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Regulation of Proteolytic Activity in Tissues

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ABSTRACT: Degradation of tissue proteins is controlled by multiple means. These include regulation of the synthesis of proteinases, activation of the zymogen forms, the activity of the mature proteinase, and the degradation of these enzymes and the substrates. Mature proteinases can be controlled by pH, calcium ions, ATP, lipids and the formation of complexes with other proteinases, proteoglycans, and inhibitors.

KEY WORDS: proteinases, zymogen activation, proteinase inhibitors, proteasomes.

I. INTRODUCTION

Proteinases are involved in many cellular and extracellular processes. Careful regulation of these enzymes is required to prevent abnormal tissue damage. Proteinases serve a variety of functions at the cellular, tissue, and systemic level. Under normal conditions, specific intracellular proteinases cleave the signal peptide found on most proteins and the propeptide found on many hormones, cytokines and enzymes.[1] The proteinase calpain probably acts as a mediator in signal transduction pathways.[2] Lysosomal and ubiquitin-mediated intracellular protein turnover is carried out by proteinases.[3–5] Extracellular proteinases mediate the turnover of extracellular matrix proteins.[6] These processes regulate the lifetime of critical molecules responsible for cellular and extracellular functions. Proteinases released into the lumen of the stomach and the duodenum digest dietary proteins.[7] Cell growth is modulated by proteinases.[8] These enzymes play a major role during wound healing.[9–12] Proteinases have important roles in the coagulation,[13] fibrinolysis,[14] complement,[15] and kininogen systems.[16,17] Proteinases are involved in tissue remodeling associated with normal growth and development,[18–21] and the specialized conditions observed in lower segment uterine dilation during labor,[22] uterine remodeling following labor,[6] and mammary gland involution after cessation of lactation.[23] Neurite outgrowth,[24] angiogenesis,[21,25] and normal[8] and malignant cell proliferation[26] require the involvement of proteolytic processes. Proteinases can induce cells to release active molecules such as oxygen metabolites.[27] The death of cells within a tissue can result in the release of intracellular proteinases that cause degradation of the extracellular matrix.

In addition to controlling endogenous proteinases, the tissues need to be able to control proteinases released by microorganisms, inflammatory cells, and tumor cells (Figure 1). These cells release proteinases that facilitate their invasion into tissues. Inflammatory cells (monocytes, macrophages, polymorphonuclear leukocytes, mast cells, and natural killer cells) can release proteinases that are targeted toward killing invading organisms and

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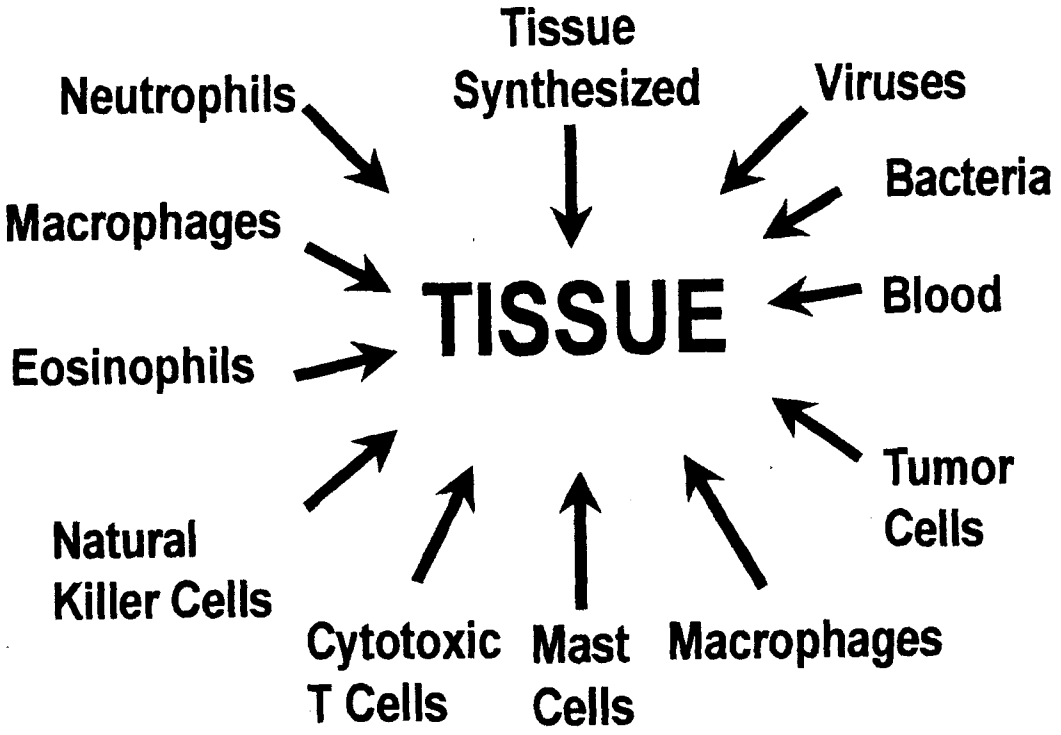

FIGURE 1. Sources of proteinases in tissues.

cells.[28–30] These enzymes also degrade host tissues.

Control of proteolysis in tissues can require the control of multiple proteinases involved in a given degradative process. Metastasis of tumor cells involves various proteinases, including cathepsins B, L, and D, plasminogen activators, and matrix metalloproteinases depending on the tumor type or oncogenic transformation.[31,32] In transformed NIH 3T3 cells, collagenase and urokinase-type plasminogen activator can substitute for each other to enable metastasis.[32] Degradation of joints in arthritis involves numerous proteinases.[9] The major proteins found in noninflamed arthritic joints are interstitial collagenase, gelatinases A and B, stromelysin, and urokinase-type and tissue-type plasminogen activators and cathepsin B.[28,33] Inflamed joints contain these proteinases plus neutrophil and macrophage proteinases.

There are multiple ways proteinases are regulated in cells and the extracellular matrix of tissues (Figure 2). Which mechanisms control a given proteinase depends upon the proteinase itself, its particular form (zymogen, active form, or modified form), and its location within a tissue. Although some proteinases are constitutively synthesized, the synthesis of many is carefully controlled at the level of gene transcription, mRNA stability, and/or translation. Proteinases, including lysosomal proteinases, are synthesized as inactive zymogen molecules that are activated by a conformational change and/or proteolytic cleavage. Often, zymogen activation involves a cascade or amplification system in which several zymogens are activated sequentially, culminating in the activation of a proteinase that plays a major physiological role (e.g., the coagulation pathway where ultimately prothrombin is cleaved, forming thrombin, and then cleaves fibrinogen to fibrin).[13] The mature forms of proteinases are controlled by pH, posttranslational modifications (phosphorylation, glycosylation, or oxidation), localization (in secretory granules or lysosomes or on membranes), reaction with activators or inhibitors, and proteolytic degradation (Figure 3). The availability of inhibitors and activators is also controlled. In addition, the proteolysis of a given protein is controlled by the availability of susceptible peptide bonds. Susceptible peptide bonds are defined by the presence of a specific amino acid sequence and posttranslational modifications (phosphorylation, glycation, oxidation, and ubiquitination) and the conformational availability of the susceptible sites.

This review is directed toward giving examples of the many different ways proteinases are regulated rather than a complete review of any given proteinase or class of proteinases. Endopeptidases rather than exopeptidases are emphasized. No proteinase is controlled by all of the mechanisms given. The reader is referred to various reviews cited throughout this discussion for more detail on specific enzymes.

