (Segundo et al., 1985). A comparison of the predicted sequence to the amino acid sequence of the mature enzyme has indentified the peptide bonds which are cleaved during the proteolytic maturation of cathepsin B.

Human and mouse mammary explants secrete a cathepsin B-like enzyme in culture that is larger but enzymatically and immunologically related to lysosomal cathepsin B (Recklies et al., 1982). Ascites fluid of patients with ovarian cancer and primary liver cancer contain a pepsin-activatable, latent proteinase, immunologically related to cathepsin B (Mort et al., 1983; Dufek et al., 1984). Normal mammary gland explants cultured in a hormone-free medium secreted a cathepsin B-like enzyme with a M_r of 39,000 that is indistinguishable from that secreted by tumor cells. In contrast, the major intracellular

form of cathepsin B from normal mammary glands and mammary tumors has an average M_r of 31,000, as compared with M_r of 27,000 for the lysosomal form in mouse liver. It has been suggested that cathepsin B is processed in an organ specific manner and its final intracellular size may depend on the tissue from which it was isolated and its final function (Recklies and Mort, 1985). It was also suggested that the larger latent secreted forms of cathepsin B, often associated with tumor cells, are the precursor forms of cathepsin B secreted as a consequence of abnormal processing (Mort and Recklies, 1986).

Cathepsin L

Cathepsin L is a lysosomal cysteine proteinase with a stronger endopeptidase activity than any other lysosomal cathepsins (Katunuma and Kominami, 1983; Matsukura et al., 1981). The detailed physiological role of cathepsin L within the cell is largely unknown. However, cathepsin L is considered to function in the degradation of tissue proteins in lysosomes and also in the breakdown of extracellular matrix proteins (Kirschke et al., 1982).

Cathepsin L is initially synthesized as an N-glycosylated, 39KDa proenzyme that undergoes multiple proteolytic processing steps post-translationally, generating mature forms of 30 and 23 KDa (Nishimura et. al., 1988). Elucidation of the determined primary structure of rat cathepsin L with that predicted from the nucleotide sequences

of isolated cDNA clones has confirmed these intracellular processing events (Ishido et al., 1987). Evidence has been obtained that an aspartate proteinase participates in the proteolytic processing of cathepsin L (Nishimura et al., 1989). Recently it has been shown that MEP, the major excreted protein of transformed fibroblasts is the precursor form of cathepsin L (Troen et al., 1987).

Biosynthetic Pathway of Lysosomal Enzymes

The targeting of lysosomal enzymes from their site of synthesis in the rough endoplasmic reticulum (RER) to their final destination in lysosomes is a multi-step process requiring a series of interactions between cellular components and protein and carbohydrate recognition signals present on the lysosomal enzymes (Von Figura and Hasilik, 1986; Nolan and Sly, 1987; Roth, 1988). In addition, many of the early steps in the biosynthesis and intracellular transport of lysosomal enzymes are the same as for membrane proteins, and secreted proteins. All these proteins contain a hydrophobic signal sequence that allows for their synthesis on membrane-bound polysomes of the RER and their translocation into the lumen of this organelle. An 11S ribonucleoprotein called a signal recognition particle mediates the interaction between the signal peptide and the ER membrane. During translocation of the polypeptide into the ER, the signal peptide is cleaved and many of these proteins acquire one or more high mannose oligosaccharide side chains linked to asparagine residues by

N-glycosidic bonds. This glycosylation step involves the transfer of a large preformed oligosaccharide (three glucose, nine mannose, and two N-acetylglucosamine residues) from a lipid-linked intermediate to the nascent polypeptide (Kornfeld and Kornfeld, 1985). The proteins then move by vesicular transport from the RER to the Golgi apparatus where they undergo a variety of post-translational modifications and are segregated from one another for targeting to their final destinations (Griffiths and Simons, 1986). While the mechanisms of transport of secretory proteins to the cell surface are unknown, the morphological pathway appears to be relatively simple and involves the budding of vesicles from the terminal compartment of the Golgi complex, commonly referred to as the trans Golgi network (TGN). These vesicles fuse directly with the plasma membrane (Griffiths et al., 1985). The mechanisms by which lysosomal enzymes are segregated from secretory proteins and membrane proteins on the biosynthetic transport pathway have been under intensive investigation. In the case of hydrolases, which comprise the bulk of the lysosomal content, selective transport is mediated by the binding of newly synthesized enzymes to receptors for mannose 6-phosphate (MPR) (Sly and Fischer, 1982; Von Figura and Hasilik, 1986).

When proteins enter the Golgi stack from the RER , they undergo a variety of posttranslational modifications which can facilitate their targeting to their final proper destination.

The simple high mannose oligosaccharides on secretory and membrane glycoproteins are converted by glycosidases and glycosyl transferases to complex-sialic acid-containing oligosaccharide units. Although some of the oligosaccharides on lysosomal enzymes undergo similar processing, most acquire phosphomannosyl residues through the concerted action of two enzymes (Kornfeld, 1986; Von Figura and Hasilik, 1986). First, UDP-N-acetyl-glucosamine-lysosomal-enzyme N-acetylglucosamine-phosphotransferase (phosphotransferase) transfers N-acetylglucosamine 1-phosphate from the nucleotide sugar UDP-GlcNAc to selected mannose residues on the lysosomal enzymes to give rise to a phosphodiester intermediate. Then, N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase removes the N-acetylglucosamine residue. The resultant phosphomonoesters serve as essential components of a recognition marker that leads to high-affinity binding to mannose 6-phosphate receptors in the Golgi (Kornfeld, 1986; Von Figura and Hasilik, 1986). The lysosomal enzyme-MPR receptor complex then exits the Golgi via a coated vesicle and is delivered to a prelysosomal compartment which is either identical or functionaly similar to the endosome. Here dissociation of the lysosomal enzyme from the MPR occurs by acidification of the compartment (Gonzalez-Noriega et al., 1980). The receptor then recyles back to the Golgi to pick up another ligand molecule, and the lysosomal enzymes which remain behind are packages into lysosomes. About 5-20% of

lysosomal enzymes escape initial recognition by the MPR and are constitutively secreted. However, a portion of these enzymes bind to MPRs on the cell surface. These are internalized by endocytosis and are then delivered to the lysosomes. This second pathway is called the salvage pathway and it accounts for 5-10% of total lysosomal enzymes delivered to lysosomes in fibroblasts (Vladutiue and Rattazzi, 1979). The various pools of receptors in the Golgi, the endosomes and the cell surface appear to be in rapid equilibrium because exogeneously added antireceptor antibodies quickly interact with all of the intracellular receptor (Von Figura et al., 1984; Sahagian, 1984).

Two kinds of mannose-6phosphate (Man-6P) receptors have been identified. They are both transmembrane glycoproteins, one is cation-independent (MPR^{CI}) with a M_r of275,000 and the other is a cation-dependent (MPR^{CD}) with a M_r of 46,000. The cation-dependent receptor appears to exist as an oligomer, probably a dimer. The receptors have similar, but not identifcal specificities toward phosphorylated oligosaccharides, preferring oligosaccharides with two phosphomonosesters to those with a single phosphomonoester (Hoflack et al., 1987). The major difference between the two receptors is that the larger one has a slightly higher affinity for phosphomonosesters. Both receptors bind ligands best at slightly acidic pH values and release the ligands at pH 5.5 or below, an important property of receptors that

transport ligands to acidified components. Recently it has been shown that the cation independent MPR is identical to the insulin-like growth factor II (IGF-II) receptor (Roth, 1988).

All lysosomal enzymes thus far studied are synthesized as preproenzymes with amino-terminal extensions (Skudlarek et al., 1984). The signal sequence which makes up the prepiece is immediately cleaved after transport into the lumen of the ER. The propeptide is cleaved later over the course of several hours to several days. This process is initiated in a prelysosomal compartment and is thought to be finished after the enzymes arrive in the lysosomes (Gieselmann et al., 1983). In some instances there may be further internal cleavages of the protein as well as carboxy-terminal processing (Erickson and Blobel, 1983).

The significance of this processing is not yet fully understood. However, in the cases of lysosomal proteinases such as cathepsins B, D, and L, the pro-sequence probably functions as an activation peptide to keep the protease inactive until it is sorted from the secretory and membrane proteins. However, in many cases the pro-forms of lysosomal enzymes appear to be active. It has been speculated that in these instances the propeptide pieces could play a role in the initial folding of the protein (Gieselmann et. al., 1983). After protein folding the propeptide would then become dispensable much like the C peptide of insulin. In addition, the cleavage of the propeptide could serve to stabilize

lysosomal enzymes in the acidic environment of the lysosomes. The propeptide might also contain sorting signals necessary for directing the enzyme to the lysosomes. However, it has been shown that the native forms of some lysosomal enzymes can be phosphorylated *in vitro* by phosphotransferase, and recently it has been shown that the amino acid sequences required for the phosphorylation of cathepsin D follow the propeptide (Baranski *et al.*, 1990).

Alternate Targeting Pathways:

The importance of the Man 6-P recognition pathway has been clearly established. However, there appear to be other mechanisms for targeting acid hydrolases to the lysosomes. Studies with patients suffering from I cell disease [mucolipidosis II (ML-II)] and Hurler polydystrophy [Mucolipidosis III (ML-III)] hinted to that fact. Those patients suffer from lack of phosphotransferase activity resulting in an inability to synthesize the phosphomannosyl recognition marker (Kornfeld, 1986; Figura and Hasilik, 1986). As a result, newly synthesized lysosomal enzymes are unable to bind to the MPRs. As expected, in some cell types from these patients (e.g., fibroblasts), lysosomal enzymes are secreted into the extracellular milieu rather than targeted to the lysosomes. On the other hand, other cell types such as hepatocytes, Kupffer cells, and leukocytes have normal levels of lysosomal enzymes even though these cells are also deficient in phosphotransferase activity (Owada and Neufeld,

1982; Waheed et al., 1982). This strongly indicates that a mechanism independent from the Man 6-P pathway must exist for targeting enzymes to the lysosomes. Attempts to identify the likely receptor-mediated targeting system has proved to be unsuccessful so far. An additional puzzling finding is that that MLII fibroblasts contain normal amounts of acid phosphatase, a soluble lysosomal enzyme known to be phosphorylated in normal fibroblasts.

Lysosomal membrane proteins are targeted by a mechanism which is in part different from that used by soluble lysosomal hydrolases. The Man 6-P recognition system does not appear to be a component of lysosomal membrane protein sorting systems in the cases thus far examined (Waheed et al., 1988). Recently, it was shown that alkaline phosphatase, a type II integral vacuolar transmembrane protein, contains a vacuolar sorting signal located in the cytoplasmic and/or transmembrane domain (Kbonsky and Emr, 1990).

A class of integral membrane glycoproteins specific to lysosomes has been identified from rat, mouse, chicken, and human cells (Kornfeld and Mellman, 1989), and the human forms are designated lysosome-associated membrane glycoprotein (lamp). At least four subclasses of lamp have been identified based on apparent molecular weight. The best studied of these sublcasses are lamps 1 and 2. Lamp-1 is a highly N-glycosylated transmembrane protein. A highly conserved tyrosine residue found in the deduced sequence of the

cytoplasmic tail of lamp-1 from chickens, rodents and humans was discovered to be necessary for efficient transport of human lamp-1 to lysosomes in COS-1 cells (Williams and Fukuda, 1990). In addition, the position of the tyrosine residue relative to the membrane spanning domain and the carboxyl terminus was also found to determine lysosomal expression.

Correlation Between Lysosomal Protease Expression and Malignancy:

Recent evidence has suggested that the cysteine proteinase cathepsin B facilitates tumor cell metastasis and may contribute to degradation of host extracellular matrices (Krepela et al., 1990). Cathepsin B has been shown to have broad proteolytic specificity for protein substrates, including myosin, actin, troponin, tropomyosin, insulin, proteoglycans, nonhelical regions of type IV collagen, and, in preliminary studies, laminin (Sloane et al., 1986).

Many studies have attempted to correlate metastatic potential with the intracellular levels of cathepsin B in the belief that the activity of this enzyme within the cell reflected its physiological function at the cell surface or in the extracellular space. Positive correlations have been observed between intracellular levels of cathepsin B and the metastatic phenotype in the murine B16 melanoma variants B16-F1 and B16-F10 (Sloane et al., 1982), in variants of B16a melanoma (Sloane et al., 1984), in three Lewis lung tumors (Sloane et al., 1984), in three cultured Lewis lung clones

(Takenaza, 1984), in human lung tumors (Krepela et al., 1990), in cultured BDX rat sarcoma variants (Koppel et al., 1990), in a rat sarcoma LW13K2 which produces spontaneous metastases (Krepela et al., 1989), in human primary liver cancer (Dufek et al., 1984), and in colonic cancer (Keppler et. al., 1988).

Of particular relevance to tumor invasion and metastasis is the increasing body of literature on the release of cathepsin B from human and animal tumors (Pietras and Roberts, 1981; Bajkowski et al., 1984). Enzymologically, the secreted enzymes are very similar to lysosomal cathepsin B in terms of their reactivity to synthetic substrates and to inhibitors. It was shown that cultured explants of malignant human breast tumors release eleven times more cathepsin B activity than normal breast tissue or nonmalignant tumors (Poole et al., 1978; Recklies et al., 1980). Other reports indicated elevation of cathepsin B activity in the pancreatic fluid of patients with pancreatic cancer (Rinderknecht and Renner, 1980), in serum of women with diverse invasive neoplastic diseases (Pietras et al., 1981), in malignant ascites fluid from patients with primary liver and ovarian cancer (Dufek et al., 1984; Mort et al., 1983), in pleural effusions from patients with breast cancer (Petrova-Skalkova et al., 1987), and in conditioned media from human and murine tumors (Quian et al., 1989; Mort et al., 1983; Recklies et al., 1982). Cathepsin B is also reported to be associated with the plasma membrane in human, rat, and murine tumors (Koppel et al.,

1984; Pietras and Roberts, 1981; Dufek et al., 1984; Mort et al., 1983; Sloane et al., 1987).

The secreted cathepsin B-like enzyme activities are different from lysosomal cathepsin B in that they have higher molecular weights, different isoelectric points and, unlike lysosomal cathepsin B, are stable above pH 7.0. A latent high molecular weight form of cathepsin B (40,000) has been identified in ascitic fluid and in culture medium of ascites cells from cancer patients. The latent enzyme could be rendered active by prior proteinase treatment (Mort et al., 1981).

The increased secretion and altered cellular distribution of cathepsin B in tumor cells may have its origins in postranslational events. However, it was also shown that cathepsin B levels are regulated at the level of synthesis. The mRNA levels for cathepsins B, L, D, H, and S in normal fibroblasts, mouse B16 melanoma cells, variants in rat carcinosarcoma , and in normal rodent tissues revealed that only the mRNA for cathepsin B is elevated in the malignant tumors (Qian et al., 1989; 1990). In the melanoma variants this elevation correlated with their metastatic phenotype as measured by their lung colony forming potential. In addition, murine melanomas contain two large cathepsin B mRNA transcripts which are not seen in normal mouse tissues. However, it has been shown that all three transcripts differ only in the length of their 3'untranslated regions (Qian et

al., 1989).

Parenteral administration of papain in mice induced antipapain antibodies that cross-react with cathepsin B and cathepsin H (Bellelli et al., 1990). In such animals, the invasion of the abdominal wall tissue (in intraperitoneally transplanted mice) by B16 melanoma and Lewis lung carcinoma tumors was clearly reduced; tumor growth rates were significantly lowered; fewer pulmonary metastases were found, and the mean survival time increased up to 180%, as compared to the controls. However, in contrast to the positive reports cited above, a lack of correlation between cathepsin B and metastatic potential was shown in a rat prostatic adenocarcinoma (Lowe and Isaacs, 1984), and in variants of a methylchloranthrene-induced sarcoma (McLaughlin et al., 1983).

Transformed mouse fibroblasts such as Kirsten-virus-transformed NIH-3T3 (KNIH) cells secrete large amounts of a 39-KDa glycoprotein called MEP (major excreted protein) (Gottesman and Sobel, 1980). MEP was later cloned from NIH3T3 cells and was shown to be the precursor form of cathepsin L (Troen et al., 1987). MEP is synthesized by transformed fibroblasts in quantities 20-100 fold greater than their nontransformed counterparts (Gottesman and Sobel, 1980). It represents up to 30% of the total protein secreted by KNIH cells, whereas it is only 0.2-0.4% of the total protein secreted by nontransformed NIH-3T3 cells. The increase in the synthesis of procathepsin L in transformed fibroblasts is due

to increased mRNA levels (Gottesman et al., 1980), but it was not known whether the increased secretion of procathepsin L was due to a defect in a cellular component of the machinery which targets enzymes to lysosomes, a change in the structure of cathepsin L, or simply a reflection of the overexpression (increased synthesis) of procathepsin L. Recently, it has been reported that the form of cathepsin L secreted by KNIH cells possesses the mannose 6-phosphate lysosomal marker but binds poorly to mannose 6-phosphate receptors (Gal and Gottesman, 1986; Dong et al., 1989).

In addition to tumors which express high levels of cathepsin B or cathepsin L, some tumors are reported to produce and secrete precursor forms of other lysosomal proteinases. A human breast carcinoma, MCF7, secretes an autoactivatable procathepsin D in cell culture in response to estrogen stimulation (Capony et al., 1989). Overexpression of human cathepsin D by a transfected rat cell line which normally did not secrete cathepsin D increased its malignant phenotype in vitro and its metastatic potency in vivo (Garcia et al., 1990). Morris hepatoma 7777 cells secrete early or precursor forms of cathepsin C (Mainferme et al., 1985).

It is possible that some tumor cells may secrete unidentified proteolytic activities that have been mistakenly attributed to known, characterized enzymes because of similar substrate and inhibitor specificities. When the conditioned media from cultures of 38 human, murine and hamster cells was

examined, they were all found to contain an enzyme activity which was attributed to cathepsin L (Yamaguchi et al., 1989). Subsequently, this activity was purified from the conditioned media obtained from the human tumor cells HPC-YP in culture. The results indicated that the enzyme produced in HPC-YP cells differed from cathepsin L; the M_r of the tumor enzyme was 68,000 as compared with 34,000 for human liver cathepsin L, and the tumor enzyme was stable to heat treatment and to extremes of pH (Yamaguchi et al., 1989). It was concluded that the enzyme secreted by the human tumor cells, HPC-YP, which appeared to have cathepsin L activity was in fact different from normal human liver cathepsin L.

At present, despite the well documented correlation between malignant transformation and increased secretion of lysosomal enzymes such as cathepsin B and cathepsin L, there remains a number of questions concerning the pathology and mechanism of expression of these enzymes in tumor cells. Among these are:

1) What is the cellular and molecular basis for the elevated secretion of cathepsin B and cathepsin L by tumors? Are tumor cells simply less efficient at targeting lysosomal enzymes so that by default many are diverted to a secretory pathway and escape proteolytic processing, or is secretion specific for a given enzyme in a given tumor type?

2) Are tumor cells defective in components of the lysosomal enzyme targeting system? Do they lack mannose 6-phosphate

receptors? Are they unable to generate a pH gradient along the biosynthetic transport pathway necessary for the recycling of MPRs?

3) Is the mistargeting of cathepsin B by tumor cells related to possible tumor cell specific differences in the glycolytic processing of oligosaccharide side chains in lysosomal enzymes? Does the secreted form of cathepsin B bear a mannose 6-phosphate signal and is it capable of binding with high affinity to mannose 6-phosphate receptors?

4) What is the structure of the secreted tumor procathepsin B-like enzyme? Is it the high molecular weight precursor form of cathepsin B, a related enzyme from a different gene locus, or a modified enzyme which lacks the ability to be efficiently targeted to the lysosomes?

In this work I have sought to address some of these questions.

First, I have examined the effects of monensin on the secretion of cathepsin B and other lysosomal enzymes by transformed cells. The assumption was that cells which are already inefficient in receptor-dependent targeting of enzymes to lysosomes cannot be further stimulated to secrete high levels of enzymes by agents which disrupt receptor-dependent transport.

Second, I have examined the immunoreactivity of the cathepsin B-like activities secreted by several tumor cells in order to determine their relationship to normal cathepsin B.

Third, I have purified and partially characterized a B16 melanoma secreted cathepsin B-like activity which does not react with antibodies against cathepsin B as a first step in determining the nature of this enzyme.

CHAPTER III

EXPERIMENTAL PROCEDURES

Materials

N-Benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-AMC), Z-Phe-Arg-AMC, H-Arg-AMC, Z-Gly-Gly-Arg-AMC, and Z-Gly-Gly-Leu-AMC were from BACHEM Bioscience Inc. (Philadelphia, PA) or Enzyme Systems Products (Livermore, CA). 4-Methylumbelliferyl-ß-D-glucuronide, monensin, cycloheximide, E64, leupeptin, diisopropyl fluorophosphate, 2,2-'dithiodipyridine, 4,4-'dithiodipyridine, and pepsin (3900 units/mg protein) were from Sigma. Nitrocellulose blotting papers were obtained from Schleicher and Schuell. Yeast phosphomannan from Hansenula holstii NRRL Y-2448 was the generous gift of Dr. M. E. Slodki, Northern Regional Research Laboratories, USDA (Peoria, IL).

The mannose 6-phosphate receptor specific ligand, pentamannosyl 6-phosphate-bovine serum albumin (PMP-BSA), was prepared by Qiuming Gong in our laboratory. Phosphomannan was hydrolyzed with dilute acid to yield pentamannosyl 6-phosphate (PMP) and a nonhydrolyzable polysaccharide core (Slodki *et al.*, 1973; Murray and Neville, 1980). PMP was then coupled to bovine serum albumin by reductive alkylation (Schwartz and Gray, 1977). The PMP-BSA product was separated form unincorporated PMP by chromatography on a 2.5 x 45 cm column

of Bio-Gel P-10 (Bio-Rad) in 20 mM Tris-HCl, 200 mM NaCl, 0.02% sodium azide buffer, pH 7.0. Assay of the effluent fractions for protein, mannose, and phosphate (Fiske and Sublarow, 1925) gave a phosphate: mannose molar ratio of 1:5.03 for PMP and a phosphate: protein molar ratio of 18.4:1 for PMP-BSA. PMP-BSA was labeled using $Na^{125}I$ (Amersham Corp.) and Iodo-Beads (Pierce Chemical Co.) according to the instructions of the manufacturer. Unreacted iodine was separated from the labeled protein by chromatography on an 8-ml column of Bio-Gel P-6 in 0.05 M sodium phosphate, 0.2 M NaCl buffer, pH 7.5. Fractions containing labeled PMP-BSA were then dialyzed for 96 h against 1 liter of the same buffer with three buffer changes.

Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) were obtained from Bio-Rad. Other reagents and buffers were purchased from commercially available sources and were of research grade purity.

Cell Culture:

Murine BALB/3T3 clone A31 cells (3T3), Moloney Murine Sarcoma Virus-transformed BALB/3T3 cells (MMSV), human breast carcinoma cells (MCF7) were obtained from the American Type Culture Collection. MMSV and 3T3 cells were propagated in 75 cm^2 tissue culture flasks in 10 ml of Dublecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 3.7 mg/ml sodium bicarbonate, 4.5 mg/ml D-glucose (MMSV cells only) and 10% calf serum. B16 melanoma

and MCF7 cells were grown in 75cm^2 flasks in 10 ml of minimal essential medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 1.27 mg/ml sodium bicarbonate, and 10% heat inactivated fetal calf serum . Cells were passed every three days by scraping them into 10 ml of serum-free medium, pelleting at 500xg, and resuspending in fresh medium containing 10% serum at a cell density of 1x10^5 cells/ml.