II. TYPES OF PROTEINASES

There are four major classes of endoproteinases based on the mechanism of catalysis: serine, cysteine (thiol), aspartic (acidic), and metalloproteinases (Table 1). In addition, several other proteinases have been identified that do not fall into these four classes. This later group includes the membrane-bound signal peptidases[34] and the proteinases present in proteasomes.[35] Recently, these groups have been divided into families based on their nucleotide and/or protein sequences.[36]

A. Serine Proteinases

Two major types of serine endoproteinases are involved in a wide range

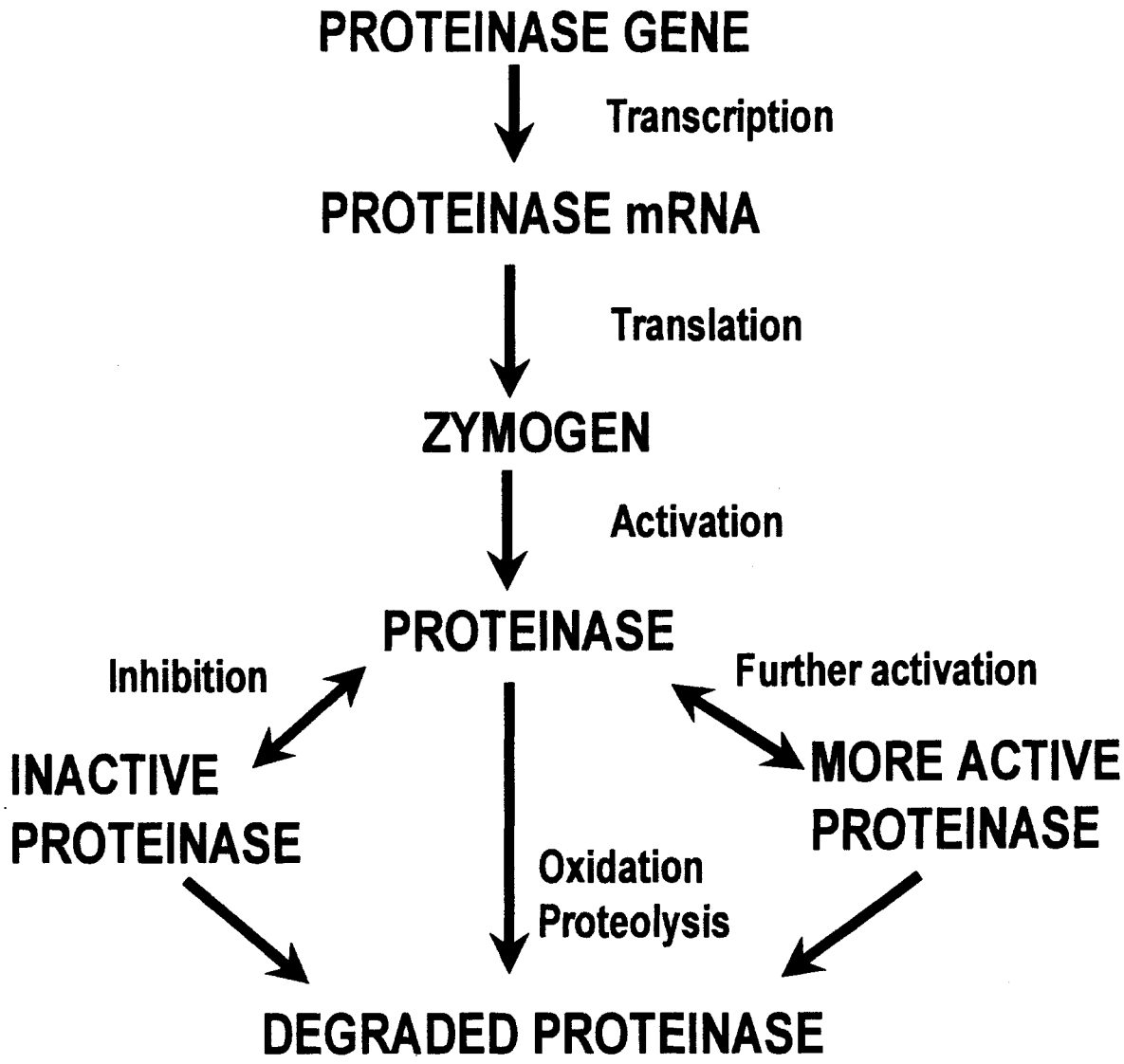

FIGURE 2. Overview of control mechanisms of proteinases.

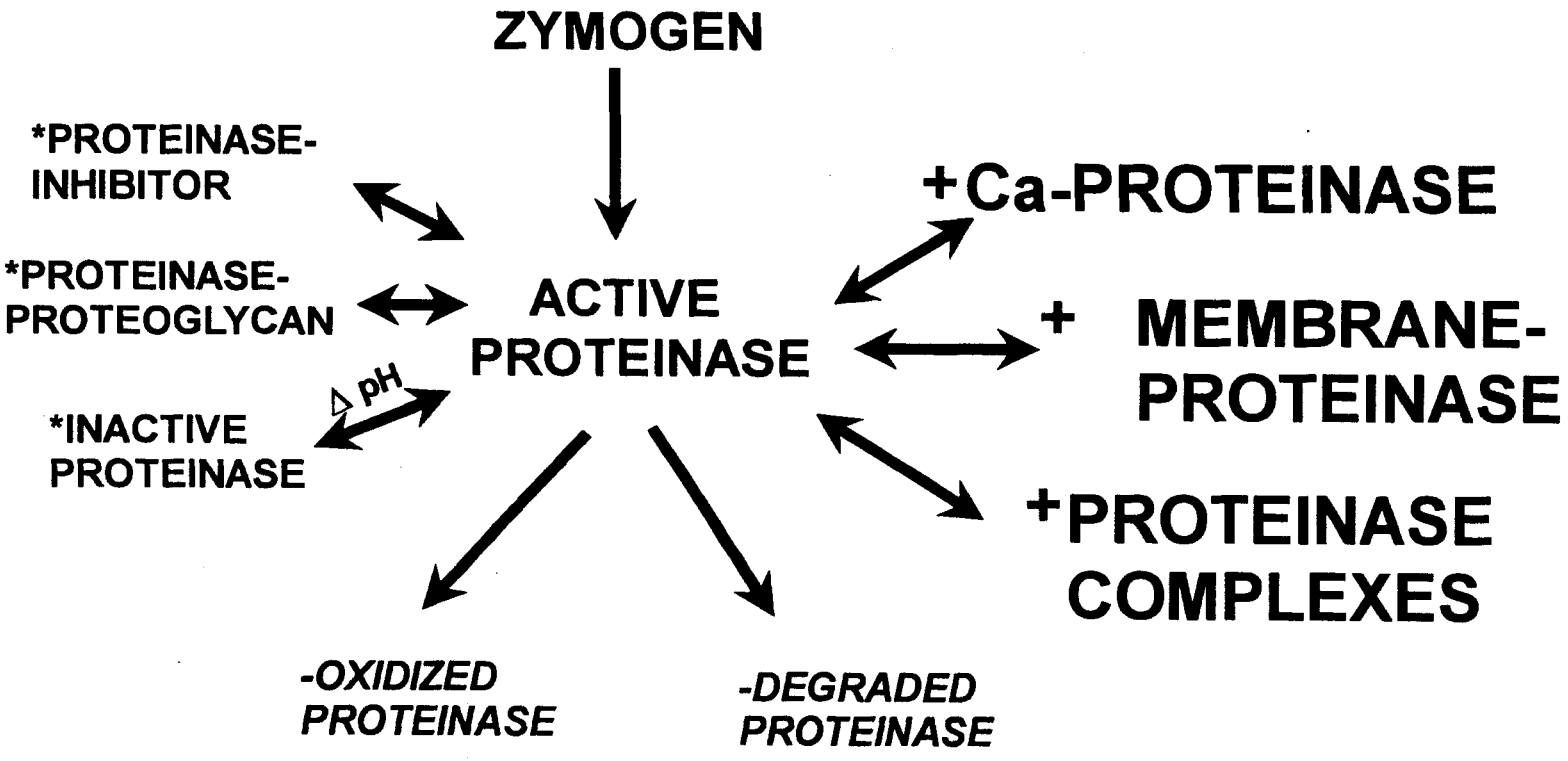

FIGURE 3. Sites of control of proteinase activity. *, reversibly inactivated proteinases; +, proteinases with enhanced activities; –, degraded or irreversibly inactivated.

TABLE 1
Proteinase Classes

Class	Examples	
Serine: tryptic-like	Chymotrypsin	Coagulation factors: thrombin, factors VII, IX, X, XI, XII
	Plasmin	Urokinase-type and tissue-type plasminogen activator
	Trypsin	
	Tryptase	
	Pancreatic elastase	Complement factors: C1r, C1s, C3, C5 convertase, factor D
	Kallikrein	Cathepsin G
	Neutrophil elastase	Neutrophil proteinase 3
	Granzymes	Chymase
Serine: subtilisin-like	Furin (PACE)	Prohormone-converting enzymes
Cysteine proteinases	Cathepsins B, H, L, S	Calpains I and II
Metalloproteinases	Gelatinases A and B	Interstitial collagenase
	Stromelysins 1–3	Neutrophil collagenase
	Matrilysin	
Aspartate proteinases	Pepsin	Cathepsins D and E
	Gastricin	Renin
	Chymosin	
Unclassified	20 S and 26 S Proteasomes	Signal peptidases

Note: See text for references.

of cleavage reactions. The first group includes digestive, lysosomal, coagulation, fibrinolytic, and immune cell secretory enzymes. Chymotrypsin, trypsin, and elastase are released from the pancreas into the duodenum and cleave dietary proteins.[7] The coagulation (factors VII, IX, X, XII and XII, thrombin, and protein C),[13] fibrinolysis (tissue plasminogen activator, urokinase type-plasminogen activator, and plasmin),[14] and complement systems (C1r, C1s, C3 convertase, C5 convertase, and factor D)[15] are all serine proteinases. Urokinase-type and tissue-type plasminogen activators are involved in embryogenesis, ovulation, neuron growth, wound healing, vascular injury, inflammatory injury, smooth muscle cell proliferation, tumor growth, and tumor cell invasion.[37] Mast cells granules contain two proteinases, chymase and tryptase, that have cleavage specificities similar to pancreatic chymotrypsin and trypsin.[29] These mast cell proteinases are released into tissues from the histamine granules during IgE-mediated allergic reactions. $Cd4^{+}$T cells contain a tryptase, TL2, that cross-reacts with the mast cell tryptase.[38] This enzyme is involved in the formation of syncytium upon binding HIV-1 protein gp 120. Cytolytic granules found in natural killer cells, cytotoxic T cells, and lymphokine-activated killer cells contain multiple serine proteinases called granzymes.[30] Natural killer cell granules contain ly-tryptase, ly-chymase,

Met-ase, Ser-ase, and Asp-ase.[30,39,40] Human polymorphonuclear neutrophils contain three major serine proteinases, cathepsin G, elastase, and proteinase 3, all of which are involved in the degradation of phagocytosed materials.[41]

The mechanism for the hydrolysis of peptide bonds by these serine proteinases has been studied extensively using various techniques that include solution assays of native and chemically modified enzymes[42,43] and NMR spectrometry and X-ray crystallography of proteinases in complex with transition state analogs.[44] The active site contains a catalytic triad composed of serine, histidine, and aspartic acid residues. These residues are part of a hydrogen-bonding system. Peptide bond cleavage involves a nucleophilic attack by the active site serine γ-oxygen on the carbonyl carbon of the susceptible substrate peptide bond. An ester is formed between the serine residue of the enzyme and the acyl portion of the peptide bond of the substrate. This involves the formation of a tetrahedral intermediate and the rearrangement of protons between the histidine and serine residues of the catalytic triad. The developing oxygen anion is hydrogen bonded to two α-NH groups from the protein backbone of the proteinase. Upon formation of the ester bond, the amino portion of the peptide bond is released. A water molecule is added back with the formation of a second tetrahedral intermediate. The acyl enzyme is hydrolyzed, the acyl portion of the peptide bond is released, and the active proteinase is regenerated.

The second major group of serine proteinases are homologous to the bacterial proteinase subtilisin. These proteinases evolved by a convergent mechanism relative to the chymotrypsin-serine proteinase family.[45] The structures of the two sets of enzymes are different and the catalytic residues are ordered differently. In this subtilisin group the order of the catalytic residues is Asp-His-Ser rather than His-Ser-Asp.[45] This group of propeptide-cleaving enzymes include furin (PACE) and the prohormone-converting enzymes PC-1 to -6.[46,47] Furins are expressed in most tissues, but PC-1 and PC-2 are only expressed in neuroendocrine tissues.[48] PC-4 is expressed only in the testis.[47]

B. Cysteine Proteinases

There are two major groups of cysteine proteinases: the lysosomal proteinases, cathepsins B, H, L, and S, and the cytosolic calpains, I and II. Cathepsins B, H, L, and S are lysosomal proteinases that are found in many tissues. Their levels vary significantly from one tissue to another, from one cell type to another,[49,50] and between cells within a tissue.[51] For example, in rat gastroduodenal mucosa, all cells except goblet cells contain these cathepsins localized to lysosomes.[51] Cathepsin H is found mainly in the lysosomes of gastric parietal cells and macrophages in the lamina propria. Cathepsin L is present in some gastric mucous cells, enterocytes, and macrophages found in the lamina propria.

Abnormal conditions can result in elevated levels of cysteine proteinases. For example, the synovial cells that are attached to cartilage and bone sites affected by rheumatoid joint erosion display an enhanced transcription of cathepsin B when compared with normal fibroblasts.[33] Cysteine proteinases also can be released from cells into the extracellular matrix. These enzymes are involved in the extracellular degradation associated with periodontal diseases.[52] Many tumor cells also synthesize and secrete cathepsins B and L.[53]

Calpains are a special group of cysteine proteinases that require calcium for activity.[54–57] Most cells contain two forms of this enzyme, calpain I (μ-calpain), which requires

micromolar levels of calcium for activity, and calpain II (m-calpain), which requires millimolar levels of calcium. Skeletal muscle contains a third member of the calpain family n-calpain 1 (p94 or ncl-1) which requires a high level of calcium for activity.[54,57] Several other members of this family have been identified based on sequence homology. These include n-calpain 2 and 2′ which are only found in the stomach.[57] The relative levels of the two forms of calpain differ between cell types and between species. The highest levels of these enzymes are found in platelets.[58] Calpain II also has been localized extracellularly in the cartilaginous matrix of growth cartilage.[59]

Each type of calpain contains a minimum or two subunits, a large unique subunit (80 kDa for calpain I and II and 94 kDa for n-calpain-1) and a small identical subunit of 28 kDa.[54,56] The large subunits are homologous and consist of four domains, an N-terminal hydrophobic domain that is involved in activation of the enzyme, a catalytic domain with homology to the typical cysteine proteinases, a ligand-binding domain, and a calmodulin-like calcium-binding domain. The small subunit that modulates the catalytic activity contains a hydrophobic domain with a glycine-rich sequence and a calmodulin-like calcium-binding domain with four sets of sequences analogous to the E-F hand of calmodulin. Five or more Ca^{2+} ions are required for activity, although there are eight potential Ca^{2+}-binding sites per molecule.[54] n-calpain 1 and 2 have three extra regions which are not found in calpains I and II.[60]

Multiple roles have been proposed for calpains based on the types of molecules cleaved by these proteinases. These include regulation of cytoskeleton structure through degradation of neurofilament proteins (microtubule-associated proteins 2 and τ-protein, vimentin, spectrin, fodin, tubulin, actinins, talin, desmin, and cytokeratins);[61–63] modulation of ion transport and down-regulation of receptors by degradation of calcium ATPase, hormone, and growth factor receptors;[56] modulation of signal transduction through cleavage of protein kinase C, phospholipase C, calmodulin-dependent kinase, phosphatase, and the transcription factors c-Jun and c-Fos;[56,64,65] and specific degradation of Z lines of muscle fibers by cleaving titin, nebulin, C-protein, tropomyosin, and troponin without the cleavage of actin or myosin.[54] Based on functional inhibition[66] and evolutionary relationships,[36] interleukin converting enzyme is identified as a cysteine proteinase. Thuis enzyme activates interleukin-1β.