Preparation of Medium for the Purification of the Melanoma Cathepsin B-like Activity:

To obtain medium for the purification of the cathepsin B-like enzyme, B16 F-10 cells from five confluent T_{75} cm^2 were scrapped and added to a stir-bottle containing 1 liter of minimal essential medium, 10% fetal calf serum, and 1 g of Cytodex 2 beads (Pharmacia). The cells attached to the beads and grew. Just prior to confluency, the media containing serum was discarded and the beads rinsed with incomplete medium 5 times allowing for a 30 min incubation at 37° C with each rinse. The beads were then incubated in serum-free minimal essential medium for 18h after which the media was collected, centrifuged at 500xg and filter sterilized. The cells were allowed to recover by incubating them with minimal essential medium containing 10% fetal calf serum for 24 h. This cycle was repeated 4 times.

Preparation of Media and Cell Extracts for Measurement of Lysosomal Enzymes:

After achieving a confluency of about 70-80% in T_{75} flasks

the cells were washed once with phosphate-buffered saline (calcium-and magnesium-free) and incubated for 18 h in medium without serum. In some cases this medium also contained monensin (0.03-10 μM) and/or cycloheximide (100 μM). At the end of this incubation period this medium was collected, centrifuged at 15,000xg for 10 min and the pellet discarded. The adherent cells were washed with phosphate-buffered saline, detached with a cell scraper, and counted with a model ZBI Coulter counter. The cells were pelleted by centrifugation and extracted by sonication for 30 sec. in 50mM sodium acetate/1.0 mM EDTA/0.02% Triton X-100, pH 5.2, as described previously (Qian et al., 1989). Cell lysates and media samples could be quick-frozen and stored at -20^{0}C for later analysis without significant effect on the activities of the enzymes measured or on the proportions of latent and active forms.

Measurements of Enzyme Activities

Latent forms of cathepsins B and L were activated by a 30-min preincubation with pepsin in activation buffer (30 mM dithiothreitol and 15 mM EDTA, pH 4.2) at 37°C (Qian et al., 1989). The ranges of pepsin concentrations which produced optimal activations of cathepsins B and L during the 30-min incubation period were 0.41-3.3 and 0.83-5.0 mg/ml, respectively. To measure levels of active forms of cathepsins B and L, samples were preincubated for 30 min at 25°C in activation buffer alone (Qian et al., 1989; Bajkowski and

Frankfater, 1975). Latent enzyme was defined as the difference in the activity of samples when preincubated with and without pepsin. Cathepsin B was measured with Z-Arg-Arg-AMC (final concentration 94 μM) in 0.2 M citrate/phosphate buffer, as described previously (Qian et al., 1989). Cathepsin L was estimated with Z-Phe-Arg-AMC (final concentration 5 mM) under identical conditions. The AMC products were quantified with an SLM/Aminco SPF-500C spectrofluoromoter at excitation and emission wavelengths of 370 and 460 nm, respectively. While Z-Arg-Arg-AMC is not hydrolyzed by cathepsin L and other known lysosomal cysteine proteinases, both cathepsin B and cathepsin L can catalyze the hydrolosis of Z-Phe-Arg-AMC. However, based on the k_{cat} and K_m values reported for these substrates with the two enzymes from humans and rats (Mason, 1986; Baricos et. al; 1988), the individual contributions of cathepsin B and cathepsin L to the hydrolysis of Z-Phe-Arg-AMC could be estimated. Thus, at the substrate concentrations used, the Z-Phe-Arg-AMC/Z-Arg-Arg-AMC activity ratio for cathepsin B was calculated to be 0.13 and the cathepsin L/cathepsin B activity ratio for Z-Phe-Arg-AMC was calculated to be 1.9 (Mason, 1986; Baricos et. al., 1988).

ß-Glucuronidase was measured with 1 mM 4-methylumbelliferyl-ß-D-glucuronide in 0.1 M citrate buffer, pH 4.8, essentially as described previously (Robbins, 1979). The 4-methylumbelliferone product was quantified by fluorescence measurements at excitation and emission wavelengths of 365 and

448 mm, respectively.

Cell Surface Receptor Binding Assays

Cell surface mannose 6-phosphate binding activity was measured by a modification of the procedure of Brown and Farquhar (1984). MMSV and 3T3 cells were grown to near confluence in 9 cm tissue culture dishes as described above. The cellular monolayer was then washed with serum-free medium 3 times and equilibrated at 4°C for 10 min. The cells were then incubated at 4°C with increasing amounts of ^{125}I-labeled PMP-BSA for 1 h in the absence and presence of either mannose 6-phosphate or pentamannosyl 6-phosphate (PMP). The monolayer was then washed 5 times with medium and extracted with 1 ml of 1 N NaOH. Radioactivity was determined with a Tm Analytical model 1191 gamma counter and protein was determined by the Bio-Rad micro protein assay kit according to the instructions of the manufacturer. Binding data were fit to the equation $(cpm)_{bound}=B_{max}$ [PMP-BSA]/(k_d+[PMP-BSA]) with the program Enzfitter (Elsevier-Biosoft, Cambridge, UK).

Purification of the Secreted Procathepsin B-like Enzyme

Media from B16F-10 cells were collected as described above. The sample (4 l.) was then concentrated to a volume of 25 ml and dialyzed against 50 mM phosphate buffer pH 6.5 overnight. It was then applied on to a concanavalin-A-Sepharose (Sigma) column (7 ml bed volume) previously equilibrated with 50 mM phosphate buffer pH 6.5. The column was then washed with 4 volumes of buffer to remove nonabsorbed

proteins. The cathepsin B-like activity that bound to the column was eluted first by 0.1M α-methyl-D- mannoside in 50 mM phosphate buffer, pH 6.5, and the eluted proteins were labeled pool #1. The column was then incubated with 0.1M α-methyl-D- mannoside in 50 mM phosphate buffer, pH 6.5, for 1.5 h and eluted with the same buffer. These fractions were labeled pool #2. The concentration of α-methyl-D- mannoside was then increased to 1 M and the proteins eluted were labeled as pool #3. The column was then incubated overnight with 1 M α-methyl-D- mannoside and then eluted the next day with the same buffer (pool #4). Each pool was dialyzed against 20 mM Tris-HCl pH 7.9. Pool 4 (opprox. 25 ml) which had the highest specific activity was applied to a polywax weak anion exchange HPLC column (The Nest Group) using multiple injections. The cathepsin B-like enzyme activity was eluted with a 0 to 1.0 M gradient of increasing sodium acetate, pH 7.9 at a flow rate of 0.5 ml/min.

Generation of a Polyclonal Antibody against ß-Galactosidase-cathepsin B Fusion Protein:

Polyclonal antibodies to cathepsin B were generated by Qiuming Gong in this laboratory. Briefly, a cDNA clone of mouse cathepsin B (Segundo et al., 1985) comprising the region coding for the mature form of cathepsin B was subcloned into a bacterial expression plasmid pWR590. E. coli strain HB101 was transformed with this vector and grown to an O.D. of 0.4. ß-Galactosidase was induced with IPTG (isopropylthio-ß-

galactoside) and the expressed ß-galactosidase-cathepsin B fusion protein with a molecular weight of 98,000 was isolated by SDS-PAGE and electroelution.

A New Zealand white rabbit was immunized with the purified fusion protein in complete Freund's adjuvant. The fusion protein injection was repeated four times with incomplete Freund's adjuvant at three-week intervals. Then the rabbit was bled and its serum was collected and stored at -20°C.

Western (protein) Blotting:

SDS-PAGE was performed on samples of cell extracts, culture media and fractions obtained during the purification of the secreted cathepsin B-like activity as described previously (Laemmli, 1970). The gels were then electroblotted onto nitrocellulose in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol [pH 8.3]) using the Bio-Rad miniblotter, according to the instructions of the manufacturer. The nitrocellulose was washed in 10 mM Tris hydrochloride (pH 8.0)/150 mM NaCl/0.05% Tween 20 (TBST) and incubated for 1 h (room temperature) in TBST plus 5% instant nonfat milk. Next the nitrocellulose filter was incubated overnight at 4°C in TBST and milk which also contained immune serum at a final dilution of 1:500. The following day, the nitrocellulose paper was washed in TBST three times for 10 min each and the filter incubated in TBST + milk containing anti-rabbit-immunoglobulin G alkaline phosphatase antibody at a

dilution of 1:10,000 for 1 h at room temperature. This solution was removed and the nitrocellulose filter was washed 3 times with TBST. The filter was blotted semi-dry and a solution containing Nitro Blue Tetrazolium (300 mg/ml), 5-bromo-4-chloro-3-indolyl phosphate (165mg/ml) in 100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5, was added to the filter for color development. Two minutes later, the reaction was stopped with 20 mM Tris-HCl containing 5 mM EDTA.

Protein Alkylation:

The purified procathepsin B-like enzyme was reduced and S-carboxymethylated as described (Takio et al., 1983). Briefly, the protein was denatured by dissolving it in 0.4 ml of 6 M guanidine hydrochloride in 1.5 M Tris-HCl, pH 8.6, containing EDTA (5mg/ml). Dithiolthreiotol (DTT) (5 mg) was then added to reduce the protein, and the solution was incubated for 1 h at room temperature. Iodoacetic acid, 13.4 mg in 0.1 ml of 0.5 N NaOH was then added and the solution incubated for 30 min at room temperature in the dark. The reaction was stopped by acidifying the solution to about pH 5.0 with 6N HCl. The sample was then dialyzed against distilled water and lyophilized.

Treatment with Endoglycosidase F/N-Glycosidase F:

The purified, alkylated enzyme was dialyzed against 50 mM sodium acetate pH 5.0. Endoglycosidase F/N-glycosidase F (1 unit) was added to this solution and the mixture incubated for 18 h at 37°C (Twining, 1984).

HPLC Gel Filtration:

The apparent molecular weights of the procathepsin B-like enzyme and its pepsin-activated form were determined by gel filtration on a Bio-Sil TSK 250 column (600 x 7.5 mm). The column was equilibrated with 50 mM phosphate buffer, pH 6.0. The flow rate was 0.5 ml/minute. The column was standardized by the injection of 200 μl of each of the following standards: Blue dextran; B-Galactosidase, M_r 116,000; bovine serum albumin, M_r 67,1000; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 29,000; calmodulin, M_r 17,000 ; and aprotinin, M_r 6,500.

The void volume of the column, V_0, was set equal to the elution volume of blue dextran and the total liquid volume of the column, V_T was set equal to the position of the solvent front obtained after each injection of a standard. The sieve coefficient, K_D, for each standard was calculated from its elution volume with the following equation (Tan et al., 1975).

$$K_D = \frac{(V_e)\ \text{standard} - V_0}{V_T - V_0} \quad (1)$$

A standard curve was then prepared according to the relationship (Tan et al., 1975)

$$K_D = A \log M_r + B. \quad (2)$$

Either the latent or pepsin-activated procathepsin B-like enzyme (see previous section) (200 μl) was applied and 200 μl fractions were collected. Enzyme assays were performed on both the latent and the active forms of the enzyme (see

previous section) to determine their elution volume. Sieve coefficients were then calculated according to equation 1 and molecular weights were estimated from the standard curve (eq. 2).

Equilibrium Dissociation Constant for Leupeptin with the Cathepsin B-like Enzyme

At pH 6.2, the equilibrium dissociation constant, K_I, for leupeptin with the pepsin activated latent cathepsin B-like enzyme was determined from the inhibition of hydrolysis of Z-Arg-Arg-AMC under first-order conditions (K_m>>[S]) and enzyme concentrations on the order of (K_I) obsd. The preactivated enzyme was preincubated with increasing concentrations of leupeptin in the assay buffer at pH 6.2 for 45 min. The reaction was then initiated by adding Z-Arg-Arg-AMC at a final concentration of 50 uM. K_I was then determined.

$$\frac{[I_0]}{1-v_i/v_0} = \frac{K_I}{v_i/v_0} + [E_0] \quad (3)$$

by a least squares linear regression analysis of

$$\frac{[I_0]}{1-v_i/v_0} \text{ vs } \frac{1}{v_i/v_0}$$ (Frankfater and Kuppy, 1981).

In this equation v_i and v_0 are the inhibited and uninhibited rates, respectively.

Measurements of Association and Dissociation Rate for the Reaction of Leupeptin with the Pepsin-activated Cathepsin B-like Enzyme

The inhibition of the pepsin-activated cathepsin B-like enzyme by leupeptin was determined against the substrate Z-

Arg-Arg-AMC as described in the preceeding section. In the absence of inhibitor, the formation of product was linear with time. In the presence of inhibitor the rate progressively decreased in a first-order manner until the final inhibited rate was achieved. First-order rate constants, k', were calculated by fitting the fluorescence data to the equation $F = A(1-e^{kt}) + Bt$ by a least squares von linerar analysis using the program enzfit (elsevier, Biosoft).The rate constants k_{on} and k_{off} were determined according to equation (4)

$$k' = k_{on}[I] + k_{off} \quad (4)$$

from a linear regression analysis of k' vs [I] (Schultz et al., 1989). Values of k_{on} were corrected for the presence of inactive inhibitor forms, the leupeptin hydrate and the leupeptin cyclic carbinolamine, which together represent 98% of the total inhibitor concentration (Schultz et. al., 1989).

Active Site Titration of the Pepsin-activated Cathepsin B-like Enzyme

The latent cathepsin B-like enzyme was incubated with increasing concentrations of the stoichiometric thiol proteinase inhibitor E64 while simultaneously activating with pepsin. After a 30 minute incubation period, enzyme activity was measured with Z-Arg-Arg-AMC at a final concentration of 200 μM. The concentration of the enzyme was determined by a least square linear regression analysis of the hydrolase activity remaining vs the concentration of the inhibitor E64. The intercept of the extrapolated linear portion of this graph

with the x-axis is the estimated concentration of the enzyme.

Determination of the Kinetic Constants kcat and Km

The kinetic constants k_{cat} and K_m of the pepsin-activated cathepsin B-like enzyme for the hydrolysis of Z-Arg-Arg-AMC and Z-Phe-Arg-AMC were determined by measuring the initial hydrolysis rate at different concentrations of substrates [(Z-Arg-Arg-AMC: 100, 200, 500, 1000 μM); Z-Phe-Arg-AMC: 5, 25, 50, 100 μM)]. K_m and V_{max} values were determined by a least square non-linear regression analysis of the hydrolase activity vs the concentration of the substrate using the following equation.

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (5)$$

In this equation v is the hydrolysis rate and [S] is the final substrate concentration.

To calculate k_{cat}, V_{max} was divided by the enzyme concentration deduced from the active site titration (see previous section), according to the following equation

$$V_{max} = k_{cat} \times [E] \quad (6)$$

Determination of Specificities (k_{cat}/K_m) of the Pepsin-activated Cathepsin B-like Enzyme with Different Substrates

The values of k_{cat}/K_m of the cathepsin B-like enzyme with different substrates (H-Arg-AMC, Z-Arg-AMC, Z-Gly-Gly-Arg-AMC, Z-Gly-Gly-Leu-AMC) was determined by measuring its hydrolase activity at final substrate concentrations of 10 and 20 μM. Under those conditions $[S] << K_m$, and according to the first

order kinetic equation,

$$v = \frac{k_{cat}}{K_m} [E][S] \quad (7)$$

dividing the rate, v, by the enzyme concentration [e] and the substrate concentration [S] gave an estimated value for $\frac{k_{cat}}{K_m}$.

The pH Dependency of k_{cat}/K_m for the Hydrolysis of Z-Arg-Arg-AMC by the Pepsin-activated Cathepsin B-like Enzyme

Under conditions of $[S] << K_m$ (see eq. 7), the hydrolysis rate of Z-Arg-Arg-AMC (25 μM) by the cathepsin B-like enzyme was measured at different pHs using the assay buffers; 3.5, 0.2M NaCl/0.1M Na Ac, pH 3.5-5.5; 0.2M NaCl/0.1M Na_2 PHO_4, pH 6.0-7.5; 0.2M NaCl/0.1M Tris-HCl, pH 7.0-8.0. pKa values were determined by a least square non-linear regression analysis of the hydrolase activity vs the pH of the assay buffer using the following equation

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{(k_{cat}/K_m)\ \lim}{1 + \frac{[H^+]}{Ka_1} + \frac{Ka_2}{[H^+]}} \quad (8)$$

Inhibition Studies

The pepsin-activated cathepsin B-like enzyme was incubated in the presence of 1mM concentrations of diisopropyl fluorophosphate, 2,2'-dithiodipyridine and 4,4'-dithiodipyridine for 45 min. The hydrolysis rate of Z-Arg-Arg-AMC (50 μM) by the enzyme was then measured as described before.

CHAPTER IV

RESULTS

Effects of Viral Transformation on Lysosomal Enzyme Levels: Table 1 shows the levels of intracellular and secreted Z-Arg-Arg-AMC hydrolase (cathepsin B-like), Z-Phe-Arg-AMC hydrolase (cathepsin L-like) and ß-glucuronidase activities in 3T3 and MMSV cells. Latent enzyme activity is defined as the difference in activity observed after a 30-min preactivation in the absence and presence of pepsin. Virtually all the secreted cathepsin B and cathepsin L-like activities were latent in the MMSV cells. On the other hand, about 30% of the secreted cathepsin B and L-like activities from BALB/3T3 cells were active in the absence of pepsin pretreatment. From the reported substrate specifities and rate constants for cathepsin B and L (Mason, 1986; Baricos et. al., 1988), and assuming these are the only enzymes present which could hydrolyze the two substrates, virtually all the observedZ-Arg-Arg-AMC hydrolase activity was due to cathepsin B, while at least 70 and 90% of the Z-Phe-Arg-AMC activity in cell lysates and media samples, respectively, was due to cathepsin L.

From the results in Table 1, it can be seen that viral transformation of BALB/3T3 fibroblasts caused an increase in

Table 1.Intracellular and Secreted Forms of Lysosomal Enzymes in Normal and Transformed Cells in Culture[a].

	Cell-associated activities (pmol/min/million cells)				
	Cathepsin B-like[b]		Cathepsin L[c]		B-glucuronidase
	Active	Latent	Active	Latent	
3T3	11.8 (1.0)	ND[d]	5.0 (.6)	1.9 (.22)	45.7 (1.2)
MMSV	31.0 (2.7)	ND	16.1 (2.2)	27.1 (2.8)	90.3 (10.8)
B16F-1	128.5 (9.5)	4.2 (0.2)	62.8 (3.1)	15.3 (1.2)	126.8 (20.3)
B16a	345.1 (25.5)	10.7 (0.7)	129.9 (11.3)	16.1 (1.4)	197.7 (14.9)
MCF7	15.0 (1.1)	ND	3.6 (0.1)	5.3 (0.3)	415.4 (14.4)

	Secreted[e] activities (pmol/min/million cells)		
	Cathepsin B-like	Cathepsin L	B-glucoronidase
3T3	7.9 (0.2)	62.6 (2.9)	43.2 (2.5)
MMSV	131.1 (17.5)	1717.4 (40.1)	88.3 (2.8)
B16F-1	92.8 (1.2)	265.9 (21.9)	35.0 (1.9)
B16a	146.4 (2.0)	181.4 (6.9)	12.8 (0.9)
MCF7	2.7 (0.1)	19.1 (1.3)	16.3 (1.6)

[a]3T3, BALBc/3T3 fibroblasts; MMSV, Moloney Murine Sarcoma Transformed BALB 3T3 cells; B16F-1 and B16a, B16 murine melanoma variants having low and high metastatic potentials; MCF7, human breast carcinoma. The numbers in parenthesis are the standard errors of three determinations.

[b]Cathepsin B-like activity determined with 94 μM Z-Arg-Arg-AMC

[c]Cathepsin L activity determined with 5 μM Z-Phe-Arg-AMC

[d]Latent cathepsin B-like is defined as the increase in Z-Arg-Arg-AMC activity after pepsin pretreatment. However, treatment of cell extracts with pepsin generally reduced the Z-Arg-Arg-AMC activity by 10-20%. Thus latent cathepsin B could not be estimated in 3T3, MMSV, and MCF7 cells.

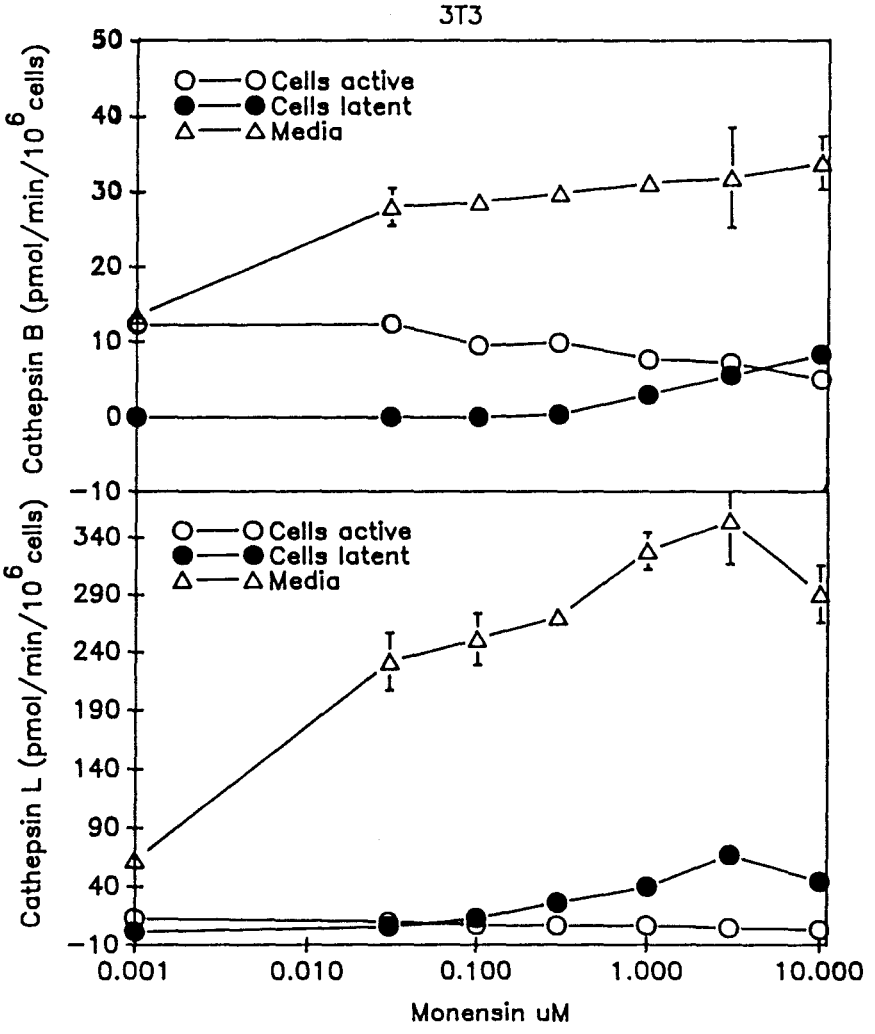
[e]All secreted cathepsin B-like and cathepsin L activities are latent except in the BALBc/3T3 cells where about 30% of the secreted enzymes are active in the absence of pepsin pretreatment.

cellular levels of cathepsin B, cathepsin L, and ß-glucuronidase, and a dramatic increase in the amounts of latent cathepsin B (17-fold) and latent cathepsin L (27-fold) secreted into the culture medium. A less dramatic (2-fold) but statistically significant increase in ß-glucuronidase secretion was also seen. Although it has been well documented that fibroblasts secrete increased amounts of procathepsin L in response to malignant transformation (Gottesman, 1978; Gal and Gottesman, 1986; Denhardt et al., 1986; Troen et al., 1987), it has not been previously reported that cathepsin B and ß-glucuronidase secretion are also elevated after viral transformation. While it could be estimated that cell extracts contained similar amounts of active cathepsin B and cathepsin L, the media from both transformed and nontransformed 3T3 cells contained much more latent cathepsin L than latent cathepsin B (perhaps as much as 50- and 30-fold more, respectively). MMSV cells also contained measureable levels of latent cathepsin L but cellular latent cathepsin B could not be detected.