The mechanism of peptide hydrolysis by cysteine proteinases is similar to that of the serine proteinases except that the nucleophilic sulfur of the active-site cysteine attacks at the si face of the substrate peptide bond rather than at the re face, which is attacked by the serine proteinases.[67] With these enzymes, a thioester intermediate is formed.[42,43] A histidine residue also is involved in shuttling a proton from the cysteine residue in the formation of the thioester intermediate through a tetrahedral transition-state. This histidine acts as both a general acid and a general base. Proteinase backbone α-NH groups also stabilize the transition-state. In contrast to serine proteinases where the residue in the P_1 site is a major contributor to substrate specificity, in cysteine proteinases the residue at the P_2 site is the major residue for the determination of substrate specificity.[68]

C. Metalloproteinases

The metalloproteinases include the matrix metalloproteinases and the astacin-

like proteinases. The matrix metalloproteinase (MMP) family includes interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), gelatinase A (72 kDa, MMP-2), gelatinase B (92 kDa, MMP 9), stromelysin (MMP-3), stromelysin-2 (MMP-10), and matrilysin (PUMP, MMP-7).[6,19] The mamalian astacin-like proteinases include meprin (rodents) and PABA-peptide hydrolase (human) are found on the brush boarder membranes of the intestine and kidney.[69]

The matrix metalloproteinases are homogeneous enzymes, however, their structures vary depending upon which domains are present.[6,19] All members of this family contain a propeptide, a catalytic domain, and a zinc-binding domain. Matrilysin consists of only these three regions. Stromelysin, stromelysin-2, and interstitial collagenase have an added hemopexin-like domain on the C-terminal end. Gelatinases A and B have the C-terminal hemopexin-like domain and a fibronectin-like domain added between the active enzyme and the zinc-binding site. Gelatinase B also has a type V collagen-like domain between the zinc-binding domain and the hemopexin domain. Substrate specificity differs among these enzymes.

Most cells synthesize and immediately secrete matrix metalloproteinases into the extracellular matrix.[6] Inflammatory cells, however, store proteinases of this class (i.e. neutrophil collagenase and gelatinase B). Tissue distribution of these proteinases varies widely. Some are constitutively synthesized (i.e., gelatinase A) by many cells, while others are synthesized mainly upon stimulation (i.e., collagenase).[70,71] Matrix metalloproteinases are involved in tissue remodeling during development, wound healing, and involution of organs, and in the invasion of metastatic cells across basement membranes.[6,23,72] Some matrix metalloproteinases can be located on cell membranes.[73] Gelatinase B binds to the invadopodia of transformed cells.

A metal ion, zinc, is found at the active site of metalloproteinases.[74–76] The recently reported structure for the catalytic subunit of human fibroblast collagenase bound to a small inhibitor shows the active-site zinc atom coordinated to three histidine residues of the enzyme.[63] These three histidine residues are found in the conserved Zn-binding consequences sequence, Ala-Ala-His-Glu-hydrophobic residue-Gly-His-X-X-Gly-X-X-His, present in the zinc-binding domain of the matrix metalloproteinases.[6,74] In the inhibited form, the active-site histidine is ligated to the carboxylate ion on the inhibitor.

Stromelysin 1[75] and collagenase[74] contain a second bound atom of zinc that is bound to three histidine residues and an aspartic residue in a tetrahedral manner.[63] These residues are found in the catalytic domain of the proteinases rather than in the binding domain in which the first zinc atom is located. In addition, a calcium ion is ligated to collagenase in an octahedral manner.[74] Both ions stabilize the tertiary structure of the molecules.

In the proposed catalytic mechanism of matrix metalloproteinases,[42,43] the zinc ion at the active site acts as an electrophile, assisting in the attack on the carbonyl carbon of the substrate scissile bond by the oxygen of a water molecule. An intermediate is formed through hydrogen bonding of a histidine residue to the catalytic water molecule. The water molecule is displaced from the metal ion by the carbonyl oxygen of the substrate. The scissile peptide carbonyl group is polarized by the zinc atom. A glutamic acid residue at the active site acts as a general base that catalyzes the addition of a water molecule and hydrolysis of the peptide bond.

D. Aspartate Proteinases

The major proteinases in this class in the human are cathepsins D and E, renin, and the digestive enzymes pepsin and gastricin.[77] Cathepsin D is a lysosomal proteinase found in most cells, while cathepsin E is a nonlysosomal enzyme found in specific cells in differing locations.[78–80] (Although most cathepsins are lysosomal, the term cathepsin originally was coined to refer to a nonpepsin proteinase.[69]) In addition to the lysosome, cathepsin D can be found in large clear vacuoles, small vesicles, and endosomes in rabbit alveolar macrophages.[81] Cathepsin D can be released from the cell and recovered through high mannose receptors on the same cells or adjacent cells.[82] Cathepsin E is localized in the canaliculi of gastric parietal cells, renal proximal tubule cells, and bile canaliculi, in the cytoplasmic matrix and cisternae of the rough endoplasmic reticulum, and in the dilated perinuclear envelope of gastric foveolar epithelial cells and parietal cells.[80] Pepsin in the form of pepsinogen is packaged into secretory granules in chief cells of the fundus.[83] It is secreted as the zymogen form into the stomach lumen, where it is activated under acidic pH. Renin is produced in the kidney, pituitary, and submaxillary gland.[83] It specifically cleaves angiotensinogen.

At the active site of these aspartate proteinases, a water molecule is hydrogen bonded equally to two aspartic groups (Asp 32 and Asp 215 in pepsin).[83,84] Although the catalytic mechanism of aspartic proteinases has been extensively studied, some aspects remain controversial. The first step in the reaction mechanism is polarization of the carboxyl oxygen of the peptide bond. This may be either through hydrogen bonding to the water molecule or to Asp 32.[43,83,85] The oxygen of a second water molecule probably attacks the substrate carbonyl carbon. A tetrahedral intermediate is formed and the peptide bond is cleaved. Asp 215 serves as the proton donor.

III. CONTROL OF PROTEINASE SYNTHESIS

The first level of proteinase control is through regulation of proteinase gene expression. The expression of various proteinase genes is regulated throughout prenatal and postnatal development,[78,86] aging,[87] ovarian cycling,[88] and wound healing.[89,90] Proteinase synthesis in a given tissue is altered in response to injury,[90,91] viral infection,[92] transformation,[72,93–95] and other disease conditions such as arthritis.[96,97] The mechanism by which the synthesis of various proteinases is controlled *in vivo* probably reflects the role a specific proteinase plays in a given tissue. For example, collagenase (MMP-1) and stromelysin genes are differentially regulated in synovium and cartilage.[97] In the synovium, the mRNA and protein for two enzymes are found at equal levels, while in cartilage, collagenase is significantly higher than stromelysin.[97] Similar proteinases of a given class are often differently regulated, such as the cysteine proteinases cathepsins B, L, and S,[98] the aspartic proteinases cathepsin D vs. cathepsin E,[79,80] and the serine proteinase urokinase-type plasminogen activator vs. tissue-type plasminogen activator.[37] The regulation of these enzymes also differs from one cell type to another.

Under abnormal conditions, cells can express proteinases that they do not normally synthesize or they synthesize specific proteinases at higher or lower levels than under normal conditions. Osteoarthritic cartilage, but not normal cartilage, expresses gelatinase B (MMP-9). This tissue also synthesizes elevated amounts of interstitial collagenase (MMP-1) and stromelysin.[96] This pattern of

matrix metalloproteinase expression can be induced by treatment of normal cartilage with IL-1α.[96] Elevated levels of metalloproteinase expression also are observed with rheumatoid and osteoarthritis.[96,97] Reduced amounts of gelatinase B are expressed by human papilloma virus-infected ectocervical cells when compared to the amount expressed by normal ectocervical cells.[92] Malignant transformation is often associated with increased synthesis of cathepsin D,[99,100] cathepsin L,[101] cathepsin B,[102] urokinase-type plasminogen activator,[100] and the matrix metalloproteinases collagenase, gelatinases A and B, matrilysin, and stromelysin.[72] The metastatic potential of tumor cells is correlated with increased synthesis of matrix metalloproteinases,[72,103] urokinase-type plasminogen activator,[100] and cathepsin D in breast cancer.[104] Mammary carcinomas and cysts, but not normal mammary cells, synthesize pepsinogen C.[105] This enzyme is identical to gastric pepsinogen C.

Transformed cells can induce the synthesis of proteinases by adjacent normal cells. Stromelysin-3 mRNA is expressed in normal dermal fibroblasts adjacent to malignant basal cells[106] and in stromal cells adjacent to malignant breast cells.[107] This proteinase is not expressed by these normal cells unless malignant cells are present, nor is it expressed in benign tumors.[93]

Some proteinases are constitutively synthesized by cells; for example, progelatinase A is synthesized constitutively by corneal fibroblastic type cells.[108] Other proteinases such as collagenase and stromelysin are synthesized only after stimulation of corneal fibroblastic cells.[70] Other proteinases are both constitutively synthesized via one mechanism and synthesized upon stimulation by another mechanism. For example, cathepsin D is constitutively synthesized from one mRNA transcript and induced by estrogen stimulation from a longer mRNA transcript.[109,110]

Cytokines, polypeptide hormones, steroid hormones, and other growth regulators (Table 2) influence the synthesis of proteinases by various cells through signal transduction pathways or direct binding to nuclear receptors. Many cytokines have the potential to stimulate proteinase synthesis through induction or enhancement of expression. For example, IL-1α and/or β induce the synthesis of the matrix metalloproteinases, gelatinase B, stromelysin, and collagenases,[96,111,112] and the serine proteinases, urokinase-type, and tissue-type plasminogen activators.[113,114] Some cytokines inhibit the expression of constitutively synthesized proteinases. TGF-β and retinoic acid both inhibit collagenase[115,116] and tissue-type plasminogen activator[113,117] synthesis in many cells. The effect, however, depends on the cell type. For example, in human umbilical vein endothelial cells, retinoic acid stimulates rather than inhibits the synthesis of tissue-type plasminogen activator.[118,119]

In addition, one cytokine can modulate the effects of another cytokine on the synthesis of proteinases. For example, interferon-γ reduces the stimulation of urokinase-type plasminogen activator by IL-1α in human foreskin microvascular endothelial cells.[120] Stimulation of the synthesis of stromelysin and collagenase by IL-1α is augmented by IL-6 in rheumatoid synovial fibroblasts.[121] This mechanism probably plays a role in fine-tuning the control of proteinase synthesis.

Interaction of cells with proteinases can regulate proteinase synthesis. Active plasmin and thrombin stimulates the synthesis of tissue-type plasminogen activator in human endothelial cells[122] and lung fibroblasts.[123] Extracellular matrix molecules (fibronectin, ostonectin, and E-cadherin) can modify the pattern of proteinase synthesis. When fibronectin binds to a cell membrane, the synthesis of urokinase-type plasminogen activator is induced to two to three times that

TABLE 2
Effect of Cytokines, Hormones, and Other Growth Regulators on the Synthesis of Proteinases

Proteinases	Induce/stimulate synthesis	Inhibit/decrease synthesis	No effect on synthesis
Serine proteinases			
Urokinase-type plasminogen activator	b-FGF, EGF, IF-α, IF-γ, IL-1, IL-4, LPS, CSF-1, FSH, TGF-α, TNF-β, TGF-β, retinoic acid[113,116,131,442–445]	TGF-β[116]	
Tissue-type plasminogen activator	IL-1, IL-4, TGF-α, TGF-β, retinoic acid[116,442,446,447]	TGF-β, retinoic acid[116,117]	
Cysteine proteinases			
Cathepsin B	IL-1, thyrotropin[448]		
Cathepsin L	b-FGF, EGF, PDGF[449,450]		
Matrix metalloproteinases			
Fibroblast collagenase	IL-1α, β, IF-α, -β, -γ, EGF, TGF-α, PTH, PAF, TNF-α, PDGF, EGF, PTH, LPS[9,96,112,113,451–455]	EGF, TGF-β, insulin, glucocorticoids, retinoic acid, estrogen, progesterone[6,134,456]	
Gelatinase A			IL-1α and β, estradiol, progestin[96,111,152]
Gelatinase B	IL-1, IL-2, EGF, TGF-α, TGF-β[96,111,112,457,458]	IL-4[459]	LPS[454]
Stromelysin-1	IL-1α and β, EGF, b-FGF, IF-α, -β, -γ, PDGF, TNF-α, LPS[6,96,111,121,453]	EGF, TGF-β, insulin glucocorticoids, retinoic acid, estrogen, progesterone[6,152,453]	
Stromelysin-2	TNF-α, EGF, TGF-α[460]		IL-1β[460]
Aspartic proteinase			
Cathepsin D	a-FGF, b-FGF, ILGF, insulin, estrogen[109,149,151]		

Note: Some factors can stimulate the synthesis of proteinases in some cells and inhibit the synthesis of the same proteinase in another cell.

of cells bound to vitronectin.[124] Macrophage synthesis of collagenase (MMP-1) is induced by the presence of its substrates, collagen types I and III.[125] At the same time, synthesis of gelatinase B is left unaffected. Ostonectin (SPARC), a molecule that modifies cell-extracellular matrix interactions, can alter proteinase synthesis. The addition of ostonectin to synovial fibroblast cells cultured on collagen types I, II, and V or vitronectin increases the expression of collagenase, stromelysin, and gelatinase B.[126] No increase in collagenase synthesis is observed upon the addition of ostonectin to synovial fibroblast cells cultured on type IV collagen. This indicates that the effect of ostonectin is specific. Disruption of E-cadherin-dependent cell-cell adhesion with a cadherin antibody results in stimulated urokinase-type plasminogen activator expression.[127] This indicates that E-cadherin-mediated cell-cell adhesion downregulates the expression of this proteinase.

Proteinase synthesis can be altered in normal cells by stress. Singlet oxygen upon reaction with plasma membrane lipids induces the synthesis of collagenase (MMP-1) by human fibroblasts.[128] Calcium phosphate crystals found in degenerated joints induce the synthesis of stromelysin and collagenase in human fibroblasts.[129] Mechanical injury to vascular smooth muscle cells also induces the synthesis of collagenase and stromelysin.[91]

Cytokines and hormones that bind to membrane-bound receptors initiate proteinase synthesis through signal transduction pathways. IL-1α stimulation of stromelysin and collagenase is mediated through a protein kinase C pathway.[130] Some cytokines act by stimulating the synthesis of gene transduction factors, which then bind to genes coding for proteinase genes. IL-1 and TNFα stimulate the synthesis of c-Fos, which binds with c-Jun to the AP-1 site and is essential for the induction of collagenase synthesis.[6] Follicle-stimulating hormone regulates the tissue-type plasminogen activator gene by inducing the formation of CREB dimers that interact with the CRE site, stimulating transcription.[131]

Glucocorticoids inhibit the synthesis of some proteinases and have no effect on others. Glucocorticoids inhibit the synthesis of urokinase-type plasminogen activator but have no effect on tissue-type plasminogen activator.[132] They can also inhibit EGF-stimulated synthesis of collagenase and stromelysin in human skin fibroblasts.[130] Glucocorticoid receptors act by binding to the AP-1 protein complex (c-Jun/c-Fos), thereby preventing the activation of genes through the AP-1 site such as the procollagenase gene.[134]

Like the synthesis of most proteins, the synthesis of proteinases is controlled by various regulatory elements on the gene (Table 3). The absence of a TATA box in some proteinase genes suggests that the proteinases are housekeeping genes.[110,131,135] Some proteinase genes have a CAAT box, while others do not.[110,135–138] Other regulatory sites observed on proteinase genes include the AP-1 binding site for the c-Jun-c-Fox complex; the AP-2 cAMP response element (CRE), NF-1, and SP-1, PEA-3 and the glucocorticoid response element.[131,137,139] Most genes have multiple response elements, which allows for fine tuning of the expression of the proteinases by a number of hormones, growth factors, and cytokines.

Similar proteinases (i.e., urokinase-type and tissue-type plasminogen activator[140]) often have different promoter regions so that they can be controlled independently. The promoter regions for stromelysin and stromelysin-2 differ significantly despite a 71% homology in their amino acid sequences.[141] Gelatinases A and B also have very different promoter regions.[139] Gelatinase B has a TATA box and AP-1

TABLE 3
Potential Nuclear Regulation Sites on Proteinase Genes

Proteinases	TATA	CAAT	SP-1	AP-1	AP-2	CRE	NF-1	NF-κB	GT	PEA-3	Ref.
Tissue-type plasminogen activator	None		2		1	1	1				131
Urokinase	1	1		1							131
[a]Cathepsin B (transcription site A)	None	None	2								135
Cathepsin D (transcription site 1)	None	None	5	2							136
Cathepsin D (transcription site 2)	1	1									110,138
Cathepsin L	None	1	6	2	3	1					137
Fibroblast collagenase	1			1						1	461, 462
Stromelysin-1	1			1							110
Gelatinase A	None			None	1						110
[b]Gelatinase B	1			1	1			1	1		139

Reported nuclear regulatory sites on various proteinases genes.