Effects of Monensin and Cycloheximide on Lysosomal Enzyme Levels in BALB/3T3 and MMSV-transformed BALB/3T3 Cells

Figure 1 shows the effects of an 18 h preincubation of 3T3 cells with increasing concentrations of monensin on cellular and secreted cathepsin B and cathepsin L activities. It can be seen that cathepsin B secretion increased with increasing monensin concentrations between 0.03 μM and 0.1 μM,

Fig. 1. Dose dependency of monensin on cellular and secreted levels of cathepsin B and cathepsin L in BALB/3T3 fibroblasts. The monensin concentrations varied from 0.03 to 10 μM. Active and latent forms of cathepsin B and cathepsin L were measured with 94 μM Z-Arg-Arg-AMC and 5 μM Z-Phe-Arg-AMC, respectively, before and after pretreatment with pepsin as described under "Experimental Procedures". About 30% of cathepsin B and cathepsin L secreted by BALB/3T3 cells were already active. Pepsin sometimes caused a 10-20% decrease in cathepsin B in cell lysates. In those cases the levels of latent cathepsin B could not be determined. Some of the error bars in this figure were smaller than the symbols.


3T3
Cells active
Cells latent
Media
Cathepsin B (pmol/min/10^6 cells)
Cathepsin L (pmol/min/10^6 cells)
50
40
30
20
10
0
-10
340
290
240
190
140
90
40
-10
0.001
0.010
0.100
1.000
10.000
Monensin uM

and then plateaued between 0.3 μM and 10 μM. Figures 2 and 3 compare the effect of monensin on the secretion of cathepsins B and L by 3T3 and MMSV cells. It can be seen that treatment of 3T3 cells with monensin causes a (4.3- and 7.3-fold increase) in the amounts of latent cathepsin B and cathepsin L, respectively, in serum-free medium. Monensin also caused an increase in the amount of latent enzymes recovered from 3T3 lysates. These increases were greatly reduced (85-90%) by 100 μM cylcloheximide, indicating that the latent enzymes secreted in the presence of monensin were newly synthesized. In contrast, the already high levels of secretion of latent cathepsin B and latent cathepsin L by MMSV cells could not be further stimulated by monensin. However, this secretion did remain cycloheximide-inhibitable (90% or greater). Fig. 4 shows the results of a similar experiment with ß-glucuronidase. Again it can be seen that monensin stimulated ß-glucuronidase release from 3T3 cells but not from MMSV cells. In this case, however, cycloheximide produced only a 55% inhibition of the monensin-stimulated secretion of ß-glucuronidase by 3T3 cells and a 5% inhibition of enzyme secretion by MMSV cells, raising the possibility that a significant portion of the secreted ß-glucuronidase activity was due to protein which had been synthesized prior to the 18 h incubation period in serum-free medium.

The lysosomotropic amines, ammonium chloride and chloroquine, at concentrations of 10 mM, exhibited similar

Fig. 2. Effects of monensin and cycloheximide on cellular and secreted levels of cathepsin B in BALB/3T3 and Moloney murine sarcoma virus-tranformed BALB/3T3 fibroblasts. The monensin and cycloheximide concentrations were 3.0 and 100 μM, respectively. Active and latent forms of cathepsin B were measured with 94 μM Z-Arg-Arg-AMC before and after pretreatment with pepsin as described under "Experimental Procedures". Virtually no active cathepsin B could be detected in media of MMSV cells, while about 30% of the enzyme secreted by BALB/3T3 cells was already active. Pepsin sometimes caused a 10-20% decrease in cathepsin B activity in cell lysates. In those cases the levels of latent cathepsin B could not be determined.

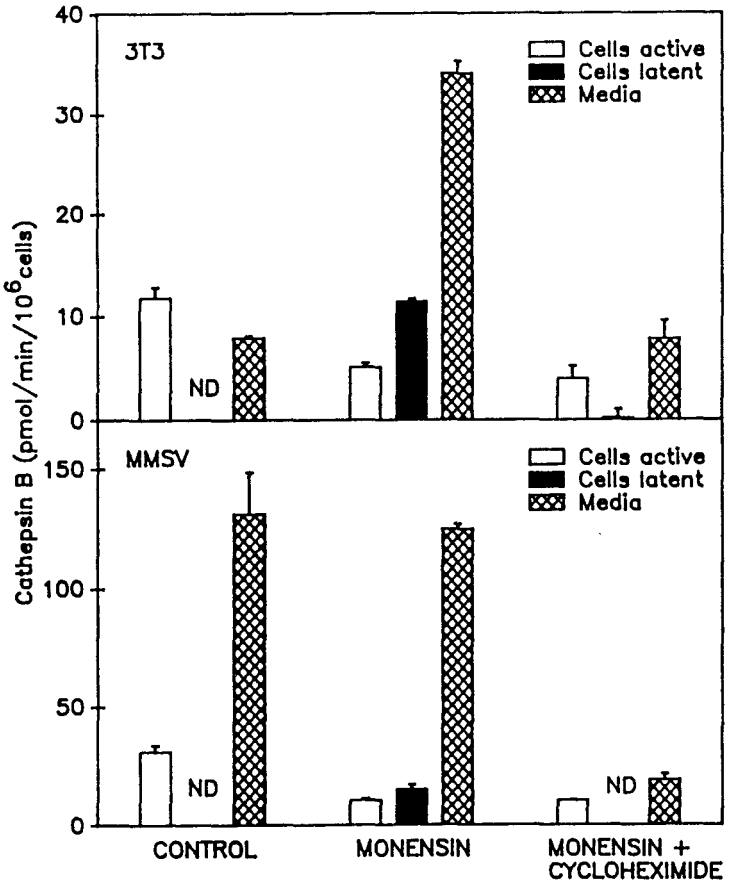

3T3
Cells active
Cells latent
Media
ND
MMSV
Cells active
Cells latent
Media
ND
ND
Cathepsin B (pmol/min/10^6cells)
40
30
20
10
0
150
100
50
0
CONTROL
MONENSIN
MONENSIN + CYCLOHEXIMIDE

Fig. 3. Effects of monensin and cycloheximide on cellular and secreted levels of cathepsin L in BALB/3T3 and Moloney murine sarcoma virus-transformed BALB/3T3 fibroblasts. Monensin and cycloheximide concentrations were identical to those in Fig. 2. Active and latent forms of cathepsin L were measured with 5 μM Z-Phe-Arg-AMC before and after pretreatment with pepsin as described under "Experimental Procedures". It was possible to estimate that the majority of the Z-Phe-Arg-AMC activity measured in the cellular and media samples was due to cathepsin L (see "Experimental Procedures"). About 30% of the cathepsin L activity in the media samples from BALB/3T3 cells was already active while virtually no cathepsin L activity could be detected in media samples of MMSV cells in the absence of pepsin pretreatment. Because of the scale of the figure, some of the values cannot be estimated from the graph and are given in parenthesis. ND, not determinable.

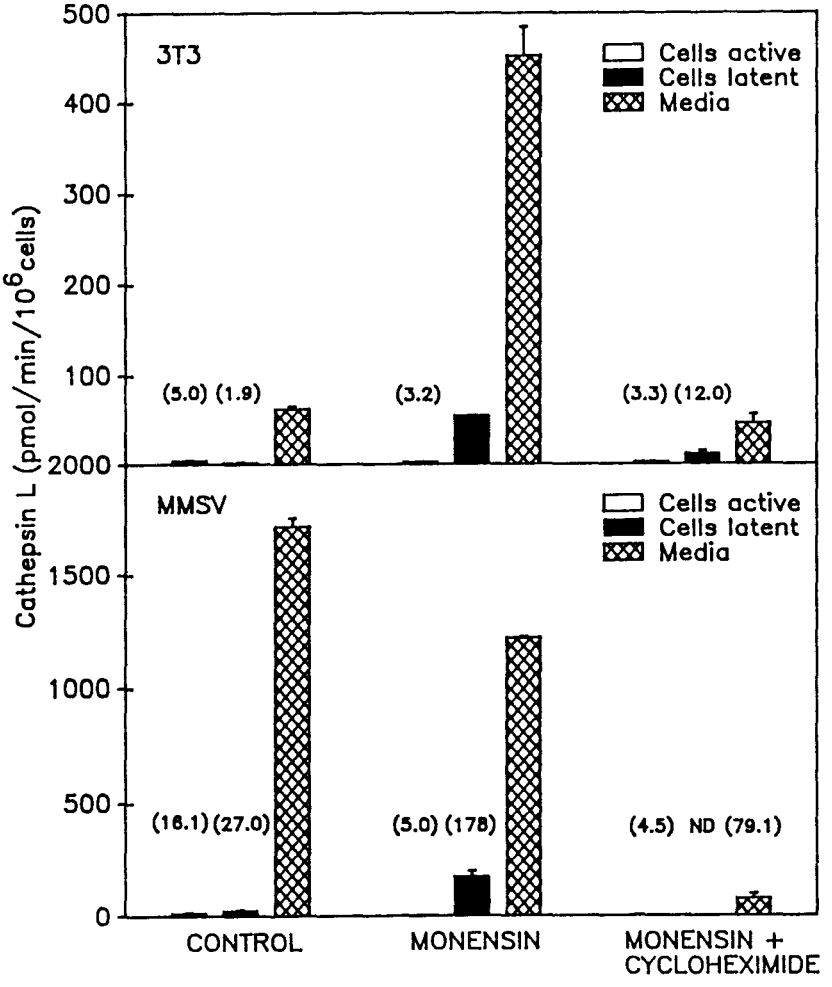
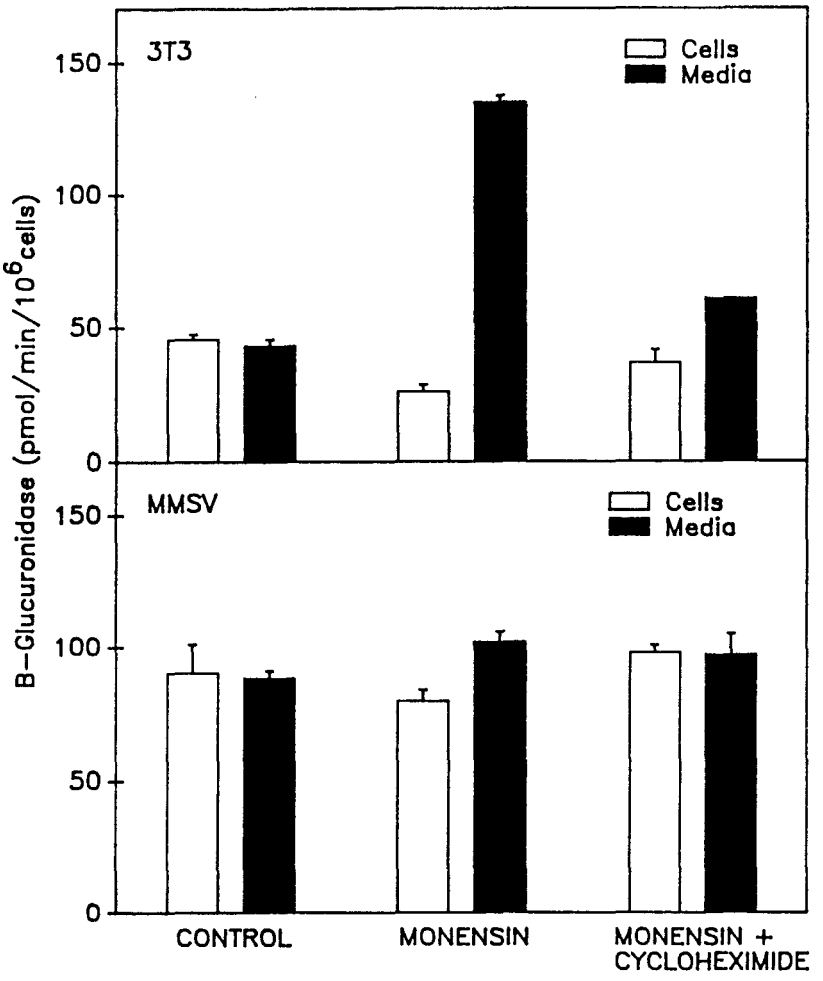

3T3
Cells active
Cells latent
Media
(5.0) (1.9)
(3.2)
(3.3) (12.0)
MMSV
Cells active
Cells latent
Media
(16.1) (27.0)
(5.0) (178)
(4.5) ND (79.1)
Cathepsin L (pmol/min/10^6cells)
500
400
300
200
100
2000
1500
1000
500
0
CONTROL
MONENSIN
MONENSIN + CYCLOHEXIMIDE

Fig.4. Effects of monensin and cycloheximide on cellular and secreted levels of ß-glucuronidase in BALB/3T3 and Moloney murine sarcoma virus-transformed BALB/3T3 fibroblasts. Monensin and cycloheximide concentrations were identical to those in Fig. 2. ß-Glucuronidase was measured with 1 mM 4-methylumbelliferyl-ß-D-glucuronide as described under "Experimental Procedures".

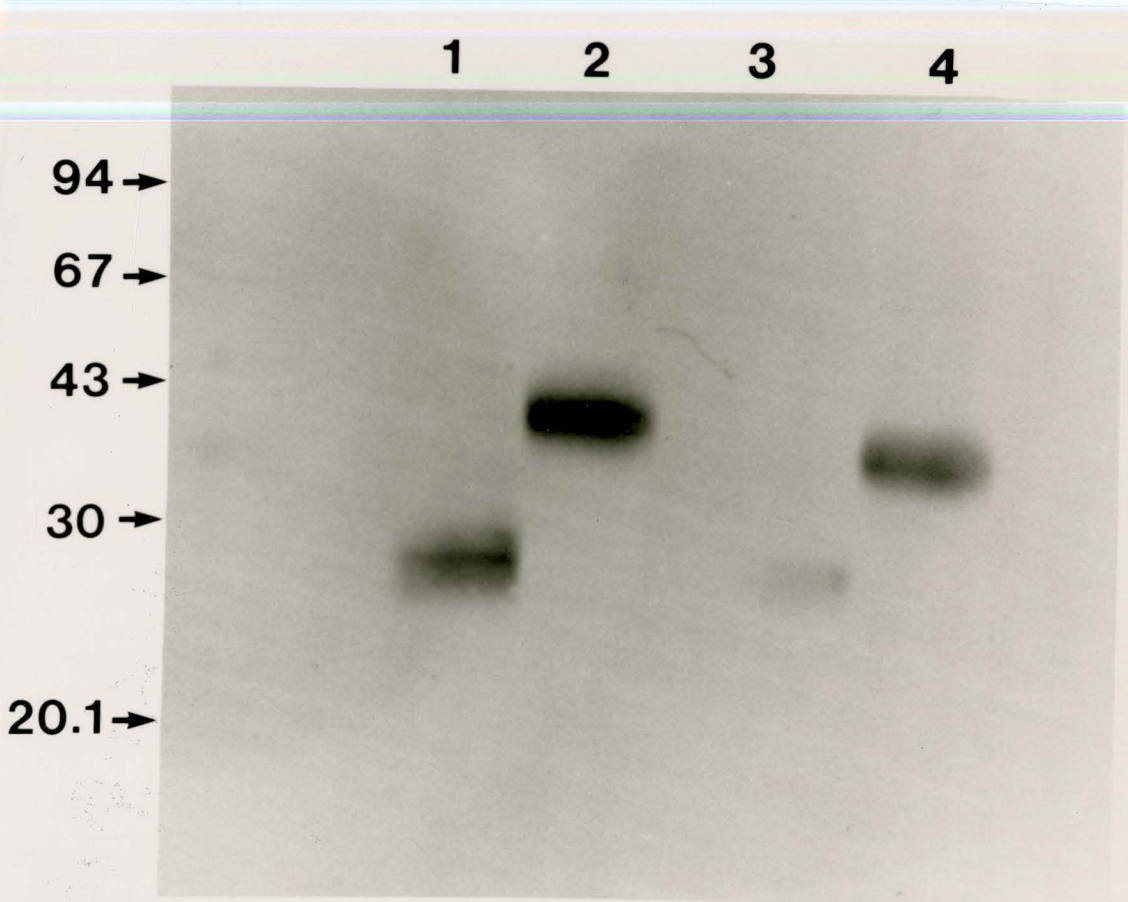

3T3
Cells
Media
150
100
50
0
MMSV
Cells
Media
150
100
50
0
B-Glucuronidase (pmol/min/10^6cells)
CONTROL
MONENSIN
MONENSIN +
CYCLOHEXIMIDE

effects on the secretion of the latent cathepsin B and cathepsin L activities from 3T3 cells. Ammonium chloride and chloroquine are believed to have this effect by raising the intravesicular pH and disrupting the biosynthetic transport of the newly synthesized lysosomal enzymes (Nishimura et al., 1988).

Immunodetection of Mature and Procathepsin B in MMSV-transformed 3T3 Cells

Western blots were performed on cell lysates and media samples in order to confirm that the cathepsin B-like activity secreted by MMSV cells was due to authentic cathepsin B. The antibody used was prepared by Qiuming Gong in our laboratory against a ß-galactosidase-cathepsin B fusion protein expressed in E coli. Fig. 5 shows the immunodetection of mature and precursor forms of cathepsin B in cell lysates and in cultured media from MMSV-transformed 3T3 cells in the presence and absence of monensin. In the lysate of the cells untreated with monensin (lane 1) a 28 kDa band was detected which corresponds to the reported molecular mass of the mature form of cathepsin B (Barrett, 1977). The media in which these cells were cultured (lane 2) was found to contain a 40 KDa protein which reacts with the anticathepsin B antibody. This band corresponds to the expected size of procathepsin B based on its amino acid sequence deduced from the molecular sequence of the mouse cathepsin B cDNA (Segundo et al., 1985). When MMSV cells were treated with monensin for 18 h in serum-free

Fig. 5. Immunodetection of Cathepsin B in Cell Lysate and Culture Media from Moloney Murine Sarcoma Virus-transformed BALB/3T3 Fibroblasts. Extracts and media samples from cells incubated for 18 hr in serum-free medium in the absence or presence of monensin were concentrated, separated by SDS PAGE and transferred to a nitrocellulose filter. Cathepsin B was visualized by the indirect antibody method using a polyclonal antibody directed against a cathepsin B-ß-galactosidase fusion protein as described in "Experimental Procedures". Lanes 1 and 2 (50 and 130 pmoles/min Z-Arg-Arg-AMC hydrolase activity, respectively) show cellular extracts and media samples from MMSV cells in the absence of monensin. Lanes 3 and 4 (10 and 115 pmoles/min Z-Arg-Arg-AMC hydrolase activity, respectively) show cell extracts and media samples from cultured MMSV cells pretreated for 18 hr with 1.0 μM monensin.


1
2
3
4
94
67
43
30
20.1

medium, the mature form of cathepsin B in the cell lysate diminished in quantity (lane 3) while the levels of procathepsin B secreted into the medium remained essentially unchanged (lane 4). These results closely paralleled the effects of monensin on the cellular and secreted levels of the cathepsin B-like activity from MMSV cells (see figure 2).

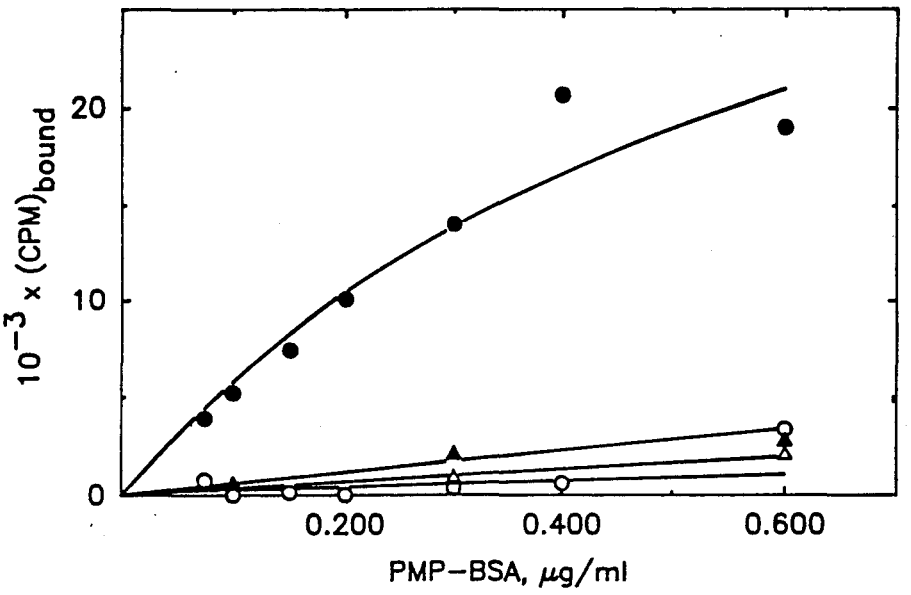
Cell Surface Mannose 6-Phosphate Receptor Binding Activity

The finding that MMSV cells secrete increased amounts of three different lysosomal enzymes prompted two of my fellow student collaborators to examine the basis for the decreased efficiency of lysosomal enzyme targeting on viral transformation.

Qiuming Gong found (Fig. 6) that the neoglycoconjugate PMP-BSA binds to 3T3 cells with a high affinity and in a mannose 6-phosphate and PMP-inhibitable manner. In contrast, MMSV-transformed 3T3 cells showed no measurable cell surface PMP-BSA binding activity, suggesting that MMSV cells either lack the mannose phosphate receptor required for lysosomal enzyme targeting, or that the receptor was no longer associated with the plasma membrane in MMSV cells. As an acidic vacuolar compartment is required for mannose phosphate receptor recycling to the cell surface and efficient lysosomal enzyme targeting, and monensin acts to alkalinize acidic compartments, Valerie Pracht undertook to measure vacuolar pH in MMSV cells.

Measurement of Vacuolar pH:

Fig. 6. Binding of ^{125}I-labeled PMP-BSA to BALB/3T3 (●) and Maloney sarcoma virus-transformed BALB/3T3 (○) fibroblasts at 4°C. The solid line for binding to the nontransformed fibroblasts was found to give a K_d = 9.14 x 10^{-9} M and B_{max} = 43 x 10^3 cpm/mg of cellular protein. The remaing curves are: (▲), binding to BALB/3T3 cells in the presence of 10 mM mannose 6-phosphate; and (Δ), binding to BALB/3T3 cells in the presence of 10 mM PMP. Binding studies were performed as described under "Experimental Procedures". The specific activity of ^{125}I-labeled PMP-BSA was 44,460 cpm/ g of BSA. Data obtained by Qiuming Gong.


10^{-3} x (CPM)$_{bound}$
20
10
0
0.200
0.400
0.600
PMP–BSA, μg/ml

BALB/3T3 and MMSV-transformed 3T3 cells were allowed to internalize fluorescein isothiocyanate dextran by endocytosis for varying periods of time. The excitation spectrum of the cells was then measured between 400 mm and 500 mm. From the 495 to 450 fluroescence ratio and the help of a standard curve, the pH of endosomal and lysosomal compartments in 3T3 and MMSV cells were estimated. From the results in Table 2 it can be seen that these are about 0.5 pH units higher in MMSV cells than in their non-transformed counterparts.

Activity of Lysosomal Enzymes in Murine B16 Melanoma Variants

In Table 1 are collected the cellular and secreted Z-Arg-Arg AMC, Z-Phe-Arg-AMC, and ß-glucuronidase activities from murine B16F-1 and B16a melanoma variants. As before, latent enzyme activity is the difference in activity observed before and after pepsin pretreatment. Virtually all the secreted cathepsin B- and cathepsin L-like activities from the B16F-1 and B16a cells were latent.

From the results in Table 1, it can be seen that the cellular and secreted cathepsin B-like activities were elevated in the melanoma variant having the higher metastatic potential as measured by ability to form lung colonies after tail vein injection (Qian et al., 1989). Cellular levels of cathepsin L and ß-glucuronidase activities were also higher in the more metastatic B16a cell line than in the B16F-1. However, the more metastatic B16a variant secreted less cathepsin L and ß-glucuronidase activities than the less

Table 2. Estimated Vacuolar pH in BALBc/3T3 Fibroblasts and Moloney murine Sarcoma virus-transformed BALBc/3T3 Fibroblasts[a].