[a] Two additional putative start sites have been identified which contain TATA and CAAT sites.[86]
[b] Gelatinase B may have more than one transcription start site.

binding site, while gelatinase A does not have these sites.

Suppression of the synthesis of collagenase and stromelysin involves inhibition of the activation of the AP-1 site in the 5′ flanking region of the genes.[142] Two mechanisms are involved in inhibition by retinoic acid. First, retinoic acid decreases the synthesis of c-Jun and c-Fos, which bind to the AP-1 site. Second, retinoic acid-retinoic acid receptor complexes form non-AP-1 binding complexes with c-Jun. Retinoic acid receptor-α-, -β, and -γ inhibit collagenase expression induced by cytokines.[143] Retinoic acid receptor-γ also regulates basal collagenase expression.[143] TGF-β can downregulate collagenase gene expression by a mechanism in which Jun-B synthesis is induced; Jun-B is a protooncogene-negative regulator of c-Jun.[115]

Some proteinase genes have several transcription start sites. Differential regulation mechanisms determine which one is used. The cathepsin D gene has two transcription start sites, one without associated TATA and CCAT sequences that is constitutive and one with consensus TATAAA and CCAT sequences that is estrogen regulated.[109,110] Constitutive transcription can start at five major sites within a 52-bp region.[109]

There are multiple mRNAs for cathepsin B present in cells that are generated by alternative splicing. In human kidney and human hepatoma cells (HEPG2), multiple cathepsin B mRNAs are expressed that differ in the 5′ and 3′ untranslated region.[144] Human tumors produce a cathepsin B variant that lacks exon 2. Breast and colon carcinomas and melanomas produce a cathepsin B mRNA lacking both exon 2 and 3 in addition to one lacking exon 2. The rate of transcription varies with these two transcripts, suggesting that mRNA processing contributes to regulation of the synthesis of cathepsin B. Other proteinase genes that have multiple mRNAs include cathepsin L,[145] cathepsin E,[146] and calpains I and II.[147]

There are cases where a given factor induces an increase in the mRNA levels for a given proenzyme, but there is no increase in the level of synthesized protein. For example, LPS induces an increase in the synthesis of tissue plasminogen activator mRNA, but no increase in the protein is observed.[148]

Methylation of genes may play a role in the tissue-specific expression of some proteinases. Cathepsin E is expressed predominantly in the stomach, but is expressed also at low levels in the spleen. The relative expression in these tissues is correlated with the degree of hypomethylation found on CCGG and GCGC sites within the gene.[149]

Although many studies of the control of proteinase synthesis have been carried out using cultured cells or tissues in organ culture, some studies have elucidated the effects of growth factors and other modulators on the whole animal. β-Adrenergic agonists induce skeletal muscle hypertrophy in animals. This treatment increases calpain II activity and decreases calpain I activity, with concomitant changes in the mRNA levels of these molecules.[150] LPS injected in mice results in a marked decrease of urinary and renal urokinase-type plasminogen activator.[148] This is attributable to a reduction in urokinase-type plasminogen activator mRNA. Intravenous administration of a-FGF increases the level of cathepsin D in the thyroid, liver, and bone of rats.[151] Animals deficient in vitamin A have significantly reduced levels of urokinase- and tissue-type plasminogen activator.[119]

The resulting effect of induction or inhibition of proteinase synthesis must be considered relative to the induction or inhibition of synthesis of their inhibitors (see Section V.I). An increase in the steady-state level of proteinases can occur due to a decrease in the inhibitor concentration rather than an increase in synthesis of the proteinases. Estradiol and progesterin have no effect on the synthesis of gelatinase A (MMP-2).[152] In-

stead, the synthesis of the corresponding inhibitor TIMP-2 is inhibited by 50%, thereby increasing the level of gelatinase A. Synthesis of gelatinase A, stromelysin, and tissue plasminogen activator as well as that of their inhibitors, TIMP-1 and plasminogen activator inhibitor-1, is induced during involution of the mammary gland.[23] The peaks of inhibitor synthesis precede the peaks of enzyme synthesis, resulting in a situation where the inhibitor concentration is greater than the active proteinase concentration for the first few days. A few days later, active proteinase levels are greater than the inhibitor levels. In the cervix during labor, the collagenase level is 23 times higher than the level in the cervix at term, while α2 macroglobulin and TIMP increase only twofold.[153]

IV. ACTIVATION OF THE ZYMOGEN FORM OF PROTEINASES

One of the most important controls of proteolytic activity is the synthesis and storage of proteinases as inactive zymogens. Most are stored and transported in the zymogen form. Exceptions include lysosomal proteinases and some proteinases stored in specific secretory vesicles. Synthesis of proteinases in an active form serves to protect the ribosomes, golgi, and endoplasmic reticulum from degradation.[154] While a few zymogens have a small amount of activity (e.g., chymotrypsinogen[155]), most are inactive until a competent active site is exposed. This usually involves a proteolytic cleavage, followed by a conformational change exposing and/or creating an intact active site. Alternatively, a change in conformation of the proenzyme can lead to an intramolecular or intermolecular autoproteolytic cleavage, generating a fully active proteinase. Zymogen activation is a carefully regulated process. Often, several mechanisms are used to regulate zymogen activation.

A. Zymogen Stabilization

The zymogen form of matrix metalloproteinases is stabilized by a novel mechanism. The secreted matrix metalloproteinase zymogens are held in the inactive form by the interaction of the active site Zn^{2+} on the zinc-binding domain with a cysteine residue on the propeptide. This cysteine residue is located in the conserved sequence Pro-Arg-Cys-Gly-Val-Pro-Asp-Val (residues 87 to 94), which is found near the carboxyl terminal end of the N-terminal propeptide.[156] The sulfur of the cysteine residue acts as one of the coordination ligands for the zinc atom. This mechanism has been called "the cysteine switch" mechanism.[156] Site-directed mutagenesis studies support this mechanism.[157] When Cys90 of stromelysin is replaced by other zinc-ligating residues or Arg89 is replaced with Lys, the molecule expressed in COS cells becomes fragmented. When other residues near Cys90 are mutated by conservative replacements, active molecules rather than the zymogen forms are recovered.

In vitro, disruption of the cysteine-Zn^{2+} bond by either sulfhydryl reagents or proteolytic degradation can lead to activation of the matrix metalloproteinase zymogens.[6] Sulfhydryl reagents activate by first disrupting the Cys-Zn^{2+} bond, followed by a conformational change that allows for an autoproteolytic cleavage of the propeptide to yield an active product.[158] The initial conformational change is required for activation of these molecules. Modification of Cys75 in human stromelysin (Cys90 in rat) with disulfide reagents [iodoacetamide, 5,5′-dithiobis (2-nitrobenzoate) or 4-aminomercuric acetate] without a conformational change does not activate the enzyme.[159]

Posttranslational modification of zymogens can stabilize the zymogen conformation and prevent the acquisition of proteolytic activity. The presence of sialic acid

on plasminogen holds this proenzyme in the inactive form.[160] Removal of the sialic acid residues from plasminogen results in a conformation change and the appearance of amidolytic and fibrinolytic activity in the zymogen form.[160]

B. Zymogen Activation Mechanisms

Fully zymogen activation involves the cleavage of one or more peptide bonds in the N-terminal portion of the proenzymes. Upon cleavage, the N-terminal propeptide can be released, as in the case of cleavage matrix metalloproteinases,[156] or the propeptide can remain attached via disulfide bonds, as in the case of chymotrypsin.[161] *In vivo,* the major form of urokinase-type plasminogen activator released from cells is the single chain.[162] This form is inactive until cleaved to the two-chain form by plasmin[162] or kallikrein.[132]

Activation can involve a single peptide bond cleavage or a series of sequential cleavages to yield the active form of proteinases. Zymogen activation by a single cleavage is often very specific because it is carried out by only one or a few specific proteinases. Examples include activation of prothrombin by factor Xa[13] and activation of plasminogen by urokinase-type and tissue-type plasminogen activators.[37] Zymogen activation by multiple sequential cleavages often involves an initial cleavage, by proteinases with differing substrate specificities, at a number of sites within a given region of the propeptide. Stromelysin, *in vitro,* can be activated by plasmin, kallikrein, neutrophil elastase, cathepsin G, and mast cell tryptase, all of which are potential physiological activators.[163] The first cleavage yields a 46- to 53-kDa intermediate, followed by a bimolecular autocatalytic cleavage to a 45-kDa active form. Bimolecular autocatalytic cleavages are characteristic of matrix metalloproteinase activation.[164] This step is also part of the activation of matrilysin, the smallest matrix metalloproteinase (Section II.A.). The 45-kDa form of stromelysin can undergo a further intramolecular autocatalytic cleavage to a 28-kDa form that is fully active. This 28-kDa form is similar to matrilysin in that part of the hemopexin domain is cleaved.[163] Procathepsin B is also activated by multiple cleavages. This proenzyme can be cleaved by cathepsin D or L or the active form of cathepsin B.[165] This form is further processed by dipeptidylpeptidase I to yield the mature form.[165]

Some zymogens are activated by a wide variety of enzymes, while others are activated only by one or several enzymes. Progelatinase B can be activated by stromelysin, plasmin, kallikrein, thrombin, neutrophil elastase, and cathepsin G.[166] Progelatinase A is not activated by these enzymes; however, urokinase-type plasminogen activator[167] and a bacterial metalloproteinase, *Pseudomonas* elastase,[168,169] convert the proenzyme into its active form. Because gelatinase A is a housekeeping proteinase, strict control over its activation is probably required.

The processing of a proenzyme can depend upon the tissue in which it is present. Cathepsin B in cultured human hepatoma cells exists in three states: an inactive 44-kDa zymogen form, a partially active 30- to 33-kDa single-chain intermediate form, and a fully active 25- to 27-kDa mature form of two subunits of 24 and 5 kDa.[65,170] In contrast, cathepsin B in sputum has two active forms, a 40- and a 37-kDa form.[171] The 37-kDa form is generated by neutrophil elastase cleavage and has five times the activity of the 40-kDa form.

A novel means of zymogen activation is the oxidation of the propeptide cysteine residue of some matrix metalloproteinases. This is the cysteine residue of the "cysteine switch".[156] Neutrophil procollagenase is activated by hydrogen peroxide or hypochlor-

ous acid.[172] Hydrogen peroxide activation is inhibited by mannitol and desferoxamine. This suggests the involvement of a Fenton-type reaction that can generate hydroxy radicals.[173] Additional evidence for this oxidation mechanism of zymogen activation is observed in cultures of Walker 256 cells.[174] These cells release progelatinase B and hydrogen peroxide with the generation of active gelatinase B. Catalase inhibits the appearance of the active form of this enzyme, which supports the activation of progelatinase B by hydrogen peroxide.[174] In contrast to neutrophil collagenase, fibroblast procollagenase is poorly activated by this mechanism.[173] Whether matrix metalloproteinase activation by oxidation occurs extensively *in vivo* is not known.[172,175]

C. Zymogen Activation Control

A variety of mechanisms are used for the control of zymogen activation. These include the involvement of protein inhibitors, glycosaminoglycan, and pH, localization of the activation reaction to membranes, extracellular matrices, or blood clots, or the use of an amplifying cascade of proteolytic activations (Table 4). Several mechanisms are usually involved in control of the activation of most zymogens.

1. Inhibitors

Zymogen activation is controlled by the availability of specific active proteinases. These mechanisms for controlling active proteinases are given in Section V. They are important in controlling the generation of additional active proteinases. An unusual mechanism for controlling zymogen activation is the interaction of tissue inhibitors of metalloproteinases-1 and -2 (TIMP-1 and -2) with the zymogen forms of gelatinases A and B.[72,158,176] This mechanism is not used for other matrix metalloproteinases. TIMP-2 binds to progelatinase A and TIMP-1 binds to progelatinase B. These complexes are found in conditioned media from cultured cells that produce both the proenzymes and their corresponding inhibitors.[176] The zymogens bind their respective inhibitors at the carboxyl terminal end in the hemopexin-like domain rather than at the active site.[72,176] TIMP-1 cannot substitute for TIMP-2 on progelatinase A and TIMP-2 cannot substitute for TIMP-1 on progelatinase B.[176] The presence of the inhibitor stabilizes the proenzyme forms of these two zymogens. When bound to TIMP-1, progelatinase B cannot be activated by stromelysin, form dimers with itself or form a heterodimer with interstitial procollagenase.[176]

TABLE 4
Control of Zymogen Activation

Activation type	Inhibition mechanism	Stimulation mechanism
Autolysis	Protein inhibitors	pH, membrane binding
Bimolecular	Protein inhibitors	Glycosaminoglycans, membrane binding, extracellular matrix binding, calcium
Oxidation	Inflammatory control antioxidants	Neutrophil activation, ischemia

2. Glycosaminoglycans

In some cases, glycosaminoglycans can stimulate zymogen activation. The k_{cat} for plasminogen activation by urokinase-type plasminogen activator is three- to fivefold greater in the presence of the glycosaminoglycans heparin, heparin sulfate, and chondroitin 6-sulfate.[177] This stimulation of activation is inhibited by the lipoproteins lipoprotein (a) and low-density lipoprotein.[177] Heparin, when bound to the C-terminal hemopexin region of progelatinase A (72-kDa gelatinase), increases autocatalytic activation eightfold.[178]

3. pH

A conformational change in some zymogens leads to the exposure of a competent active site. When aspartic proteinase zymogens are exposed to low pH, they undergo a conformational change that leads to their activation. Procathepsin E is converted to the active enzyme at pH 3.5 by a one-step autocatalytic process.[179] In contrast, pepsinogen and procathepsin D undergo multiple-step activation processes. Upon acidification, pepsinogen and procathepsin D undergo an autocatalytic cleavage to pseudopepsin and pseudocathepsin D, respectively.[180] Pseudopepsin is an active intermediate that catalyzes intermolecular cleavages that yield pepsin.[71] Pseudocathepsin D is converted to cathepsin D by a cysteine proteinase.[181,182] Cathepsin B can also be activated by a unimolecular autocatalytic mechanism *in vitro*.[183]

4. Localization

Binding of zymogens and active enzymes to membranes, extracellular matrix, or blood clots, or the inclusion of these molecules in lysosomes can localize zymogen activation to the site where the activated enzymes are required. Binding of proenzymes to membranes enhances both autocatalytic activation and activation of zymogens active proteinases. This can be through binding of the zymogens to membrane phospholipids or specific receptors. Inhibitor-free progelatinase A (but not gelatinase B) binds to membranes via the C-terminal hemopexin domain of the proenzyme.[184,185] This binding facilitates progelatinase A activation, most likely by an autocatalytic mechanism.[185]

Urokinase-type plasminogen activator binds to specific receptors on cell membranes. When bound to these receptors, urokinase-type plasminogen activator activation of plasminogen is dramatically enhanced.[186] The K_m of the reaction is decreased twofold, and the k_{cat} is increased sixfold.[37] Activity is enhanced further upon cleavage of the bound single-chain urokinase-type plasminogen activator to the two-chained form by various proteinases, including cathepsin B.[132,162] Plasminogen and urokinase-type plasminogen activator receptors are present at high densities in the same area on the cell surface.[37] This localizes the activation of plasminogen to the cell membrane at the sites of cell-cell and cell-substratum contact sites.[186] Metastasizing cells utilize this membrane-associated plasminogen activation mechanism as an invasion mechanism.[186]

Cell-surface plasminogen activation is regulated by plasminogen activator inhibitors-1 and -2. These inhibitors bind to the receptor-urokinase-type plasminogen activator complex inhibiting enzymatic activity.[186] This trimolecular aggregate induces internalization and degradation of urokinase-type plasminogen activator.[186] A second method of controlling plasminogen activation is cleavage of the urokinase-type plasminogen receptor by active urokinase-type plasmino-

gen activator and plasmin, yielding a truncated molecule. The first of three homologous domains is lost, and the receptor no longer has the ability to localize plasminogen activation.[186]