	Estimated Vacuolar pH		
Incubation time	15 min	2 hr	24 hr
BALBc/3T3 cells	6.40	6.05	5.30
MMSV cells	7.00	6.40	5.75

[a]Cells were incubated for the indicated period with 1 mg/ml fluorescein isothiocyanate dextran. The cells were then washed, collected by scraping and resuspended in PBS buffer, pH 7.4 . The average vacuolar pH was determined from ratio of cellular fluorescence measured at the excitation wavelengths of 495 nm and 450 nm with the help of a standard curve. Cellular fluorescence was first corrected for light scattering by the cells and for leakage of fluorescein dextran. Significant leakage was not observed. Data obtained by Valerie Pracht.

metastatic B16F-1 cells.

Effects of Monensin and Cycloheximide on Lysosomal Enzyme Levels in B16F-1 and B16a Cells.

Fig. 7 shows that an 18 h incubation of B16F-1 cells with the proton ionophore monensin (0.3 μM) produced a relatively moderate increase (2-fold) in the amount of latent cathepsin B-like activity in serum-free medium. Under the same conditions, B16a cells also show modest monensin induced (1.8 fold) of latent cathepsin B-like activities secreted into serum-free medium. Monensin also caused only a modest increase in the apparent amount of procathepsin L (Fig. 8) secreted by both B16F-1 and B16a cells (about 2.5-fold). These increases were largely independent of monensin concentrations between 0.1 μM and 1 μM. Monensin also caused an increase in the amount of latent cathepsin B and L detected in cell lysates from both B16F-1 and B16a cells. Media levels of latent cathepsin B- and cathepsin L-like activities secreted in the presence of monensin were substantially reduced (85-95%) by 100 μM cycloheximide. Cellular levels of latent cathepsin B- and cathepsin L-like activities which accumulated in the presence of monensin were also almost completely abolished by cycloheximide. These results indicate that the latent activities which accumulated in the cells and media in the presence of monensin were newly synthesized. Fig. 9 shows the results of the same experiment with ß-glucuronidase. In contrast to both the cathepsin B- and

Fig. 7. Effects of monensin and cycloheximide on cellular and secreted levels of cathepsin B-like activities in murine B16 F-1 and B16a melanoma cells. The monensin and cycloheximide concentrations were 0.3 and 100 μM, respectively. Active and latent forms of cathepsin B-like activities were measured with 94 μM Z-Arg-Arg-AMC before and after pretreatment with pepsin as described in "Experimental Procedures". Although virtually no cathepsin B-like activity could be detected in media samples in the absence of pepsin pretreatment, pepsin sometimes caused 10-20% decrease in cathepsin B-like activity in cell lysates. In those cases the levels of latent cathepsin B could not be determined. Because of the scale of the figure some of the values cannot be estimated from the graph and are given in parenthesis. ND, not determinable.

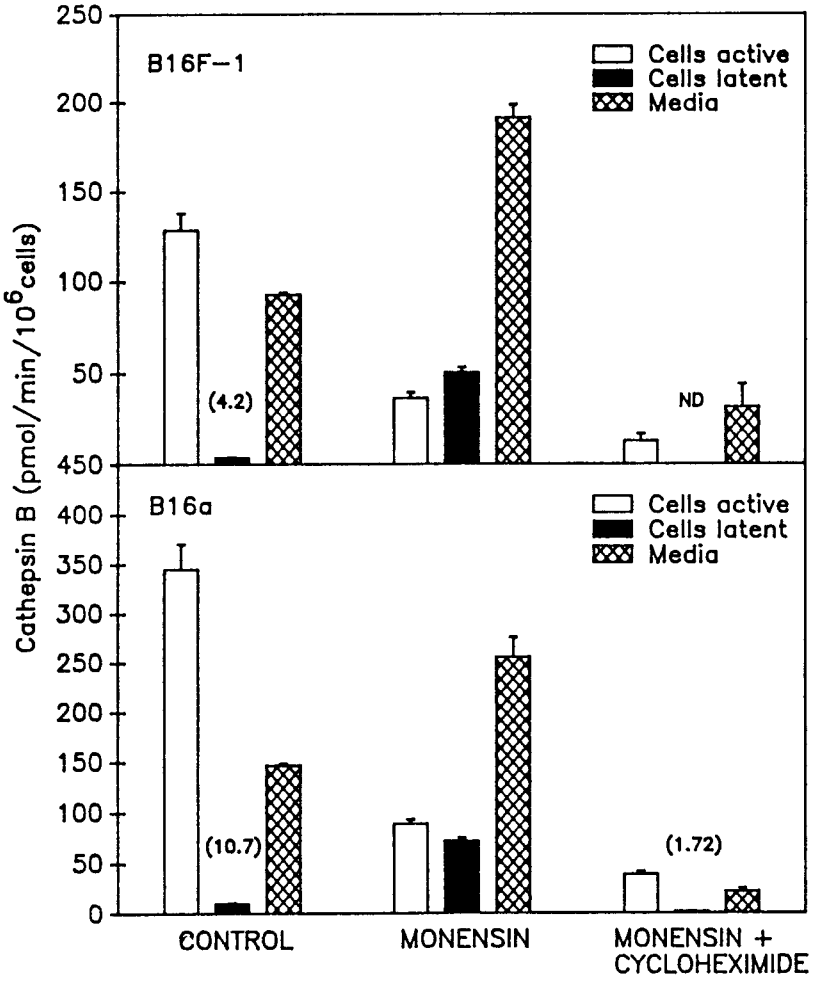

B16F−1
Cells active
Cells latent
Media
(4.2)
ND
B16a
Cells active
Cells latent
Media
(10.7)
(1.72)
Cathepsin B (pmol/min/10^6cells)
250
200
150
100
50
450
400
350
300
250
200
150
100
50
0
CONTROL
MONENSIN
MONENSIN + CYCLOHEXIMIDE

Fig. 8. Effects of monensin and cycloheximide on cellular and secreted levels of cathepsin L in murine B16 F-1 and B16a melanoma cells. Monensin and cycloheximide concentrations were identical to those in Fig. 4. Active and latent forms of cathepsin L were measured with 5 μM Z-Phe-Arg-AMC before and after pretreatment with pepsin as described under "Experimental Procedure". Virtually no cathepsin L activity could be detected in media samples in the absence of pepsin pretreatment. Because of the scale of the figure some of the values cannot be estimated from the graph and are given in parenthesis.

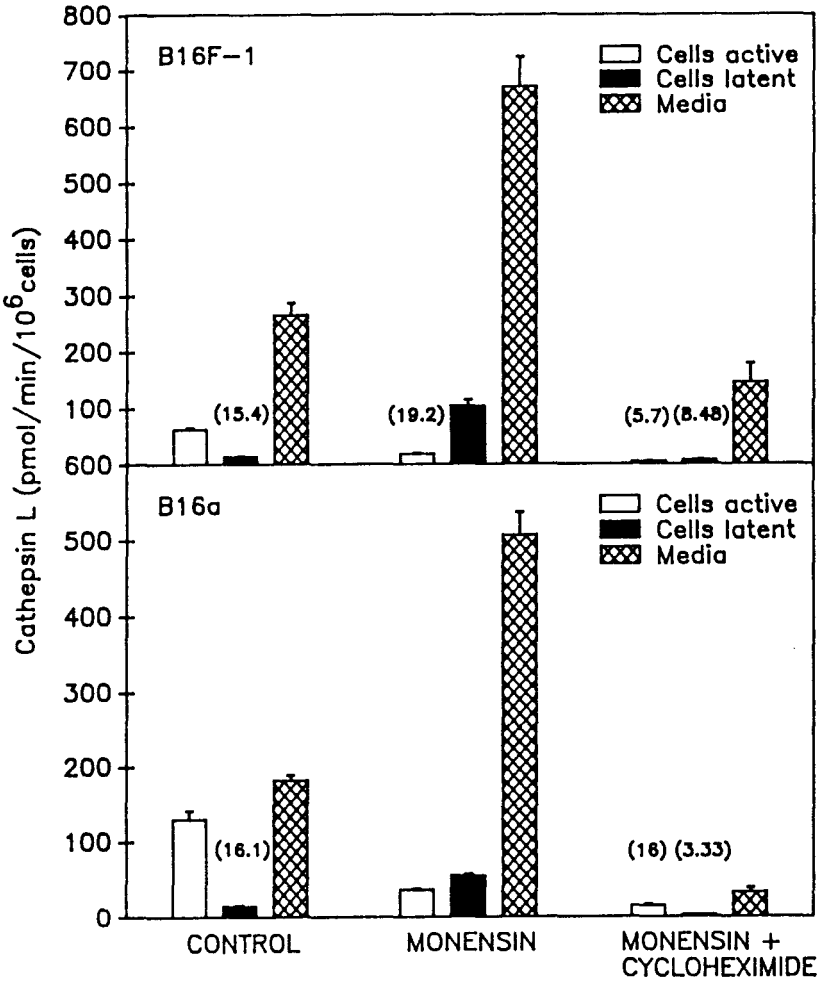
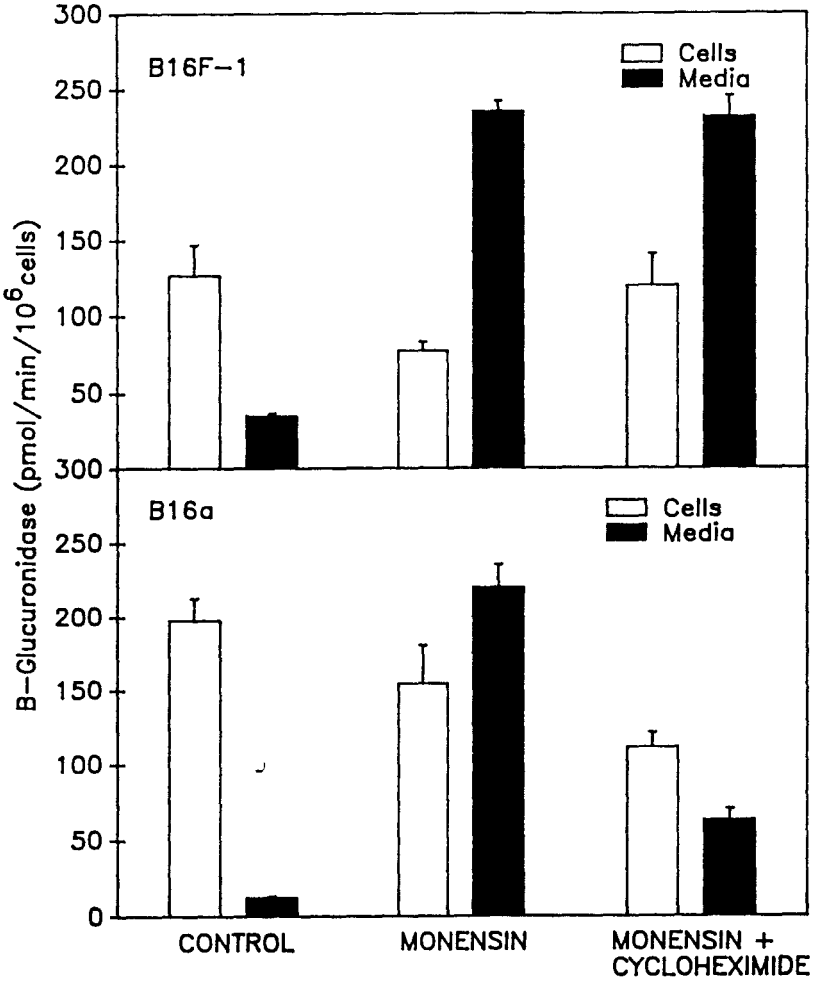

B16F−1
Cells active
Cells latent
Media
(15.4)
(19.2)
(5.7)
(8.48)
B16a
Cells active
Cells latent
Media
(16.1)
(16)
(3.33)
Cathepsin L (pmol/min/10^6cells)
800
700
600
500
400
300
200
100
600
500
400
300
200
100
0
CONTROL
MONENSIN
MONENSIN + CYCLOHEXIMIDE

Fig. 9. Effects of monensin and cycloheximide on cellular and secreted levels of ß-glucuronidase in murine B16 F-1 and B16a melanoma cells. Monensin and cycloheximide concentrations were identical to those in Fig. 4. ß-glucuronidase was measured with 1 mM 4-methylumbelliferyl-ß-D-glucuronide as described under "Experimental Procedures".

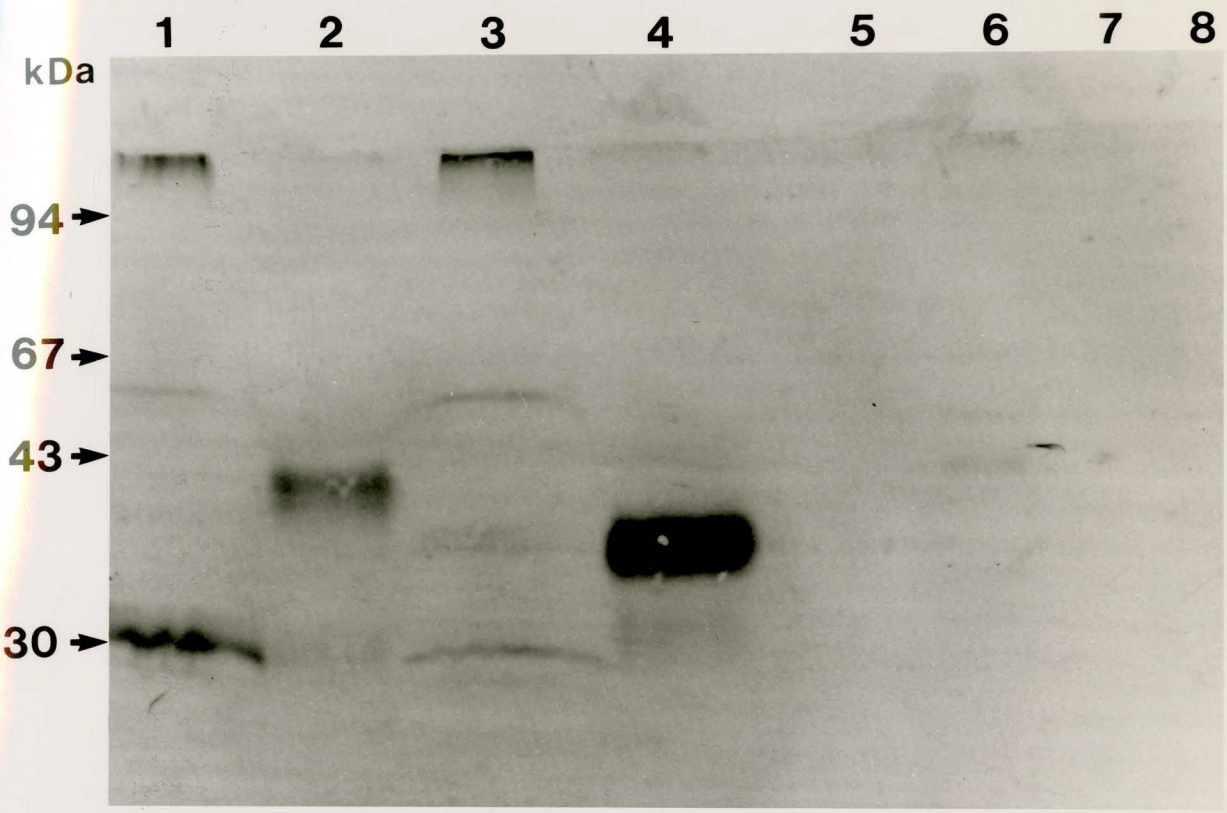

B16F−1
Cells
Media
B16a
Cells
Media
B−Glucuronidase (pmol/min/10^6cells)
300
250
200
150
100
50
0
CONTROL
MONENSIN
MONENSIN +
CYCLOHEXIMIDE

cathepsin L-like activities, the secretion of ß-glucuronidase was highly stimulated in B16F-1 and B16a cells (18- and 7-fold respectively) by monensin. However, this secretion was not strongly inhibited by cycloheximide raising the possibility that a significant portion of the secreted enzyme was synthesized prior to the 18-h incubation period in the presence of monensin.

Immunodetection of Mature and Procathepsin B in B16a Melanoma Cells

Fig. 10 shows an immunoblot of sample of cell lysates and culture media from B16a cells pretreated with and without the proton ionophore monensin. In the lysate of the cells untreated with monensin (lane 1), a 31 kDa band was detected which corresponds to the molecular weight reported for the active form in those cells (Docherty et al., 1983). The media (lane 2) in which these cells were cultured also appears to contain a small quantity of an immunoreactive protein of 42 kDa which may be procathepsin B (Segundo et al., 1985). Upon treatment with monensin in a serum-free medium for 18 h, the mature form of cathepsin B in the cell lysate is greatly diminished (lane 3) while a higher molecular weight form (40 kDa) secreted into the medium appears to be elevated (lane 4). It should be noted that lane 2 and 4 were heavily overloaded with similar amounts of latent Z-Arg-Arg-AMC hydrolase avtivity. The marked difference in the intensity of the immunoreactive bands stongly suggests that a

Fig. 10. Immunodetection of cathepsin B in Cell Lysate and media samples from B16 murine melanoma cells. Extracts and media samples from cells incubated for 18 hr in serum-free medium in the absence or presence of monensin, were concentrated, separated by SDS PAGE and transferred to a nitrocellulose filter. Cathepsin B was visualized by the indirect antibody method using a polyclonal antibody directed against a cathepsin B-ß-galactosidase fusion protein as described in "Experimental Procedures". Lanes 1 and 2 (250 and 220 pmoles/min Z-Arg-Arg-AMC hydrolase activity, respectively) show cellular extracts and media samples from B16a in the absence of monensin. Lanes 3 and 4 (75 and 220 pmoles/min Z-Arg-Arg-AMC hydrolase activity) show cell extracts and media samples from cultured B16a cells pretreated for 18 hr with 0.3 μM monensin. Lane 5 is the purified latent cathepsin B-like enzyme. Lane 6 is the partially purified latent cathepsin B-like enzyme from pool #4 of the con-A-Sepharose column. Lanes 7 and 8 show the purified latent secreted cathepsin B-like enzyme after pretreatment with either pepsin or a mixture of N-glycosidase F/endoglycosidase F as described in "Experimental Procedures". Lanes 5-8 were all loaded with 200 pmoles/min Z-Arg-Arg-AMC hydrolase activity.


1
2
3
4
5
6
7
8
kDa
94
67
43
30

significant amount of the latent Z-Arg-Arg-AMC hydrolase activity secreted by B16 cells in the absence of monensin is not due to an immunoreactive procathepsin B.

Effects of Estrogen Treatment on MCF_7 cells

Table 1 shows the effects of preincubating human breast carcinoma cells, MCF_7 with 10 μM estrogen. All of the secreted cathepsin B-like and cathepsin L enzymes in both the absence and presence of estrogen were latent and required pepsin treatment for activation. Forty-eight hours of estrogen treatment had no effect on both the cellular and secreted enzyme activities of cathepsin B-like, cathepsin L, and ß-glucuronidase enzymes, in contrast to the reported increase in procathepsin D secretion caused by estrogen treatment (Mainferme et. al., 1985).

Effects of Monensin and Cycloheximide on Lysosomal Enzyme Levels of MCF_7 Cells in the Presence and Absence of Estrogen

Figs. 11 and 12 show that an 18-h incubation of MCF_7 cells with the proton ionophore monensin (0.3 μM) in the absence and presence of estrogen produced the same moderate increase, 2.5- and 2.7-fold respectively, in the amount of latent cathepsin B-like and cathepsin L activities in serum-free medium. Similarly, estrogen did not alter the magnitude of the monensin-induced increase in ß-glucuronidase secretion (approximately 2-fold) by MCF_7 cells (see Fig. 13). Cycloheximide was able to negate the effect of monensin on the secretion of both latent cathepsin B-like and cathepsin L

Fig. 11. Effects of monensin and cycloheximide on cellular and secreted levels of cathepsin B-like activities in human breast carcinoma cells, MCF7 before and after treatment with 10 nM estrogen for 48 hours. The monensin and cycloheximide concentrations were 0.3 and 100 μM, respectively. Active and latent forms of cathepsin B-like activities were measured with 94 μM Z-Arg-Arg-AMC before and after pretreatment with pepsin as described under "Experimental Procedures". Although virtually no cathepsin B activity could be detected in media samples in the absence of pepsin pretreatment, pepsin sometimes caused a 10-20% decrease in cathepsin B-like activity in cell lysates. In those cases the levels of latent cathepsin B-like activity could not be determined. ND, not determinable.

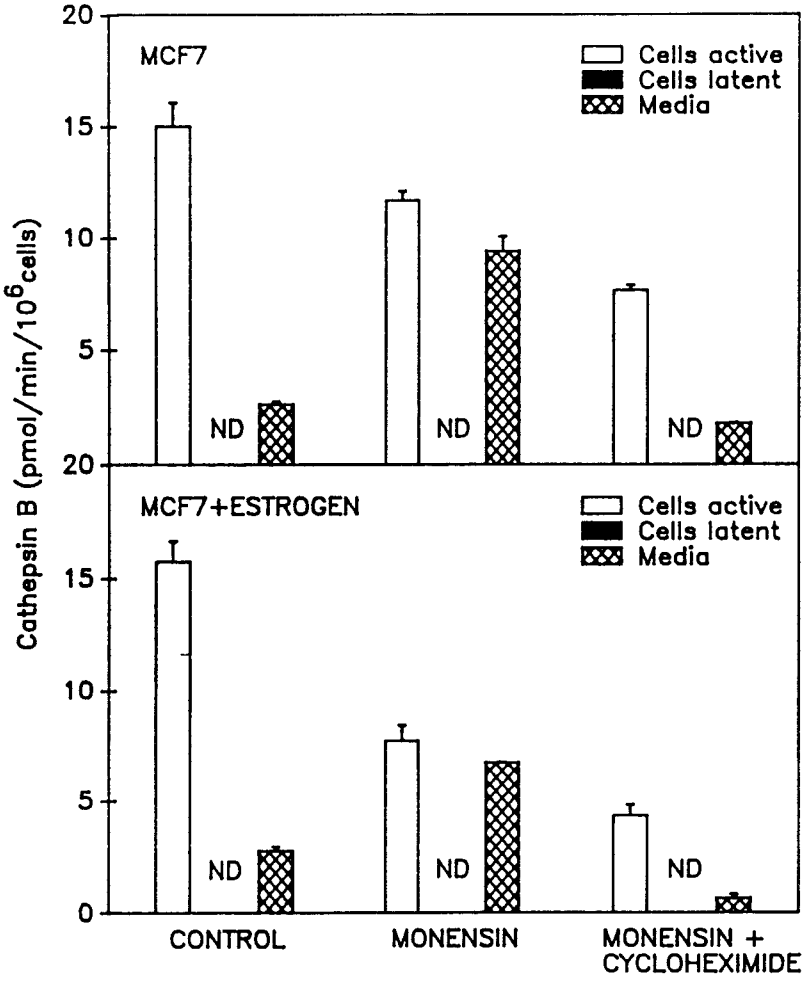

MCF7
Cells active
Cells latent
Media
ND
ND
ND
MCF7+ESTROGEN
Cells active
Cells latent
Media
ND
ND
ND
Cathepsin B (pmol/min/10^6cells)
20
15
10
5
0
CONTROL
MONENSIN
MONENSIN + CYCLOHEXIMIDE

Fig. 12. Effects of monensin and cycloheximide on cellular and secreted levels of cathepsin L in human breast carcinoma cells MCF7 before and after treatment with 10 nM estrogen for 48 hours. Monensin and cycloheximide concentrations were identical to those in Fig. 7. Active and latent forms of cathepsin L were measured with 5 μM Z-Phe-Arg-AMC before and after pretreatment with pepsin as described under "Experimental Procedures". Virtually no cathepsin L activity could be detected in media samples in the absence of pepsin treatment.

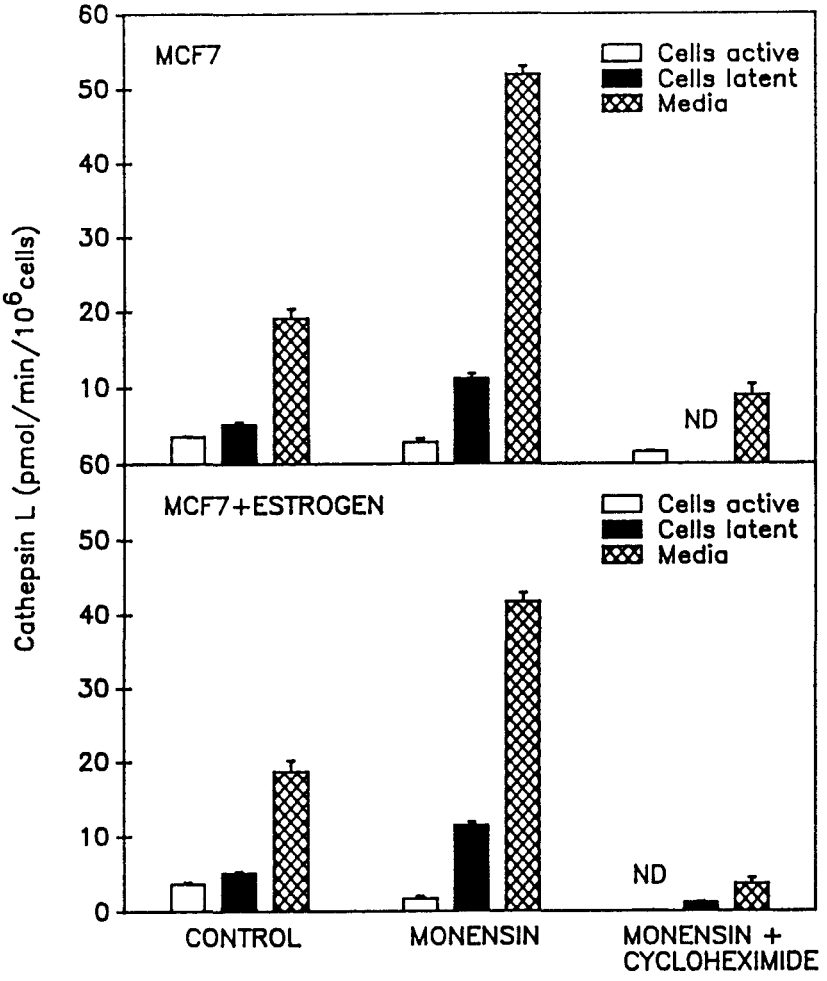
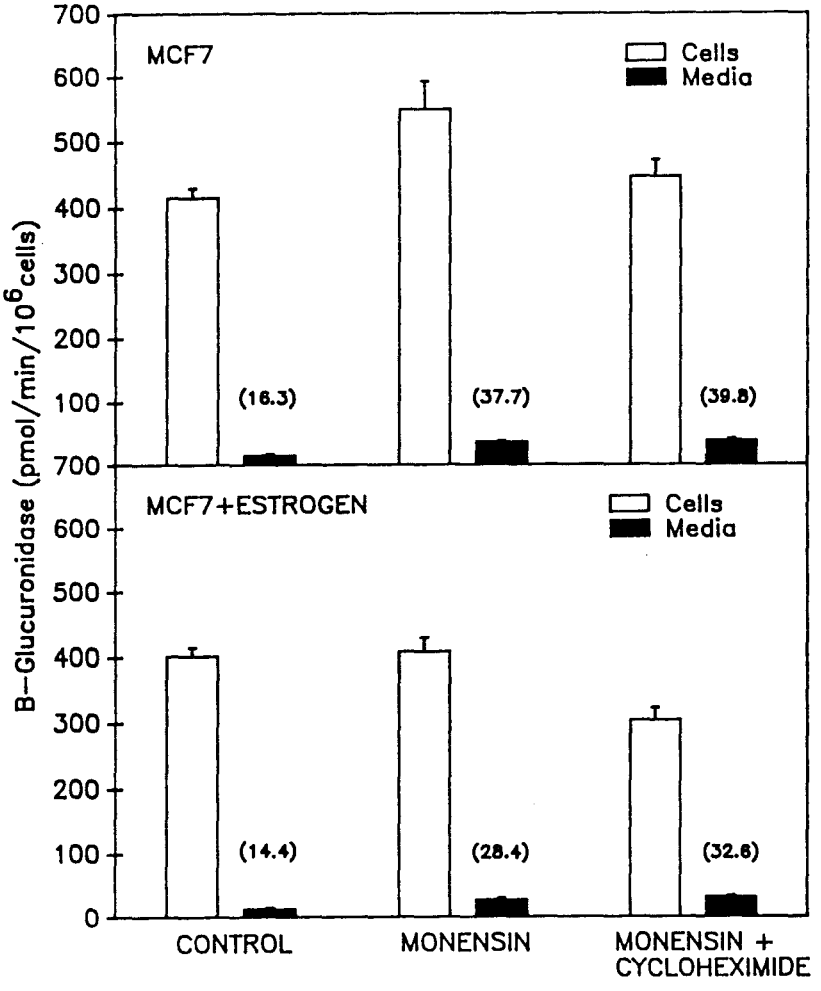

MCF7
Cells active
Cells latent
Media
ND
MCF7+ESTROGEN
Cells active
Cells latent
Media
ND
Cathepsin L (pmol/min/10^6cells)
60
50
40
30
20
10
0
CONTROL
MONENSIN
MONENSIN + CYCLOHEXIMIDE

Fig. 13. Effects of monensin and cycloheximide on cellular and secreted levels of ß-glucuronidase in human breast carcinoma cells, MCF7, before and after treatment with 10 uM estrogen for 48 hours. Monensin and cycloheximide concentrations were identical to those in Fig. 7. ß-glucuronidase was measured with 1 mM 4-methylumbelliferyl-ß-D-glucuronide as described under "Experimental Procedures".


MCF7
Cells
Media
(16.3)
(37.7)
(39.8)
MCF7+ESTROGEN
Cells
Media
(14.4)
(28.4)
(32.6)
B-Glucuronidase (pmol/min/10^6cells)
700
600
500
400
300
200
100
0
CONTROL
MONENSIN
MONENSIN + CYCLOHEXIMIDE

activities, but as in other cell lines had little effect on ß-glucuronidase secretion, raising the possibility that the ß-glucuronidase activity secreted into the media was synthesized before monensin treatment.

Purification of the Latent Secreted Cathepsin B-like Enzyme from the Murine B16 F-10 Melanoma Cells

The failure to detect significant levels of immunoreactive cathepsin B in the culture medium from B16 melanoma cells, despite the presence of relatively large amounts of a latent Z-Arg-Arg-AMC hydrolase activity, raised the possibility that the latent Z-Arg-Arg-AMC hydrolase activity secreted by B16 melanoma cells is not due to cathepsin B. Therefore I undertook to purify and characterize this enzyme. The purification of the latent Z-Arg-Arg-AMC hydrolase activity from serum free culture medium of B16-F10 cells has been described in "Experimental Procedures". Chromatography on a concanavalin A-Sepharose column was used to separate the cathepsin B-like enzyme from bovine serum albumin which appeared as a major contaminant in the media samples even though the cells had been thoroughly washed and incubated in serum-free media. BSA did not bind to the concanavalin-A-Sepharose column as determined from the recovery of immunoreactive BSA in the initial column effluent. In contrast, over 80 percent of the Z-Arg-Arg-AMC hydrolase activity did bind to the column. The latent cathepsin B-like activity was bound so tightly to the column that it was

necessary to incubate the resin for 12 hrs with 1.0 M α-methyl-D- mannoside to recover most of the bound enzyme. Table 3 lists the four different enzyme pools obtained from the con A-Sepharose column and describes the manner in which they were obtained. The fold purification calculated for these fractions ranged from 3.3 to 19.7. Pool #4, which had the highest specific activity was applied to a Poly WAX LP weak anion exchange column and then eluted with a sodium acetate gradient as described in the methods section. The size of the fractions were typically either 0.5 or 1.0 ml. Fig. 14 shows a typical elution profile for A_{280} and Z-Arg-Arg-AMC hydrolase activity. The cathepsin B-like enzyme eluted in two broad peaks suggesting that two or more protein species might be responsible for the Z-Arg-Arg-AMC hydrolase activity in the culture medium. Fig. 15 shows the results of SDS gel electrophoresis under reduced and denatured conditions of the protein fractions across the second peak which had the greater latent Z-Arg-Arg-AMC activity. Lanes 1-4 correspond to fractions 56, 57, 58 and 59. These show a predominant band with a molecular weight of about 67 kDa that correlates with the enzyme activity. These tubes contained close to 36% of the Z-Arg-Arg-AMC hydrolase activity applied to the Poly WAX LP column . Their pooled specific activity was 0.903 corresponding to a 74.7-fold purification (Table 3). Fig. 16 shows the elution profile for the Z-Phe-Arg-AMC hydrolase activity from the same column. It seems clear that the latent

Table 3. Purification Table of the Secreted Cathepsin B-like Enzyme from B16F-10 Murine Melanoma Cells.

Fraction	Total Protein (ug)	Total Activity (pmol/sec)	Specific Activity (pmol/sec/ug)	Yield	Purification
Media	70,565	3230	0.0458	100%	
ConA p#1[a]	3,899	591	0.152	_	3.3
ConA p#2[b]	1,676	624	0.372	_	8.1
ConA p#3[c]	706	517	0.733	_	16.0
ConA p#4[d]	773	698	0.903	_	19.7
ConA (All fractions)		2430		75.24%	
Ion ex[e]	54	185	3.42	35.5%[e]	74.7

[a] eluted with 0.1M α-methyl-D-mannoside.
[b] incubated with 0.1M α-methyl-D-mannoside for 1.5 h and eluted with the same buffer
[c] eluted with 1M α-methyl mannoside.
[d] incubated with 1M α-methyl-D-mannoside overnight and eluted with the same buffer.
[e] 75% (25 ml) of pool#4 was applied to the ion exchange column.

Fig. 14. Chromatography of Pool #4 from the Con-A-Sepharose column on a 4.6 x 100 mm Poly WAX weak anion exchange column in 20 mM Tris-HCl buffer pH 7.9. The column was eluted with a 0 to 1.0 M linear sodium acetate gradient. Panel A, absorbance at 280 nm; Panel B, 1.0 ml fractions assayed for latent cathepsin B-like activity with 94 μM Z-Arg-Arg-AMC as described in "Experimental Procedures".

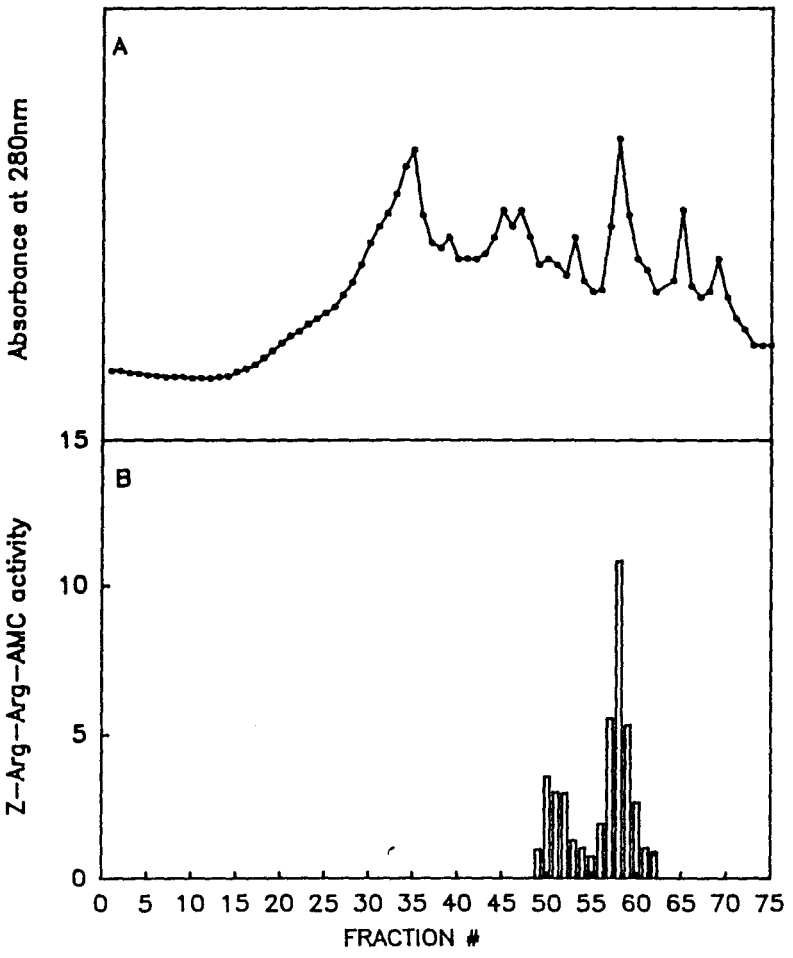

A
B
Absorbance at 280nm
Z-Arg-Arg-AMC activity
15
10
5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
FRACTION #

Fig. 15. SDS PAGE Electrophoresis of Enzyme Samples after High Performance Liquid Chromatography on a Poly WAX anion exchange column. Lanes 1 through 4 are fractions from peak 2 in Fig. 14 which had the greatest latent Z-Arg-Arg-AMC hydrolase activity, and correspond to fractions 56, 57, 58 and 59. After concentration, their Z-Arg-Arg-Arg-AMC hydrolase activities were approximately 100, 200, 400 and 100 pmoles /min, respectively.

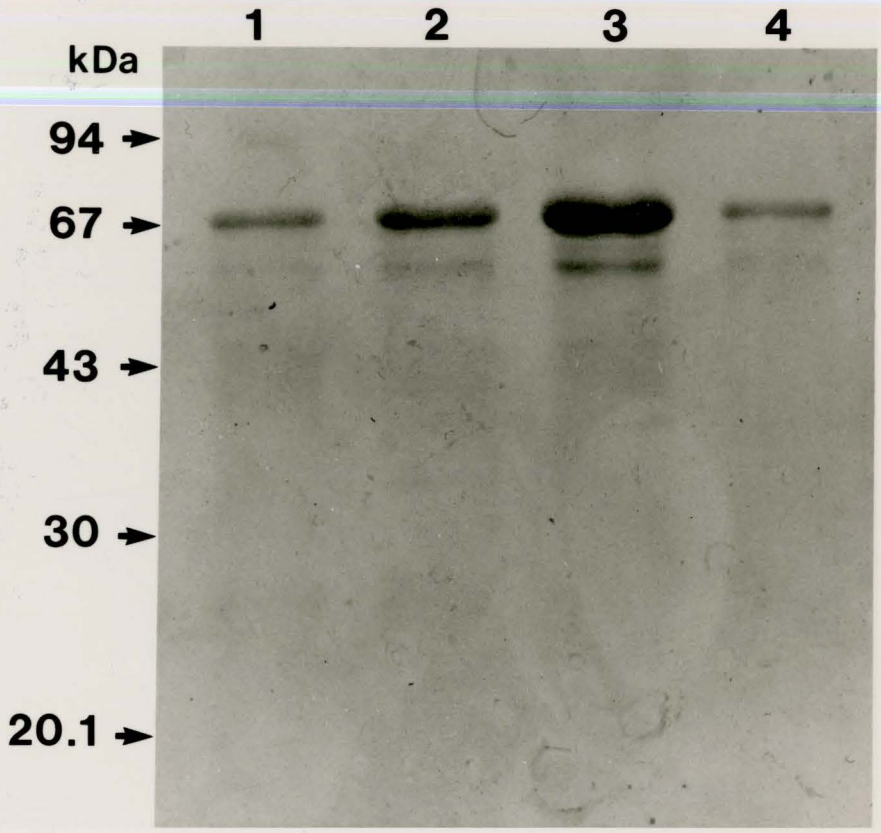
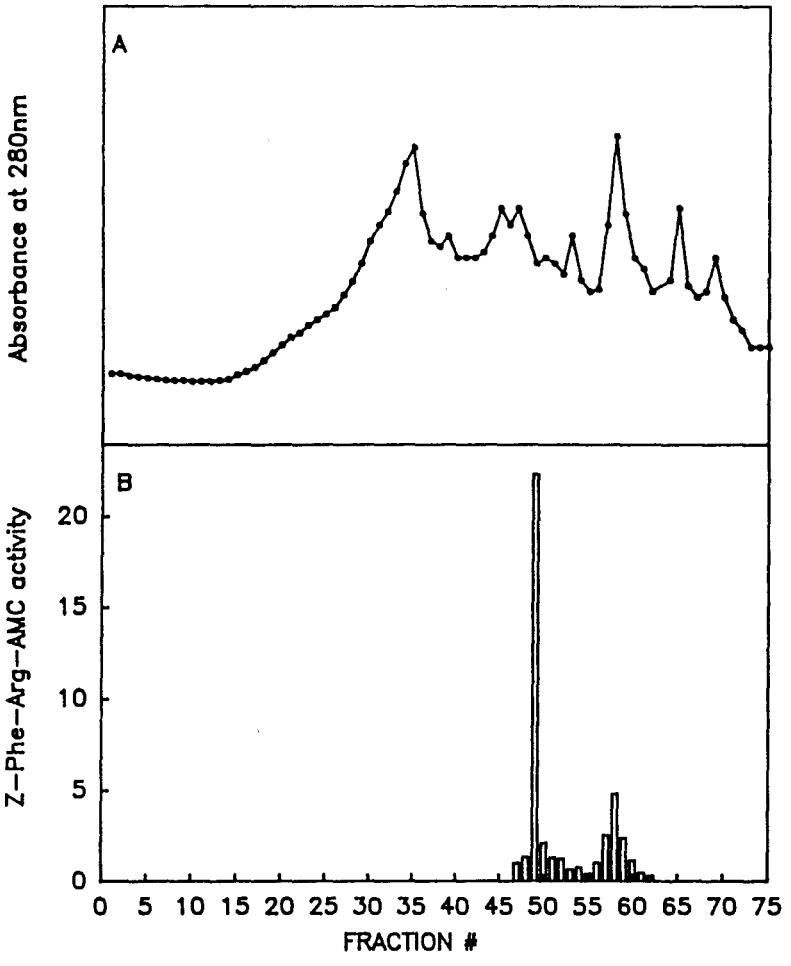

1
2
3
4
kDa
94
67
43
30
20.1

Fig. 16. Chromatography of Pool #4 from the Con-A-Sepharose Column on a 4.6 x 100 mm Poly WAX weak anion exchange column in 20 mM Tris-HCl buffer pH 7.9. The column was eluted with a 0 to 1.0 M sodium acetate gradient. Panel A, absorbance at 280 mm; Panel B, 1.0 ml fractions assayed for cathepsin B-and cathepsin L-like activities with 5 μM Z-Phe-Arg-AMC as described in "Experimental Procedures".


A
Absorbance at 280nm
B
Z-Phe-Arg-AMC activity
0
5
10
15
20
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
FRACTION #

cathepsin B-like enzyme also has Z-Phe-Arg-AMC hydrolase activity. Remarkably, one fraction in peak 1 contained an unusually large amount of latent Z-Phe-Arg-AMC hydrolase activity as confirmed by repeated measurements. The ratio of Z-Phe-Arg-AMC to Z-Arg-Arg-AMC hydrolase activities of this fraction was very high suggesting that it had very little or no Z-Arg-Arg-AMC activity. Based on this observation it is presumed that this fraction also contains procathepsin L. Fig. 17 shows an SDS gel electrophoresis of this enzymatic activity under reducing and denaturing conditions. Lane 1 corresponding to fraction 49 which contained the peak Z-Phe-Arg-AMC hydrolase activity shows a single band of approximately 38 kDa which is the size expected for preprocathepsin L (Troen et al., 1987). There was insufficient amount of protein in the remaining fractions to be clearly visualize in the gel.

Native Gel Electrophoresis

Fig. 18 shows a native gel electrophoresis (non-denaturing conditions) of the purified latent cathepsin B-like enzyme. The gel was overloaded with protein. This resulted in a major protein band and at least 2 minor bands after staining with Comassie blue. In order to determine which band contained the Z-Arg-Arg-AMC hydrolase activity, each band prior to staining was cut out of the gel, and then homogenized in 50 mM phosphate buffer, pH 6.5 containing .02% Triton X-100. Measurements of Z-Arg-Arg-AMC hydrolase

Fig. 17. SDS PAGE electrophoresis of enzyme amples after High Performance Liquid Chromatography on a Poly WAX anion exchange column. Fractions from peak 1 in Fig. 16 which had the greatest latent Z-Phe-Arg-AMC hydrolase activity were concentrated prior to electrophoresis. Lane 1 corresponds to fraction 49. Lanes 2, 3, 4 and 5 are fractions 48, 50, 51 and 52 respectively.

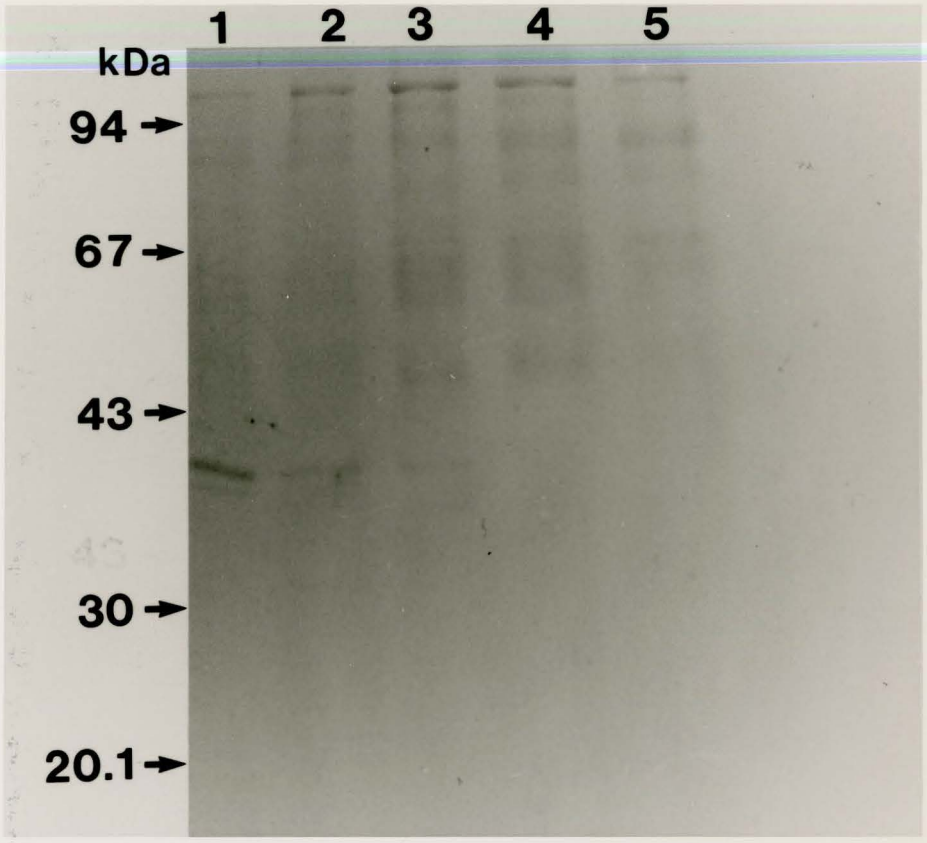
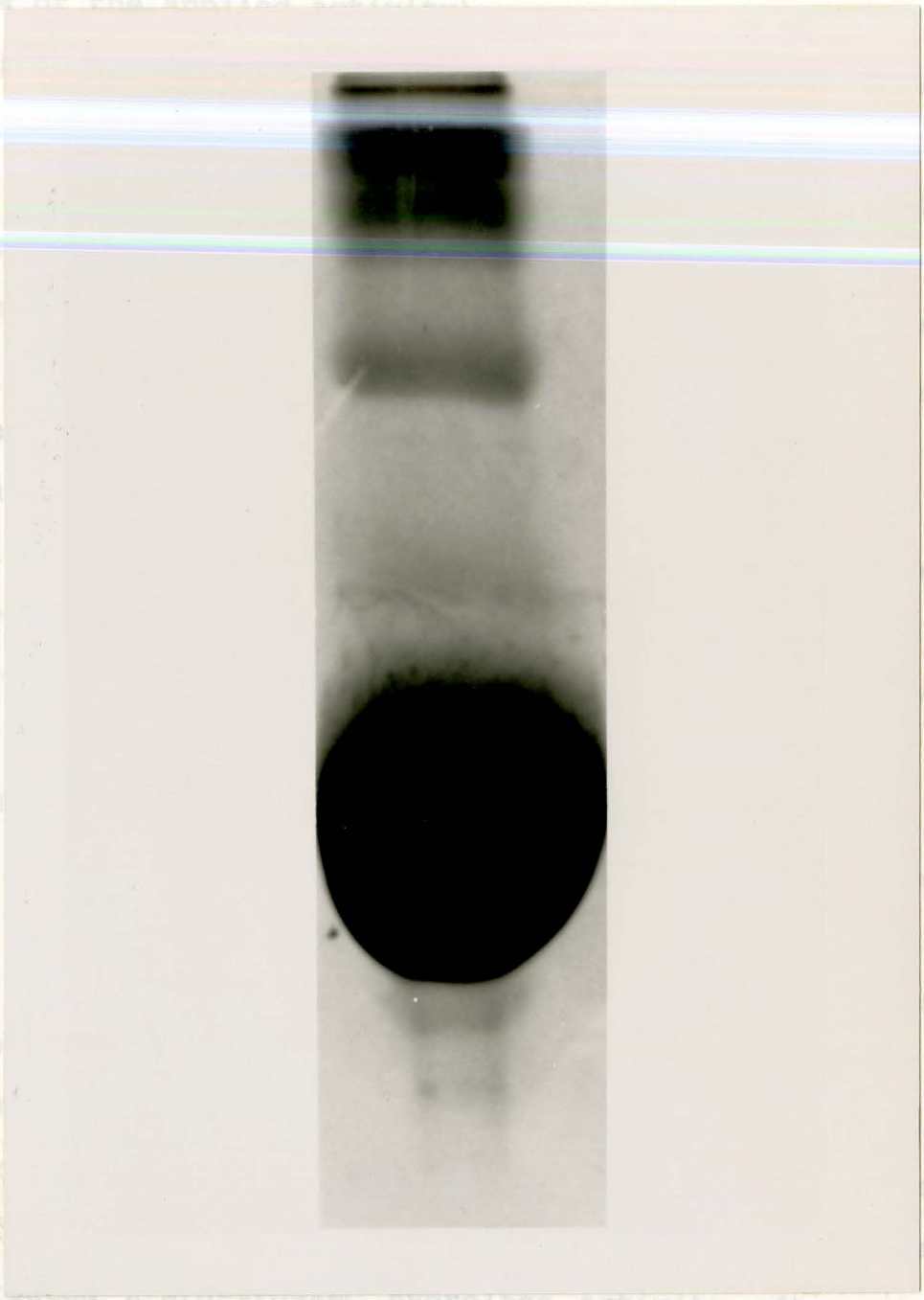

1
2
3
4
5
kDa
94
67
43
30
20.1

Fig. 18. Native polycrylamide gel electrophoresis of the purified latent cathepsin B-like enzyme from B16 melanoma cells. This gel was run under similar conditions as SDS PAGE but without SDS and reducing agents.



activity after activation with pepsin confirmed that only the major protein band contained latent cathepsin B-like activity (24% of the applied activity).

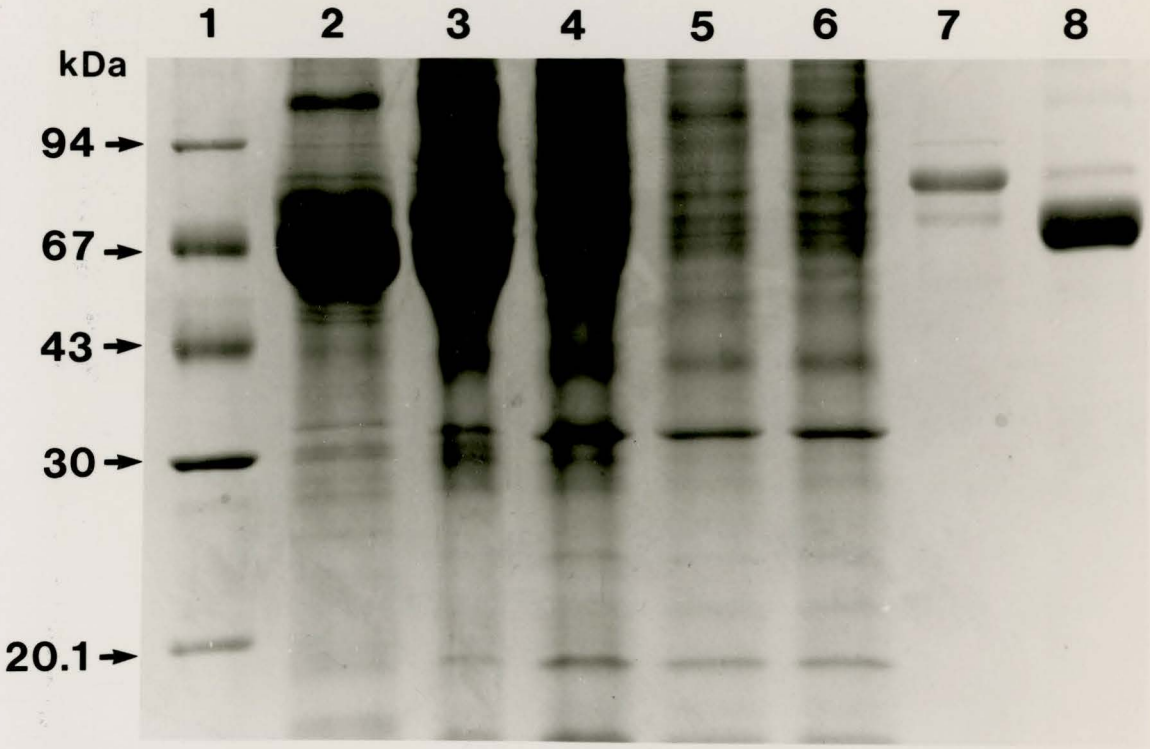
Deglycosylation of the Purified Melanoma Latent Z-Arg-Arg-AMC Hydrolase Activity

Fig. 19 shows the results of SDS PAGE of different fractions obtained during the purification of the latent cathepsin B-like enzyme. Lane 7 is the purified enzyme after the ion exchange column and after reduction and alkylation with iodoacetic acid. Alkylation caused the enzyme to be significantly retarded on the gel with respect to the unalkylated standards. Treatment of the enzyme with endoglycosidase F/N-glycosidase F resulted in about a 6 KDa decrease in the apparant molecular weight of the purified alkylated enzyme (lane 8).

Molecular Weight Determination by HPLC Gel Filtration

The molecular weights of the purified latent cathepsin B-like activity from B16 melanoma cells and its pepsin-activated form was estimated by HPLC native gel filtration on a TSK-250 (600x7.5 mm) column as described in Methods section. Fig. 20 shows the elution of a series of standards as indicated in its upper panel. The lower panel of Fig. 20 shows the superimposed elution profiles for the latent and pepsin-pretreated Z-Arg-Arg-AMC hydrolase activity after each was applied separately to the column. The elution volume corresponding to each fraction was converted to a

Fig. 19. SDS PAGE electrophoresis of fractions obtained during different stages in the purification of the melanoma cathepsin B-like enzyme. Lane 1 contains the protein standards. Lane 2 is a sample of serum-free media from cultured B16 F-10 cells (30 pmoles Z-Arg-Arg-AMC hydrolase activity/sec). Lanes 3, 4, 5 and 6 are pools #1 through #4 from the con-A-Sepharose column and contain either 30 pmoles Z-Arg-Arg-AMC hydrolase activity/min, (lanes 3 and 4), and 10 pmoles Z-Arg-Arg-AMC activity/min, (lanes 5 and 6). Lane 7 is the reduced, alkylated latent cathepsin B-like enzyme and lane 8 is the reduced, alkylated enzyme after treatment with a mixture of N glycosidase F/endoglycosidase F as described in the Methods Section.


1
2
3
4
5
6
7
8
kDa
94
67
43
30
20.1

dimensionless parameter sigma as described in the legend to Fig. 20. The data in Fig. 20 was used to construct the standard curve shown in Fig. 21. From the elution position of the latent (arrow 1) and active (arrow 2) enzyme, their molecular weights were estimated to be 67.4 kDa and 55 kDa, respectively. The broadness of the pepsin-activated enzyme peak suggests that pepsin treatment leads to the generation of several active species which differ in size

Active Site Titration with E-64

Fig. 22 shows the active site titration of the purified latent cathepsin B-like enzyme. E-64, a known thiol protease inhibitor, was added to the latent enzyme in the indicated amounts prior to the addition of pepsin. The rationale for this procedure was that this might minimize the consequences of any subsequent inactivation or autolysis of the pepsin-activated enzyme and would yield better estimates of enzyme concentration. Fig. 22 shows that up to 90% of the pepsin activatable Z-Arg-Arg-AMC hydrolase activity is inhibited by E-64. Extrapolation of the linear portion of the curve , indicates that 8 picomoles of E-64 is required to completely inhibit the enzyme activity corresponding to 0.558 μg of protein as determined with the Bio-Rad dye binding assay (Methods section). The ratio of protein to active site titrant yields a molecular weight estimate for the purified latent cathepsin B-like enzyme of 70,000, in close agreement with 67,000 as estimated by SDS PAGE.

Fig. 20. Determination of the molecular weight of the latent and pepsin-activated secreted cathepsin B-like enzyme from B16 melanoma. The upper panel is the elution profile obtained for a series of proteins of known molecular weight after high performance liquid chromatography on a Bio-Sil TSK-250 gel filtration column. The abbreviations used are : ß-Gal, ß-galactosidase; BSA, bovine serum albumin; Oval, ovalbumin; CA, carbonic anyhydrase; CAL ,calmodulin; Apn, Aprotinin. The lower panel shows the activity profile of pepsin pretreated and latent enzymes applied to the gel filtration column. Latent enzyme was measured after pepsin pretreatment (solid line) and active enzyme (pepsin pretreated before injection, dotted line) was measured with pepsin omitted from the activation buffer as described in "Experimental Procedures". Sigma, corresponding to the x-axis coordinate was calculated from the relationship

$$\text{Sigma} = \frac{V_F - V_0}{V_T - V_0}$$

where V_F is the elution volume for a given fraction, V_0 is the void volumne of the column determined with blue dextran, and V_T is the total accessible volume of the column determined from the elution volume of the solvent front. Sigma is a dimensionless parameter which is independent of the size of the gel filtration column.

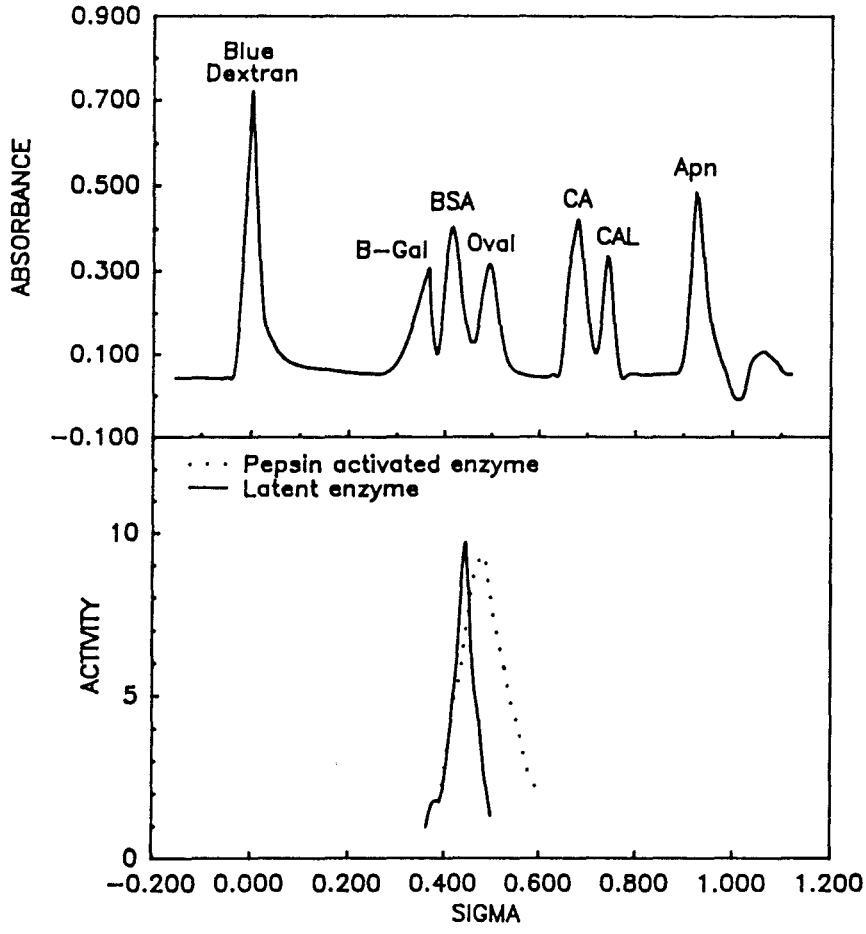

0.900
0.700
0.500
0.300
0.100
−0.100
ABSORBANCE
Blue
Dextran
B−Gal
BSA
Oval
CA
CAL
Apn
· · · Pepsin activated enzyme
—— Latent enzyme
10
5
0
ACTIVITY
−0.200
0.000
0.200
0.400
0.600
0.800
1.000
1.200
SIGMA

Fig. 21. Standard curve of molecular weight vs sieve coefficient for protein standards after high performance liquid chromatography on a Bio-Sil TSK-250 column. The experimental points correspond to the peak elution position of the standards from Fig. 20 and their molecular weight. Arrows 1 and 2 are respectively the peak elution positions of the latent and pepsin-activated Z-Arg-Arg-AMC hydrolase activities secreted by B16 melanoma cells.

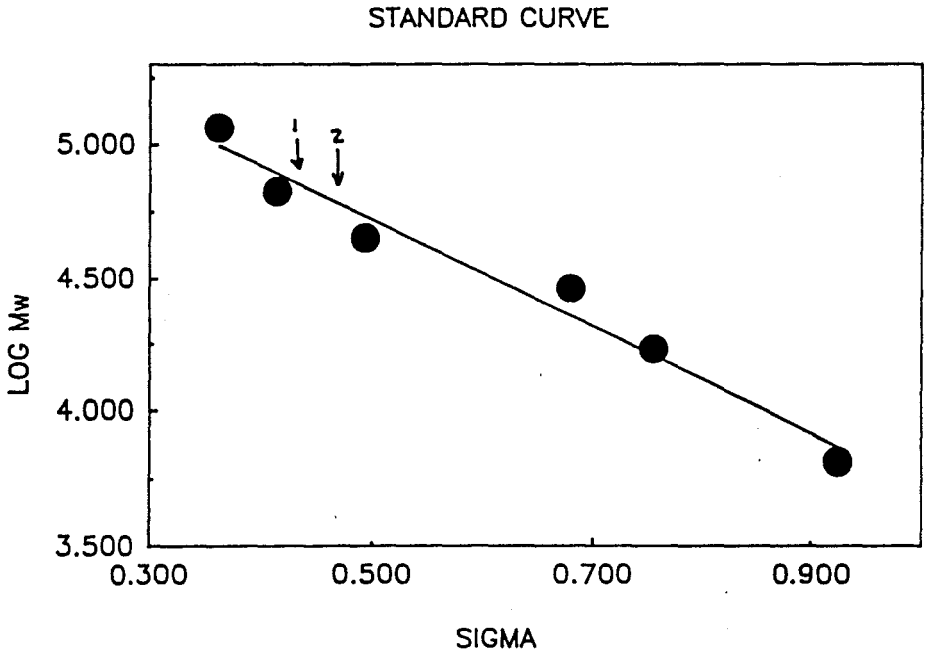
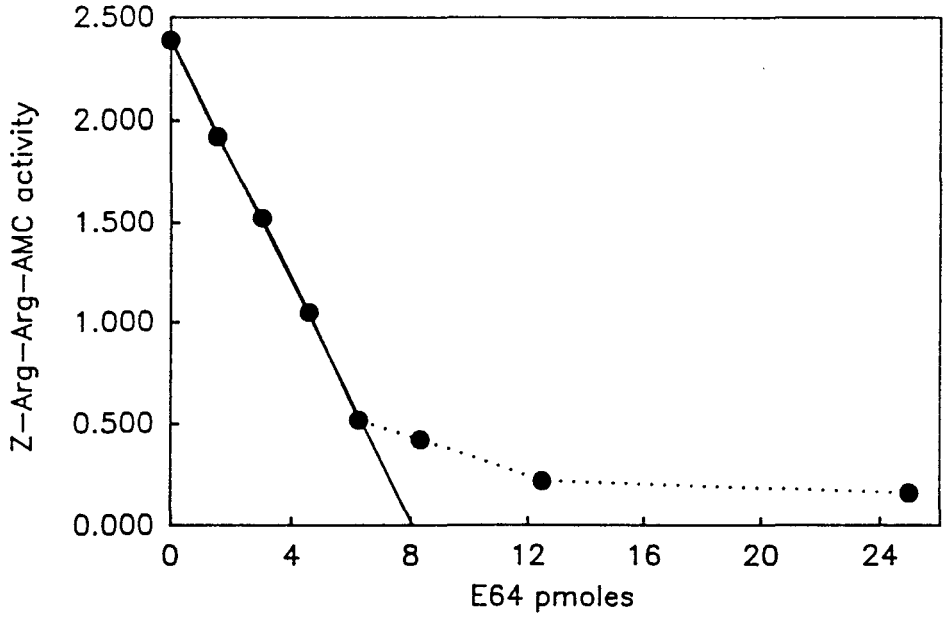

STANDARD CURVE
LOG Mw
5.000
4.500
4.000
3.500
0.300
0.500
0.700
0.900
SIGMA
1
2

Fig. 22. Active site titration of the purified latent cathepsin B-like enzyme from B16 melanoma cells. The active site titrant, E-64 at the indicated concentrations, was incubated with the purified latent enzyme during a 90 min activation period with 0.1 mg/ml of pepsin as described in "Experimental Procedures". The residual enzyme activity was measured with Z-Arg-Arg-AMC (200 μM) as the substrate.


2.500
2.000
1.500
1.000
0.500
0.000
Z-Arg-Arg-AMC activity
0
4
8
12
16
20
24
E64 pmoles

Kinetic Constants of the Latent Cathepsin B-like Enzyme

The kinetic constants k_{cat} and K_m were determined for the pepsin-activated latent cathepsin B-like enzyme from melanoma cells against the substrates Z-Arg-Arg-AMC and Z-Phe-Arg-AMC. Figs. 23 and 24 show plots of velocity vs substrate concentration for the two substrates. Each point is an average of 3 measurements. K_m and V_{max} were determined by non-linear regression fit of the rate data to the equation

$v = \frac{V_{max}[S]}{K_m + [S]}$ using the program Enzfit (Elsevier-Biosoft, Cambridge, UK). The solid lines were calculated from the best fit parameters. Dividing V_{max} by the number of moles of the enzyme determined from active site titration with E-64 yielded k_{cat}. In Table 4 is collected the kinetic constants for the hydrolysis of Z-Arg-Arg-AMC and Z-Phe-Arg-AMC by the purified cathepsin B-like enzyme from melanoma cells. For Z-Arg-Arg-AMC, K_m and k_{cat} are respectively 1.00 $\pm$ 0.29 mM and 0.188 $\pm$ 0.040 s^{-1} respectively. For Z-Phe-Arg-AMC the corresponding constants are 121 $\pm$ 0.081 μM and 0.286 $\pm$ 0.012 s^{-1}, respectively. In contrast the authentic cathepsin B secreted by MMSV cells had a K_m of 422 $\pm$ 21 μM for Z-Arg-Arg-AMC (Fig. 25). The values for the K_m of the cathepsin B-like enzyme from B16 melanoma cells and authentic cathepsin B from MMSV cells were significantly different ($P<.025$).

Values of k_{cat}/K_m for Z-Arg-Arg-AMC and Z-Phe-Arg-AMC were also determined under conditions of $[S]<<Km$. Under these

Fig. 23. Plot of velocity vs Z-Arg-Arg-AMC concentration for the secreted pepsin-activated cathepsin B-like enzyme from B16 melanoma. K_m and V_{max} values were determined by fitting the data points to the following equation

$$v = \frac{V_{max} \times [S]}{K_m + [S]}$$

by non-linear regression analysis (Enzfit/Elsevier-Biosoft, Cambridge, UK). Each point is an average of 3 measurements. The solid line represents the best fit curve. The parameter k_{cat} was calculated as being equal to V_{max}/E, where E is the enzyme concentration deduced after active site titration. K_m and k_{cat} were calculated to be 1.00 $\pm$ 0.29 mM and .188 $\pm$ 0.040 s^{-1}, respectively.

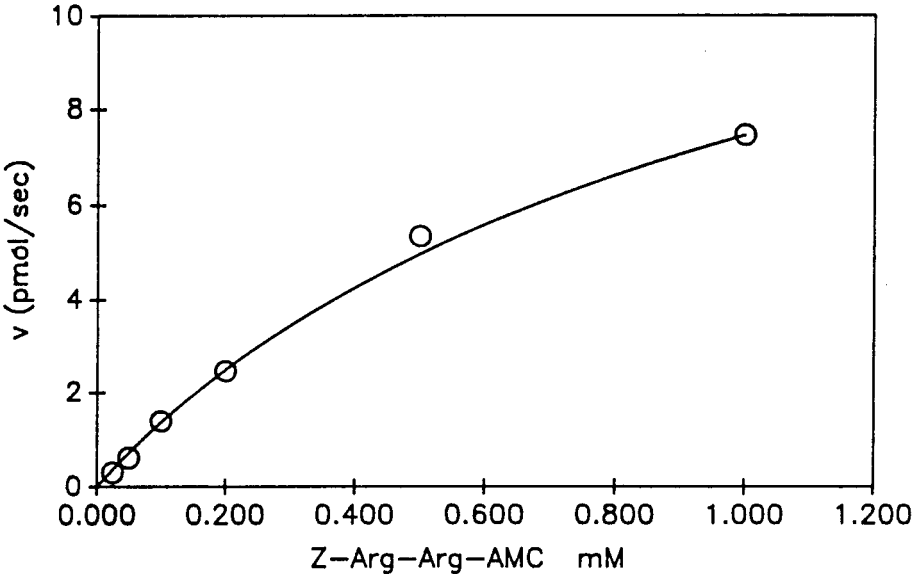

10
8
6
4
2
0
v (pmol/sec)
0.000
0.200
0.400
0.600
0.800
1.000
1.200
Z–Arg–Arg–AMC mM

Fig. 24. Plot of velocity vs substrate concentration (Z-Phe-Arg-AMC) for the secreted pepsin-activated cathepsin B-like enzyme from B16 melanoma. K_m and V_{max} values were determined by fitting the data points to the following equation

$$v = \frac{V_{max} \times [S]}{K_m + [S]}$$

by non-linear regression analysis (Enzfit/Elsevier-Biosoft, Cambridge, UK). Each point is an average of 3 measurements. The solid line represents the best fit curve. k_{cat} was calculated as being equal to V_{max}/E, where E is the enzyme concentration deduced after active site titration. K_m and k_{cat} were calculated to be 121 $\pm$ 0.081 μM and 0.286 $\pm$ .012 s^{-1}, respectively.

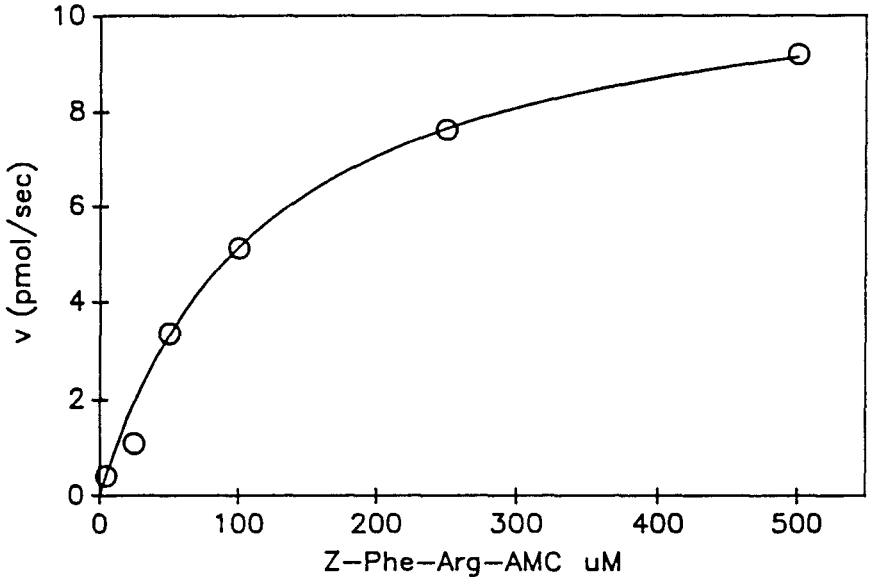
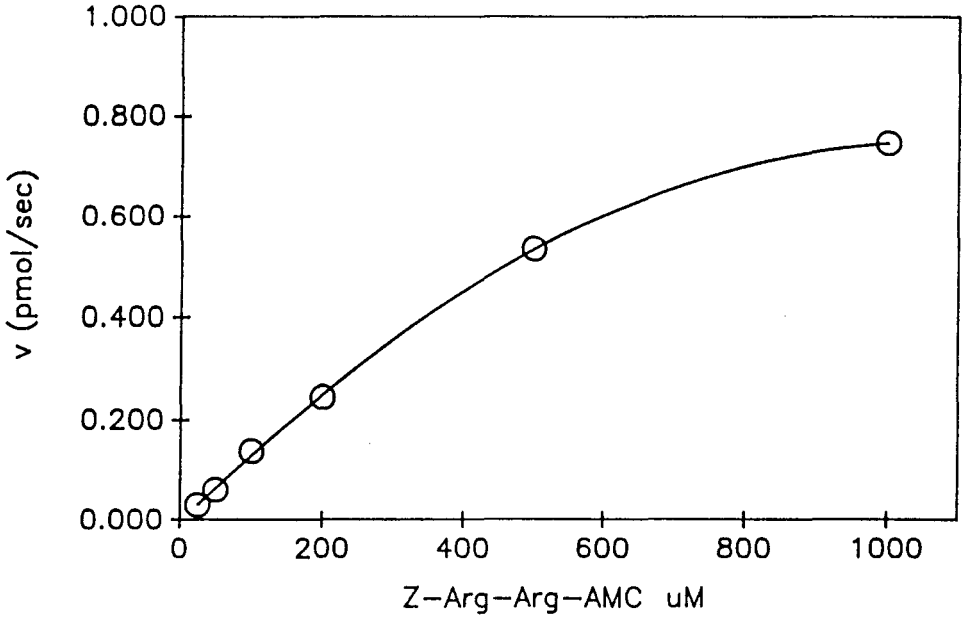

10
8
6
4
2
0
v (pmol/sec)
0
100
200
300
400
500
Z–Phe–Arg–AMC uM

Fig. 25. Plot of velocity vs substrate concentration (Z-Arg-Arg-AMC) for the secreted pepsin-activated cathepsin B enzyme from MMSV cells. Km was determined by fitting the data points to the following equation $v = \frac{V_{max} \times [S]}{K_m \times [S]}$ by non-linear regression analysis (Enzfit/Elsevier-Biosoft, Cambridge, UK). Each point is an average of 3 measurements. The solid line represents the best fit curve. K_m was calculated to be $0.422 \pm .021$ mM.


1.000
0.800
0.600
0.400
0.200
0.000
v (pmol/sec)
0
200
400
600
800
1000
Z-Arg-Arg-AMC uM

conditions reaction rates were directly proportional to substrate concentration and the parameter k_{cat}/K_m was calculated as being equal to v/[E][S]. These various kinetic constants are collected in Table 4.

Dependency of k_{cat}/K_m on pH

The rate of hydrolysis of Z-Arg-Arg-AMC was measured at a substrate concentration of 25 μM which corresponds to the condition [S]<<Km ([S]=0.025xKm). Under these conditions, k_{cat}/K_m=v/[E][S]. Fig. 26 shows the effect of pH on k_{cat}/K_m. Each experimental point is the mean and standard error of three determinations. The results, resemble a bell- shaped curve typical for cysteine proteinases such as cathepsin B (Bajkowski and Frankfater, 1975). The marked decline in k_{cat}/K_m between 7.5 and 8 may be due to an instability of the enzyme above pH 7.5. The data points from pH 3.5 to 7.5 were fit to the following equation for bell-shaped pH dependency curve

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)\ lim}{1 + \frac{H}{K_{a1}} + \frac{K_{a2}}{H}}$$

by non-linear regression analysis (Enzfit/Elsevier-Biosoft, Cambridge, UK). The solid line in Fig. 25 is the best fit curve calculated for pK_{a1}=5.05 and pK_{a2}=8.04.

Equilibrium Dissociation Constant for Leupeptin with the Secreted Cathepsin B-like Enzyme from B16 Melanoma

K_I was calculated as described by Perlstein and Kezdy (1973) for a tightly binding competitive inhibitor according to the following equation

Table 4. Kinetic Constants of the Secreted Latent Cathepsin B-like Enzyme from cultured Cells after Incubation with Pepsin[a].

Z-Arg-Arg-AMC

	k_{cat} (s^{-1})	K_m (M^{-1})[b]	k_{cat}/K_m ($M^{-1}s^{-1}$)
B16F-10	0.188±0.04	(1.00±0.290) x 10^{-3}	188
MMSV	ND	(0.422±0.02) x 10^{-3}	ND

Z-Phe-Arg-AMC

	k_{cat} (s^{-1})	K_m (M^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
B16F-10	0.286±0.012	(0.121±0.081) x 10^{-3}	2351

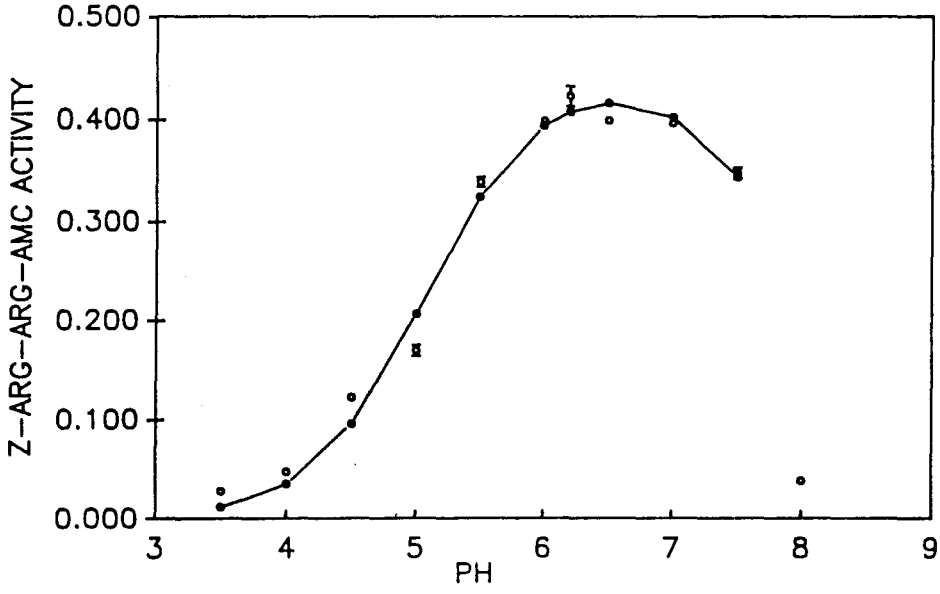
[a]B16F-10, B16 murine melanoma variant; MMSV, Moloney Murine Sarcoma Transformed BALB 3T3 cells. Values were determined by non-linear regression analysis (Enzfit / Elsevier-Biosoft, Cambridge, UK) and are presented with the estimates of the standard errors.

[b]The values for K_m are significantly different ($P<0.025$).

Fig. 26. Dependency of k_{cat}/K_m on pH of the Activated Purified Latent Cathepsin B-like Enzyme at 25°C. Rates were measured with [Z-Arg-Arg-AMC] = 25 μM. This concentration is about 50-fold less than K_m such that the rate of substrate hydrolysis is directly proportional to k_{cat}/K_m. The points are the means and standard error of the means of 3 determinations. The solid line was calculated from the equation

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{(k_{cat}/K_m)_{lim}}{1+\frac{H}{K_{a1}}+\frac{K_{a2}}{H}}$$

for the data points from 3.5 to 7.5. Best estimates of $(k_{cat}/K_m)_{lim}$, pK_{a1}, and pK_{a2} were 6.4, 5.05, and 8.04, respectively.


0.500
0.400
0.300
0.200
0.100
0.000
Z-ARG-ARG-AMC ACTIVITY
3
4
5
6
7
8
9
PH

$$\frac{A_T}{1-k_i/k_0} = \frac{K_I}{k_i/k_0} + (E_T).$$

In this equation k_0 and k_i are either first-order rate constants or initial rates for substrate hydrolysis ([S]<<Km) in the absence and presence of inhibitor, and (A_T) and (E_T) are total aldehyde and total enzyme concentration, respectively. Fig. 27 shows a plot of these data at pH 6.2. From the slope, K_I was calculated to be $(1.70 \pm .212) \times 10^{-9}$ M. As only 2% of leupeptin is present as the active (aldehyde) inhibitor form, in equilibrium with unreactive species (Schultz et al., 1989) the true K_I is 3.4×10^{-11} M.

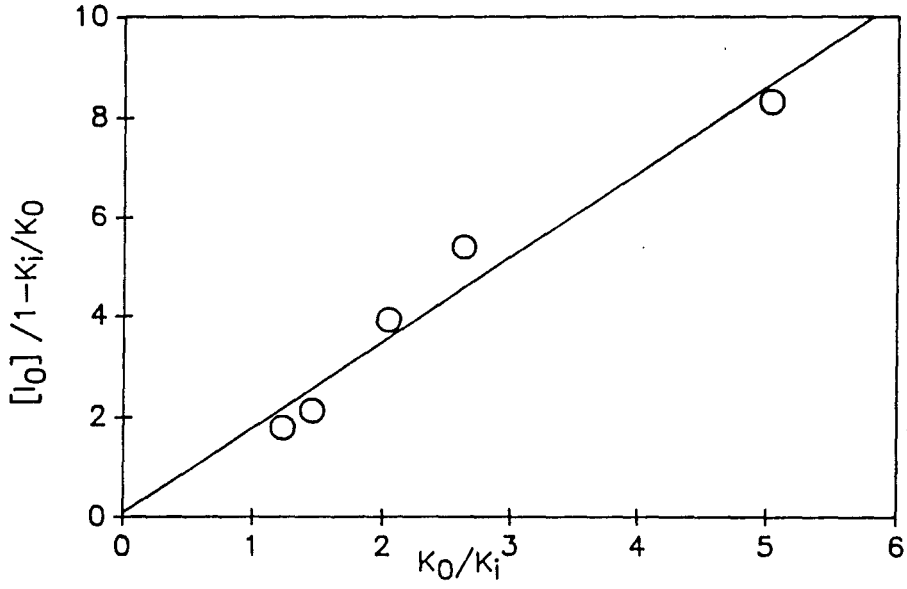
Rate Constants for the Association-Dissociation of the Melanoma Secreted Cathepsin B-like Enzyme with Leupeptin

The rate of association of leupeptin to the pepsin-activated cathepsin B-like enzyme was determined by measuring time dependent changes in the hydrolysis of Z-Arg-Arg-AMC in the presence of leupeptin. In the absence of leupeptin, the fluorescence due to the formation of the AMC product was linear with time. In the presence of leupeptin the fluorescence due to product formation obeyed the relationship

$$F = A(1 - e^{-kt}) + Bt$$

In this equation, A is the fluorescence change during the presteady state phase of inhibitor binding, B is the rate of fluorescence change during the steady state, and k is the pseudofirst order rate constant for attainment of the inhibited state. Fluorescence data was fit to this equation

Fig. 27. Inhibitions of cathepsin B-like enzyme-catalyzed hydrolysis of Z-Arg-Arg-AMC by leupeptin at 25°C in 0.2 M citrate/phosphate buffer pH 6.2. So = 50 μM, and I_0 varied from 0.2 x 10^{-9} M to 6.66 x 10^{-9} M. Each point is the average of 3 determinations. The solid line was calculated by a least squares linear regression analysis to give a K_I = 1.70 $\pm$ 0.21 x 10^{-9} M.


10
8
6
4
2
0
0
1
2
3
4
5
6
$[I_0]/1-K_i/K_0$
K_0/K_i

by a least squares linear regression analysis using the program Enzfit (Elsevier-Biosoft, Cambridge, UK). Values for k are collected in Table 5. The pseudofirst order rate constant, k, has the following meaning $k = k_{on}[I_A] + k_{off}$ where k_{on} is a second order rate constant for association of leupeptin with enzyme and k_{off} is a first order dissociation rate constant for decomposition of the enzyme leupeptin complex (Bartlet and Marlow, 1987). Fig. 28 shows a plot of k vs $[I_A]$, the concentration of the active inhibitor form. From the slope and intercept k_{on} was found to be 4.53 + 0.55 x 10^6 $M^{-1}sec^{-1}$ and k_{off} 1.31 $\pm$ 0.59 x 10^{-3} sec^{-1}. The constant k_{off} can also be more accurately calculated from the relationship

$$K_I = \frac{k_{off}}{k_{on}}$$

Using the values of 3.4 x 10^{-11} M and 4.53 x 10^6 $M^{-1}sec^{-1}$ for K_I and k_{on} respectively, k_{off} is found to be 1.54 x 10^{-4} sec^{-1}.

Inhibitor Studies

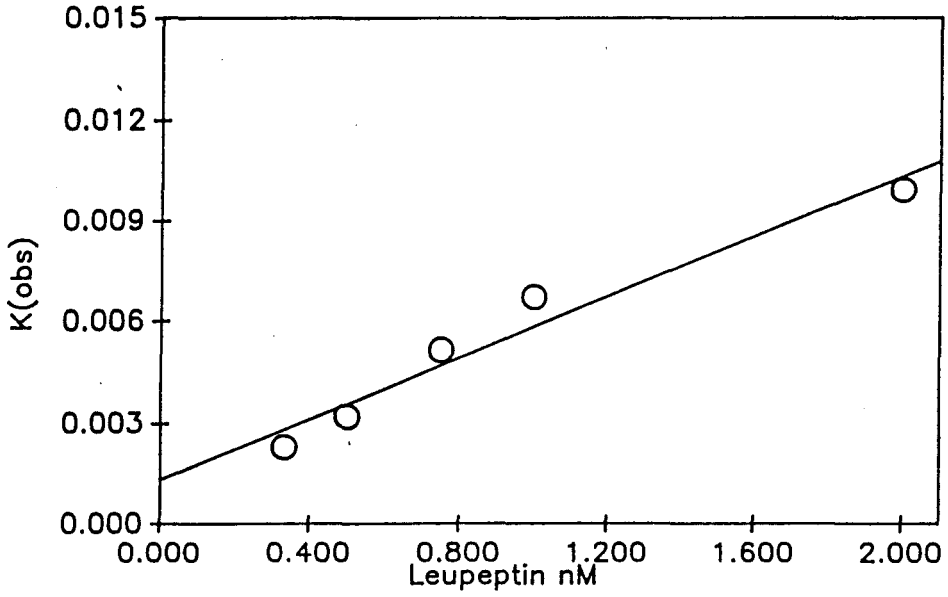
A variety of agents were tested for their effect on the hydrolysis of Z-Arg-Arg-AMC (50 μM) by the pepsin-activated cathepsin B-like enzyme. Table 6 shows that at a concentration of 1 mM, diisopropylfluorophosphate, a known serine proteinase inhibitor inhibited the rate of hydrolysis by only 12.5%. At the same concentration, 4,4'-dithiodiphridine and 2,2'-dithiodipyridine (thiol proteinase inhibitors) exhibited 72 and 100% inhibition, respectively, of the the hydrolysis of Z-Arg-Arg-AMC.

Table 5. First-order rate constants for approach to steady-state inhibition of the cathepsin B-like enzyme by leupeptin.

Active leupeptin conc.	k
form I_A x 10^{10} M[a]	sec^{-1}
3.33	2.30 x 10^{-3}
5.00	3.21 x 10^{-3}
7.50	5.16 x 10^{-3}
10.0	6.71 x 10^{-3}
20.0	9.95 x 10^{-3}

[a]Total leupetin concentration would be 50-fold greater than the concentration of the I_A form.

Fig. 28. Plot of the pseudofirst order rate constant for attainment of the inhibited state vs the concentration of active inhibitor form of leupeptin. The rate constants k_{on} and k_{off} were calculated by fitting the data points to the following equation $k = k_{on} [I_A] + k_{off}$ by a least squares linear regression analysis (Enzfit/Elsevier-Biosoft, Cambridge, UK). $[I_A]$ is the concentration of the active inhibitor form of leupeptin. The slope k_{on} was calculated to be $4.53 \pm 0.55 \times 10^6$ $M^{-1}sec^{-1}$ and the intercept koff was found to be $1.31 \pm 0.59 \times 10^{-3}$ sec^{-1}.


0.015
0.012
0.009
0.006
0.003
0.000
K(obs)
0.000
0.400
0.800
1.200
1.600
2.000
Leupeptin nM

Peptidyl-7-Amino-4-Methylcoumarin (AMC) Substrate Specificity

Table 7 shows the relative rates of release of AMC from several peptidyl-AMC substrates (all at 10 μM) as determined by fluorescence measurements. When rates were observed they were found to be directly proportional to the substrate concentration indicating that the condition ([S]<<Km) prevailed. Values of k_{cat}/K_m calculated from $v = \frac{k_{cat}}{K_m}[E][S]$, are shown in Table 7.

Table 6. Effect of various agents on cathepsin B-like activity.

Agent[a]	Concn.	% Inhibn[b]
diisopropyl fluorophosphate	1 mM	12
4,4'-dithiodipyridine	1 mM	72
2,2'-dithiodipyridine	1 mM	100

[a]The pepsin-activated enzyme was preincubated with the agent at the indicated concentration for 45 min at 25^{0}C.

[b]Each value is the mean of triplicate determinations. Activity was compared to that of the control enzyme preincubated under the same conditions but without the test agent.

Table 7. Relative rates of hydrolysis of peptidyl-7-amino-4-methylcoumarin substrates.

Substrate (10 μM)	Relative rate[a] (%)	k_{cat}/K_m[b]
Z-Arg-Arg-AMC	100	188
Z-Phe-Arg-AMC	1250	2351
H-Arg-AMC	2.4	4.5
Z-Arg-AMC	ND[c]	ND[c]
Z-Gly-Gly-Arg-AMC	ND[c]	ND[c]
Z-Gly-Gly-Leu-AMC	ND[c]	ND[c]

[a]Values are relative rates and are compared to Z-Arg-Arg-AMC (=100%)

[b]The hydrolysis rates that were observed were directly proportional to substrates concentrations. Therefore, under this condition ($[S] << K_m$), it was possible to calculate K_{cat}/K_m.

[c]Values were too small to detect.

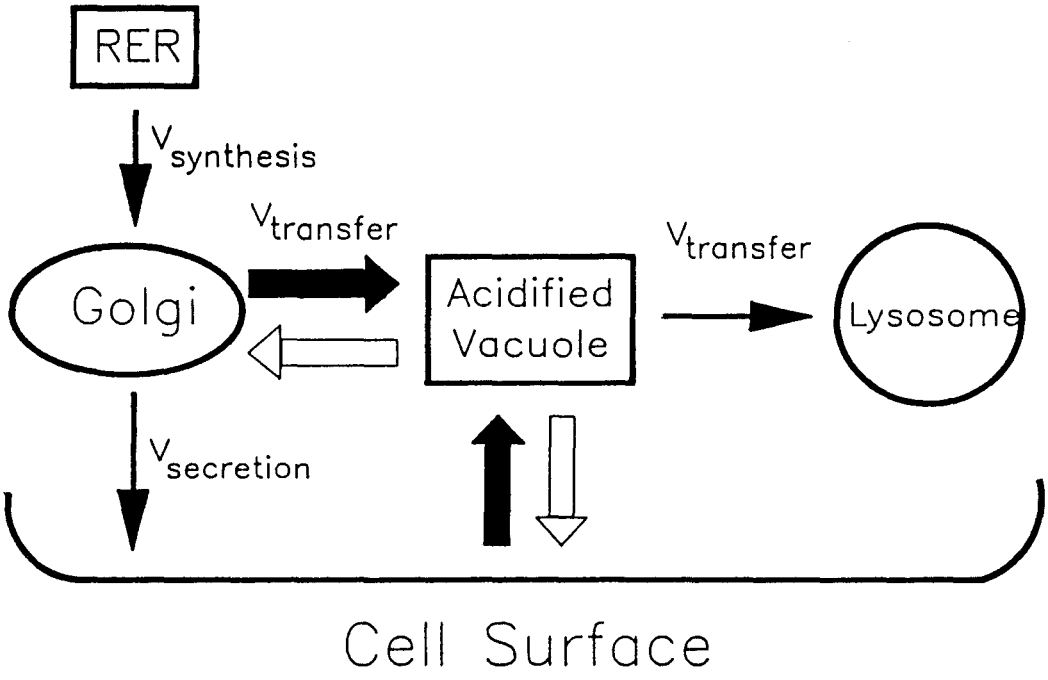
CHAPTER V

DISCUSSION

Effects of Monensin on Lysosomal Enzyme Targeting

Fig. 29 summarizes the current understanding of lysosomal enzyme targeting in fibroblasts (reviewed in more details under "Review of the Related Literature"). Mannose 6-phosphate receptors (MPRs) bind to newly synthesized lysosomal enzymes in the trans Golgi network, thereby segregating them from secretory proteins. The lysosomal enzyme-receptor complex is then delivered to an intermediate acidified compartment where dissociation of the complex occurs. Receptors are then returned to the Golgi apparatus and to the cell surface for reuse while newly synthesized lysosomal enzymes in turn are transferred to the lysosome. A small proportion of the lysosomal enzymes (5-20%) are secreted before delivery to lysosomes can occur (Kornfeld, 1987). Some of these may then be recaptured by receptor-mediated endocytosis and redirected to lysosomes via the post-Golgi acidified compartment (Kornfeld, 1987). The proteolytic processing of lysosomal enzymes is thought to be initiated in the intermediate acidified compartment (Mainferme et. al., 1985) and is completed in the lysosome (Nishimura and Kato, 1987).

Fig. 29. Biosynthetic transport of lysosomal hydrolases and mannose 6-phosphate receptors. -->, transport of unbound acid hydrolases; -->, transport of MPR-bound acid hydrolases; and ==>, recycling of unbound MPR (15, 28).


RER
$V_{synthesis}$
$V_{transfer}$
Golgi
Acidified Vacuole
$V_{transfer}$
Lysosome
$V_{secretion}$
Cell Surface

Proton ionophores such as monensin, and weak bases such as ammonium chloride, can act to prevent receptor-ligand dissociation and receptor recycling (Brown et. al., 1986; Geuze et. al., 1985) by increasing the pH of acidic compartments (Maxfield, 1982). The result can be a deficiency of cell surface (Geuze et. al., 1985) and trans-Golgi-associated (Brown et. al., 1986) MPRs, an inhibition of enzyme sorting within the trans-Golgi network, and the diversion of lysosomal enzymes to a constitutive secretory pathway (Nishimura et. al., 1988). As lysosomal enzymes are diverted from degradative compartments, proteolytic processing and turnover is inhibited (Nishimura et. al., 1988), and high levels of precursor forms should accumulate within the cell and in the culture medium. As shown in Figs. 2 and 3, the effect of monensin treatment on the secretion of cathepsin B and L by 3T3 cells conformed to this description.

If this explanation of monensin action is correct, and if as suggested monensin does not have a large effect on protein synthesis per se (Ledger and Tanger, 1984), then the rate of accumulation of latent cathepsin B and cathepsin L in cells and media exposed to monensin should provide an estimate of $V_{synthesis}$. The rate of accumulation of latent enzyme in the medium in the absence of monensin corresponds to $V_{secretion}$ (Fig. 29).

Lysosomal Enzyme Secretion by 3T3 and MMSV Cells

Using the assumptions described above, the fraction of

the newly synthesized cathepsin L which is secreted by 3T3 cells was calculated to be 0.12 using the data in FIg. 3. This is in excellent agreement with the value determined independently for NIH/3T3 cells by pulse-chase measurements (Dong et. al., 1989). The corresponding values for cathepsin B and ß-glucuronidase were 0.17 (Fig.2) and 0.30 (Fig.4), respectively. Thus in 3T3 cells, 70% or more of cathepsin B, cathepsin L and ß-glucuronidase is routed to lysosomes by a monensin sensitive pathway which is presumed to be receptor dependent.

In contrast to 3T3 cells, MMSV-transformed 3T3 cells secrete increased amounts of latent cathepsin B, latent cathepsin L, and ß-glucuronidase in the absence of monensin pretreatment. In addition, monensin is unable to further increase the levels of cellular and secreted precursor forms of the two cathepsins or to promote the secretion of ß-glucuronidase by MMSV cells. In this way MMSV cells behave like 3T3 cells which have been exposed to monesin. Thus, MMSV cells appear to be inefficient in targeting enzymes to lysosomes, and enzymes which do reach the lysosomes appear to travel a pathway which is monensin insensitive. Presumably, this pathway is either receptor independent or does not require a low pH compartment to function.

Possible Explanations for the Secretion of Lysosomal Enzymes in MMSV Cells

The observation that MMSV cells appear less efficient

than 3T3 cells in targeting enzymes to lysosomes can have several explanations. These cells may be unable to form phosphorylated mannose oligosaccharides required for high affinity binding to MPRs, MMSV cells may be deficient in functional MPRs, or MMSV cells may be unable to generate an acidic pH gradient along the biosynthetic transport pathway required for receptor-ligand uncoupling and receptor recycling. Alternatively, MMSV cells may have some other fundamental defect in the vesicular transport of enzymes from the trans-Golgi network to lysosomes.

The first explanation can probably be eliminated since cathepsin L-secreting Kirsten sarcoma virus transformed NIH 3T3 cells are capable of producing lysosomal enzymes which carry the mannose 6-phosphate targeting signal. In contrast, transformation-related defects in vacuolar acidification, reduced levels of cell surface and intracellular asialoglycoprotein receptors, and deficiencies of lysosomal hydrolases have been observed following hepatocarcinogenesis (Anderson et al., 1989). Consistent with a vacuolar acidification defect, Qiuming Gong in collaboration with the author has shown that MMSV cells are deficient in cell surface mannose 6-phosphate binding activity compared to nontransformed BALB c/3T3 fibroblasts (Achkar et al., 1990), and Valerie Pracht has observed that vacuolar pH in MMSV cells is about one half pH unit higher than in the 3T3 cells . This pH difference is of sufficient magnitude to inhibit MPR-

lysosomal enzyme dissociation and MPR recycling to the Golgi and cell surface. This would explain the lack of mannose 6-phosphate binding activity at the cell surface of MMSV-transformed cells. Thus, it seems possible that the cause of lysosomal enzyme secretion in MMSV cells is related to a failure in recycling of the MPRs.

Synthesis of Lysosomal Enzymes in 3T3 and MMSV Cells

Although the rate of synthesis of cathepsin L is much greater than that of cathepsin B in 3T3 cells as determined by the levels of the latent forms secreted in the presence of monensin, cellular levels of both enzymes were similar. This cannot be due to the greater rate of secretion of cathepsin L than cathepsin B by 3T3 cells, as the fraction of the newly synthesized enzyme which was secreted appeared similar for both (12 and 17% , respectively). Instead, the unexpected similarity in the cellular levels of cathepsin B and cathepsin L could be due to the presence of a cathepsin L inhibitor in cell extracts which caused an underestimation of active cathepsin L activity. Alternatively, the rate of turnover of cathepsin L may be greater than that of cathepsin B.

While increased secretion of procathepsin L by transformed fibroblasts is well documented (Gottesman, 1978; Gal and Gottesman, 1986; Denhardt et al., 1986; Troen et al., 1987; Dong et al., 1989), I found that nontransformed fibroblasts also secrete 3-fold more latent cathepsin L than cathepsin B. Second, I have demonstrated that transformation

causes a similar proportionate increase in the secretion of cathepsin B in addition to cathepsin L (17-fold for cathepsin B and 27-fold for cathepsin L). The transformation-induced increase in cathepsin B secretion is due in part to increased expression, as Qiuming Gong has found that the level of cathepsin B mRNA is 3-4 fold higher in MMSV cells than in 3T3 cells (Gong and Frankfater, unpublished results). Transformation also produced a 2-fold increase in ß-glucuronidase secretion.

Previous studies with Kirsten virus transformed NIH 3T3 cells (KNJH) indicated that transformation caused a selective increase in the secretion of cathepsin L. The specificity of this secretion was attributed to both an increased expression of cathepsin L and to an inability cathepsin L to bind with high affinity to mannose 6-phosphate receptors (Dong et al., 1989). In agreement, cathepsin L and ß-galactosidase are reported to be handled differently in KNIH cells, the secretion of the former being unaffected by ammonium chloride (Sahagian and Gottesman, 1982). In nontransformed fibroblasts ammonium chloride and monensin stimulate the secretion of both enzymes (Nilsen-Hamilton et al., 1981). This would suggest that mannose 6-phosphate receptor-dependent pathways target both cathepsin L and ß-galactosidase to lysosomes in 3T3 cells but only ß-galactosidase in KNIH cells. In contrast, I have found that monensin is unable to further stimulate the already enhanced secretion of the three lysosomal enzymes which I have

examined in MMSV cells, indicating that lysosomal enzyme secretion by these cells is not selective. This could indicate a general defect in lysosomal enzyme targeting in MMSV cells possibly related to a decreased recycling of MPRs. Ammonium chloride stimulation of cathepsin C secretion has also been observed in normal hepatocytes but not with Morris hepatoma cells (Mainferme et al., 1985), and hepatocytes but not hepatoma cells were found to synthesize measurable levels of cation-independent MPR (Mainferme et al. 1985). In addition, mutant Chinese hamster ovary cells with altered MPRs secrete increased amounts of precursor forms of lysosomal hydrolases (Robbins and Myerowitz, 1981).

Lysosomal Enzyme Secretion by B16 Murine Melanoma Cells

Previous reports that B16 murine melanoma cells secrete a latent cathepsin B-like activity prompted an investigation of whether these cells also had a general defect in lysosomal enzyme targeting or whether secretion was specific for cathepsin B. By comparing the levels of cathepsin B, cathepsin L and ß-glucuronidase secreted by the two murine melanoma variants, B16F-1 and B16a, in the presence and absence of monensin, I was able to calculate the fractions of the newly synthesized enzymes which were secreted. For latent cathepsin L this was calculated to be 0.34 and 0.32 for B16F-1 and B16a cells, respectively. The corresponding values for the latent cathepsin B-like activity (as defined by the substrate Z-Arg-Arg-AMC) were 0.38 for B16F-1 cells and 0.44 for B16a cells.

In contrast, the fraction of newly synthesized ß-glucuronidase which was secreted was estimated to be 0.14 and 0.05 for B16F-1 and B16a cells, respectively. Thus, it appears that ß-glucuronidase is relatively efficiently targeted to lysosomes in B16 melanoma cells by a monensin senstive (receptor-dependent) pathway. In contrast, the latent cathepsin L- and cathepsin B-like enzymes appear to be inefficiently targeted to lysosomes as 35 to 40% of the estimated newly synthesized activities are secreted in the absence of monensin. However the targeting defect in B16 melanoma cells is less absolute than in MMSV cells where nearly all the cathepsin B and cathepsin L appeared to be secreted.

Effects of Estrogen Treatment on the Synthesis and Secretion of Lysosomal Enzymes in MCF-7 Cells

In breast cancer, estrogens are known to facilitate the growth of primary and secondary tumors when these contain estrogen receptors (Lippman, 1981). In addition, estrogen is known to induce the synthesis and secretion of a Mr 52,000 protein in estrogen-dependent breast cancer cells (Rochefort et al., 1987b). This protein which displays a mitogenic effect on dormant MCF7 cells has been identified as a precursor of cathepsin D. The corresponding complementary DNA has been cloned, and its sequence indicates high homology with cathepsin D of normal tissues apart from 5 nucleotide changes (Augereau et al., 1988).

In order to determine whether the effect of estrogen on

the MCF7 cells is specific to cathepsin D or whether it affects the secretion of other lysosomal enzymes, I examined the effect of estrogen stimulation on the secretion of cathepsin B, cathepsin L and ß-glucuronidase by MCF7 cells. Figs. 11, 12 and 13 show no effect of estrogen on the levels of these three enzymes in culture media from MCF7 cells and no effect on the efficiencies by which these enzymes are targeted to lysosomes. The secretion of these enzymes ranged between 30 and 40%. Thus, it appears that these cells are relatively inefficient at targeting enzymes to lysosomes. Selective induction of cathepsin D by estrogen results in the appearance of a selective increase in cathepsin D secretion.

Immunodetection of the Cathepsin B-like Activity by MMSV and B16a Cells

Using anticathepsin B polyclonal antibody, I was able to detect the mature 28 kDa form of cathepsin B in cell lysates from 3T3, MMSV and B16 melanoma cells, and the precursor procathepsin B (40 kDa) secreted by MMSV cells. There were almost no changes in the level of secretion of the proenzyme upon monensin treatment while the level of the mature enzyme in the cell lysates decreased on incubation with monensin (Fig. 5). These results are in agreement with those obtained by measuring latent and active enzyme activities (Fig. 2). These results also indicate that the mature and proenzyme forms of cathepsin B can be detected by western blotting after SDS PAGE using a polyclonal antibody to cathepsin B. Thus, it

appears to be clear that the MMSV cells secrete authentic procathepsin B that is responsible for the latent cathepsin B-like activity using Z-Arg-Arg-AMC as a substrate.

Western blots using the anticathepsin B antibody were able to immunodetect low levels of procathepsin B released into the medium by B16a cells. However the levels of this proenzyme in the medium increased dramatically when B16a cells were treated with monensin. This is very apparant in Fig. 10 which shows the results of electrophoresis and immunoblotting of media samples obtained with and without monensin and contain the same amount of Z-Arg-Arg-AMC hydrolase activity. It is clear that very much less than expected immunoreactive cathepsin B is present in media in the absence of monensin even though a significant amount of latent cathepsin B-like activity is detected (Fig. 10). It would therefore appear that a second, immuno-unreactive protein is at least partially responsible for the Z-Arg-Arg-AMC hydrolase activity detected in B16a media in the absence of monensin. In addition, and since Fig. 10 shows a dramatic increase in the secretion of immunoreactive procathepsin B upon monensin treatment of B16a cells, it can be estimated that immunoreactive cathepsin B is efficiently targeted to the lysosomes in B16 melanoma cells as was ß-glucuronidase. Attempts to detect immunoreactive cathepsin B on a western blot in MCF7 cells and media failed due to the low Z-Arg-Arg-AMC hydrolase activity present.

Relation between Cathepsin B Expression, Secretion of a Latent

(Qian et al., 1989) examined the mRNA levels of five lysosomal proteinases, cathepsins B, D, H, L, and S, in normal mouse tissues and three melanoma variants B16-F1, B16-F10, and B16a, which were selected for differences in their ability to colonize the lung following i.v. (tail vein) injection (Fidler, 1973). In the i.v. assay, the B16a is more metastatic than the B16-F1 (Qian et al., 1989). Of the five cathepsins studied, only the mRNA level of cathepsin B was elevated in the highly metastatic melanoma and the mRNA levels in the three melanoma variants correlated positively with their metastatic potential. The ratio of cathepsin B mRNA levels in B16F-1, B16 F-10, and B16a was 1:1.5:7 (Qian et al., 1989). In agreement with those findings, I observed that the intracellular cathepsin B-like activity is 2.7 times higher in cell lysates from B16a than from B16F-1 cells (Table 1). In addition, the latent secreted cathepsin B-like activity is 1.6 times higher in media from B16a cells than from B16F-1 cells. In contrast, B16F-1 secrete 1.5 times more latent cathepsin L than B16a cells (Table 1).

Cathepsin B-like activity or cathepsin B mRNA levels have been reported to correlate with the metastatic potentials of variants of several other tumors, including rat pancreatic carcinomas (Koppel et al., 1984), human genital tract cancers (Pietras and Roberts, 1981), and murine hepatoma (Moin et al., 1989).

In view of the fact that the levels of the latent cathepsin B-like activity secreted by B16 melanoma variants was correlated with the message levels for cathepsin B in these cells, the finding that the secreted enzyme was not immunoreactive cathepsin B came as a considerable surprise. It thus became an important priority to try to identify the protein responsible for this latent cathepsin B-like activity.

The Purification of the Secreted Latent Cathepsin B-like Enzyme from B16 Cells

Because western blots of media samples from B16a melanoma cells suggested that these cells secrete a latent cathepsin B-like enzyme that does not cross-react with anticathepsin B-antibody, it was important to purify the protein responsible for this activity. The purification of this enzyme was undertaken as described in "Experimental Procedures" and "Results". The product appeared better than 95% pure based on active site titration with E-64. SDS polyacrylamide gel electrophoresis under reducing and denaturing conditions gave an apparent molecular weight of 67000 for the purified enzyme as compared with 70,000 by active site titration. In further agreement, the molecular weight of the latent enzyme estimated by high performance molecular sieve chromatography was 67,400. This cathepsin B-like enzyme was responsible for about half of the total avtivity recovered from the anion exchange column. When the latent form was preactivated with pepsin, the active species eluted at an apparent molecular weight of 55,700. If

these results are correct, then the B16 melanoma secreted cathepsin B-like activity can not be the translation product of the cathepsin B mRNA in mouse (Segunda et al., 1985). Those workers have isolated cathepsin B cDNA gene clones from rat liver and found that their translated region encodes a polypeptide having a molecular weight of approximately 39,000.

In order to investigate the possibility that the larger molecular weight of the purified enzyme was due either to dimerization or to a tight binding inhibitor which also blocked the binding of antibodies to the enzyme, a purified sample was reduced with mercaptoethanol in 6 M guanidine hydrochloride and alkylated with iodoacetate. SDS PAGE in Fig. 19 revealed that this treatment actually decreased rather than increased the mobility of the purified enzyme arguing against this explanation.

In order to investigate the possibility that oligosaccharide chains on the surface of the enzyme might be responsible for the higher apparent molecular weight on SDS polyacrylamide gels of the purified latent cathepsin B-like activity,the reduced and alkylated enzyme was treated with a mixture of glycosidase F and endoglycosidase F (see "Experimental Procedures") and then analyzed by SDS/PAGE. This treatment resulted in a 6kDa decrease in the apparent molecular mass of the purified enzyme, but its final molecular mass was still greater than that expected for authentic procathepsin B. In addition, the apparent molecular mass of

the pepsin activated enzyme (55 kDa), determined by high perfomance molecular sieve chromatography, is also much higher than the reported size of the active cathepsin B in mouse mammary gland (31 kDa) (Recklies and Mort, 1985) and rat islet cells (31.5 KDa) (Docherty et al., 1983).

The SDS treated purified latent enzyme failed to cross-react with antibodies prepared against an SDS-denatured cathepsin B-ß-galactosidase fusion protein. Pretreating the latent enzyme with pepsin prior to SDS denaturation, reduction and alkylation of the guanidine hydrochloride denatured enzyme also did not increase its immuno-reactivity toward anti cathepsin B antibodies (Fig. 10). These results provide additional evidence that the purified latent cathepsin B-like enzyme is not authentic procathepsin B. As a positive control, procathepsin B was detected in culture medium from MMSV cells and from B16a cells treated with monensin (Fig. 5) by Western immunoblotting.

Active Site Titration with E-64

E-64, a known thiol protease inhibitor was able to inhibit the Z-Arg-Arg-AMC hydrolase activity of the cathepsin B-like enzyme up to 90% in a stoichiometric manner (Fig. 22). This indicates that indeed this enzyme is a cysteine protease. Extrapolation of the initial linear portion of the inhibition curve to 100% inhibition yielded an estimate of the number of moles of enzyme which was present in the reaction mixture. From a knowledge of the amount of protein also present (as

determined by the Bio-Rad method), the molecular weight was calculated to be 70,000 as compared with 67,000 and 67,400 obtained by SDS PAGE and molecular sieve chromatography, respectively.

Kinetic Characterization of the Latent Cathepsin B-like Enzyme

Table 4 shows the values of K_m, k_{cat}, and k_{cat}/K_m for the latent cathepsin B-like enzyme after activation with pepsin. Values of k_{cat} were calculated from the relationship $k_{cat}=V_{max}/[E]$ and k_{cat}/K_m was determined from the relationship $k_{cat}/K_m=v/[E][S]$ at $[S]<<Km$. K_m and V_{max} were in turn determined by filing kinetic data to the Michaelis-Menten equation by non linear least squares analysis as described in "Results". For the substrate Z-Arg-Arg-AMC, K_m was calculated to be 1.00 $\pm$ 0.29 mM and kcat was 0.188 $\pm$ .040 sec^{-1}. In contrast, the pepsin activated procathepsin B secreted by MMSV transformed cells had a K_m of 0.422 $\pm$ .020 mM for the same substrate. These K_m values are different at a level of statistical significance of $P<.025$. The value of k_{cat} for the MMSV cell secreted procathepsin B could not be determined because the enzyme had not been purified. However, k_{cat} and k_{cat}/K_m for the activated melanoma enzyme is about 840- and 4500- fold less than for authentic cathepsin B (Deval et al., 1990). When Z-Phe-Arg-AMC was used as the substrate, K_m was calculated to be 121 $\pm$ 8 μM, k_{cat} .286 $\pm$ .012 s^{-1}, and k_{cat}/K_m 2351 $M^{-1}s^{-1}$. In contrast, these values for authentic cathepsin B are 252 μM, 364 s^{-1} and 1.44 x 10^6 $M^{-1}s^{-1}$ (Deval et al., 1990). It can be concluded

based on this data that the pepsin-activated secreted cathepsin B-like enzyme is much less efficient than normal cathepsin B in its ability to hydrolyze the synthetic substrates Z-Arg-Arg-AMC and Z-Phe-Arg-AMC.

The pH dependency of the apparent second order rate constant k_{cat}/K_m, between pH 3.5 and pH 8.0, appeared to depend on the state of ionization of at least two groups in the free enzyme having pKa values of 5.05 and 8.04. The pKa of the group controlling the activity of the alkaline limb of the titration curve could not be determined with certainty owing to the apparent instability of the enzyme above pH 7.5. These findings are characteristic of lysosomal cysteine proteinases in general, and are similar to the results obtained by Knight (1981) for the hydrolysis of Z-Arg-Arg-2-naphthylamide by human liver cathepsin B. Bajkowski and Frankfater (1983) have obtained pKa values of 4.2, 5.5 and 8.6 for bovine spleen cathepsin B with the substrate Z-Lys p-nitrophenyl ester. Overall, these results suggest that the 67kDa latent secreted cathepsin B-like enzyme from B16 melanoma cells is a latent cysteine proteinase which when activated with pepsin is a relatively poor catalyst for the hydrolysis of Z-Arg-Arg-AMC and Z-Phe-Arg-AMC.

Inhibitors of the B16 Melanoma Latent Secreted Cathepsin B-like Enzyme

Treatment of the pepsin-activated latent secreted cathepsin B-like activity with 1 mM DFP resulted in a 13%

inhibition of Z-Arg-Arg-AMC hydrolase activity while 2,2'-dithiodipyridine, a cysteine protease inhibitor gave 100% inhibition. The equilibrium dissociation constant for inhibition of the activated cathepsin B-like enzyme by leupeptin was calculated to be 1.7×10^{-9} M, a value that falls within the range for observed cysteine proteases such as papain (Frankfater and Kuppy, 1981) and authentic cathepsin B purified from B16 melanoma Cells (Shcultz et al., 1989). This value is however two to three order of magnitude smaller than those often seen for leupeptin inhibition of serine proteases.

Measurements of the rates of association of leupeptin with cathepsin B gave a value for the second order rate constant, k_{on} of $4.53 \pm 0.55 \times 10^{6}$ $M^{-1}sec^{-1}$ as compared with the value of 1.2×10^{7} $M^{-1}s^{-1}$ obtained for the active cathepsin B purified from B16 melanoma cells. These values are statistically different at a P value of < 0.025. The off rate constant was in turn found to be 1.54×10^{-4} sec^{-1} from the relationship $k_{off} = K_I \times k_{on}$. This differs from the value of 1.78×10^{-3} s^{-1} for the purified active B16 melanoma cathepsin B.

Although the kinetic data strongly imply that the pepsin-activated form of the latent cathepsin B-like enzyme from B16 melanoma is different from active cathepsin B from humans and mice, such a conclusion cannot be reached with certainty. Thus pepsin may not activate latent cathepsin B in the same manner as procathepsin B which undergoes physiological

activation *in vivo*. Recently Mort *et al.* has found that the peptide bond in procathepsin B cleaved by pepsin *in vitro* differs from the cleavage site for removal of the propeptide *in vivo*. Thus, it might not be unexpected that the physiologically activated cathepsin B would have different kinetic properties from pepsin-activated purified secreted cathepsin B precursor. The only substantial kinetic evidence for a difference between the melanoma secreted cathepsin B-like enzyme and authentic procathepsin B is the single finding that the K_m for the pepsin-activated MMSV secreted enzyme differs from the pepsin-activated B16 melanoma secreted enzyme. However, taken together with molecular weight measurements and determinations of immunoreactivity differences, it appears that the cathepsin B-like enzyme secreted by B16 melanoma cells is a novel cysteine proteinase.

The cathepsin B-like enzyme secreted from B16 melanoma cells was partially purified earlier by Bajkowski *et al.*, (1984). In agreement with the results of this study, this latent cathepsin B-like enzyme appeared as a major band on SDS PAGE with an apparent molecular weight of 68,000. However, I have observed that bovine serum albumin (M_r 68,000) is a major contaminant in media samples from B16 melanoma cells previously grown in the presence of bovine serum. Furthermore I have found that B16 melanoma cells also secrete a 65 kDa prohormone activating enzyme (unpublished results) and a 63 kDa serine protease inhibitor homologous to baboon

antitrypsin. Given uncertainties in the extent to which Bajkowski et al. purified the secreted cathepsin B-like enzyme from B16 melanoma cells, I can not ascertain whether the 68 kDa band they observed on SDS PAGE was indeed due to this enzyme.

The cathepsin B-like enzyme secreted by B16 melanoma cells may be similar to a 68 kDa secreted Z-Phe-Arg-AMC hydrolase activity recently reported to be associated with HPC-YP cells and previously mistaken for procathepsin L (Yamaguchi et al., 1989).

CHAPTER VI

CONCLUSIONS

I. Tumor cells differ in their capacity to secrete lysosomal enzymes. MMSV-transformed fibroblasts are inefficient at targeting enzymes to lysosomes, secreting high levels of cathepsin B, cathepsin L and ß-glucuronidase. In contrast B16 melanoma cells are efficient in targeting immunoreactive cathepsin B and ß-glucuronidase to lysosomes. However these cells secrete about 45% of their newly aynthsized pro-cathepsin L.

II. The cathepsin B-like activity secreted by B16 melanoma cells is due to a new enzyme species having a molecular weight of 67,000. The pepsin-activated form of this enzyme is a less efficient catalyst of Z-Arg-Arg-AMC and Z-Phe-Arg-AMC hydrolysis than is the mature form of cathepsin B from rodent tissues.

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The Dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Ph.D.

July 29, 1991
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