During blood clot formation, plasminogen is trapped within the fibrin matrix. Tissue-type plasminogen activator is released from endothelial cells at the site of injury and binds with high affinity to the fibrin clot.[187] When tissue-type plasminogen activator is bound to the fibrin clot, its activity toward plasminogen is dramatically enhanced. This reaction also is enhanced by the cleavage of native Glu-plasminogen to Lys-plasminogen. This cleaved form of the proenzyme binds to fibrin with a higher affinity and is more readily activated to plasmin by urokinase-type and tissue-type plasminogen activator.[37]

Zymogens and active proteinases can specifically bind to structural extracellular matrix molecules. Plasminogen and tissue-type plasminogen activator bind to specific sites on fibronectin and laminin.[188] Plasminogen and tissue-type plasminogen activator can also bind to type IV collagen in basement membranes.[189] Urokinase-type plasminogen activator binds to vitronectin in focal areas in the vicinity of the secreting cell.[124] Adhesion of cells to vitronectin results in the clustering of urokinase-type plasminogen activator receptors in the adhesion area. Transglutaminases can cross-link plasminogen to endothelial surfaces and to fibronectin, forming high molecular weight complexes.[190]

Proteinase activation can also be localized by compartmentalization. This occurs in the activation of lysosomal enzymes. Procathepsin D is processed from the 51- to 55-kDa species to a 44-kDa pseudocathepsin D intermediate in the endosomes.[191] This intermediate is further processed to the mature 31-kDa species in the early lysosomes. Some secretory vesicles contain both zymogens and active proteinases. In juxtaglomerular cells of the kidney, prorenin is either sorted to a nonregulated constitutive pathway for secretion as the proenzyme or into immature secretory granules for processing to the active form prior to secretion.[192] Both prorenin and cathepsin B are present in these cells. Cathepsin B processes prorenin to renin.

5. Calcium

The activation of procalpains is unusual because of the requirement of calcium. Upon binding of calcium ions, procalpain I is translocated from the cytosol to the plasma membrane and/or the granule membrane.[55,193] In the presence of Ca^{2+} and phosphatidylinositol-bis-phosphate, the membrane-bound calpain is activated. In the platelet, 160 n*M* Ca^{2+} is required for half-maximal activation of calpain I.[194] Thrombin stimulation of platelets increases calcium levels and activates calpain I.[195] The magnitude of the initial transient increase in calcium is important for calpain I activation.

Cleavage of the propeptides from calpains I and II results in the first active forms (heterodimers of 80- and 28-kDa subunits). These calpains are autocatalytically activated by cleavage of the 80-kDa large subunits to 76 kDa for calpain I and 78 kDa for calpain II and the 28-kDa small subunit to an 18-kDa product.[194] The kinetic constants of activation are increased in the presence of phospholipids such as phosphatidylinositol, phosphatidylserine, and phosphatidylcholine.[196] These phospholipids bind to the hydrophobic region of the small subunit. The second cleavage reaction reduces the calcium requirements from 7.1 to 0.6 μ*M* Ca^{2+} for calpain I and from 1000 to 180 μ*M* Ca^{2+} for calpain II for half-maximal activity.[193]

Rat skeletal muscle contains a natural calpain II activator of 35 to 45 kDa. This

protein can bind to the 80-kDa subunit in a 1:1 complex and reduce the Ca^{2+} requirement 25-fold for the autolysis reaction.[197] Even when the procalpain II is bound to the membrane surface this activator is effective.

6. Cascade Mechanism

A cascade system of zymogen activation is a means by which an initial proteolytic cleavage can be amplified.[13,16] By this mechanism, zymogens are sequentially cleaved to active proteinases. Each sequential activation step yields an exponential increase in the number of activated proteinase molecules. This type of system is used for the activation of a key proteinase in a critical reaction, such as the generation of thrombin for fibrinogen cleavage in the coagulation pathway. This coagulation cascade is also controlled by the affinity of the proenzymes for the activated platelet membrane.[13]

The coagulation pathway is initiated by several pathways. Release of kallikrein and kininogen, the cofactor for factor XII activation, from a wound site initiates the intrinsic pathway. Sequential activation of factors XII, XI, IX, X, and prothrombin then occurs. Release of tissue factor bound to phospholipids initiates the extrinsic pathway.[198] Sequential activation of factors VII, X, and prothrombin then occurs. Factor VII, IX, X, prothrombin, and protein C are localized to the activated platelet surface through their N-terminal Gla residues (glutamic acid residues containing a second γ-carboxyl group).[13] (The Gla residues are formed posttranscriptionally by a vitamin K-dependent pathway.) The Gla residues bind to calcium ions that are also bound to phospholipids on the activated platelet surface. Bound to the platelet surface through the Gla residues, the proenzyme is held in the proper conformation for efficient activation by the corresponding activating enzyme.

V. CONTROL OF THE REACTIVE FORM

Active proteinases are carefully regulated. These enzymes can be modified by phosphorylation and glycosylation, stored in vesicles, and/or localized on membranes. Their activity can be controlled by pH, calcium ions, ATP, complex formation, and reaction with inhibitors (Figure 3).

A. Posttranslational Modifications

Posttranslational modifications can influence the activity of proteinases. The most common posttranslational modification is proteolytic processing, as given in Section III. Phosphorylation and glycosylation of some proteinases can also alter their activities. In some cells, urokinase-type plasminogen activator is phosphorylated on at least two serine residues.[199] This form of urokinase-type plasminogen activator is much less sensitive to plasminogen activator inhibitor-1 even though the K_m and k_{cat} for plasminogen activation is the same. A fourfold higher concentration of plasminogen activator inhibitor 1 is needed to achieve 50% inhibition of the phosphorylated form. Full inhibition is not observed at 20 n*M* for the phosphorylated form, while complete inhibition of the nonphosphorylated form is observed at 1 n*M*.

Posttranslational addition of carbohydrate groups to proteinases can target these molecules to vesicles and lysosomes (Section V.B), regulate the half-life of these enzymes (Section VI), or modify their activities. Tissue plasminogen activator is glycosylated at either two (type II) or three (type I) asparagine residues.[200] Each type has a subpopulation of cell-specific glycoforms. These forms differ in affinity for fibrin and in the rate of fibrin-dependent plasminogen activation. The k_{cat} of plasminogen activation in the presence of a fibrinogen fragment is five-

fold greater for type II tissue-type plasminogen activator than for the type I tissue-type plasminogen activator. Type II tissue-type plasminogen activator is about 25% more active than type I tissue-type plasminogen activator.

B. Vesicle Sequestration

Zymogens and active proteinases can be sequestered in vesicles or localized on membranes. Sequestration of active proteinases is a major means of storing these enzymes intracellularly (in lysosomes and secretory granules) and extracellularly (in vesicles and bound to extracellular matrix molecules) until needed. Enzymatic activity inside the vesicles can be controlled by pH and the presence of proteoglycans.[201,202] This sequestration mechanism protects both cytoplasmic and extracellular matrix proteins from degradation. Release of the proteinases from vesicles is carefully regulated.[41,203] Often, this involves the fusion of a vesicle with another membrane compartment such as a phagosome or another cell. This localizes proteolytic reactions onto membranes or in degradation vesicles.

Lysosomes are acidic cytoplasmic vacuoles that contain proteinases; these include the aspartic proteinase cathepsin D and the cysteine proteinases B, H, L, and S. There are several pathways by which these proteinases can reach lysosomes. The best studied are those involving mannose 6-phosphate receptors to direct acid hydrolases to the lysosomes.[82,204,205] Two receptors bind mannose 6-containing proteins: the cation-independent mannose 6-phosphate receptor/insulin-like growth factor II receptor and the cation-dependent mannose 6-phosphate receptor. Lysosomal enzymes synthesized on membrane-bound polysomes are cotranslationally glycosylated in the rough endoplasmic reticulum at specific asparagine residues. Glycosylation is directed by the primary sequence of the enzymes.

Cathepsin D, a lysosomal aspartic proteinase, has a conformation-dependent recognition domain that contains two sorting sequences for lysosomal targeting.[206] When this domain is engineered into pepsin, a secretory aspartic proteinase, the modified pepsin is found in lysosome rather than in secretory granules. From the endoplasmic reticulum, the lysosomal zymogens are transferred to the early Golgi compartment.[204] Here, mannose 6-phosphate is added to proteinases that are targeted for lysosomes. Mannose 6-phosphate-modified proteins are bound to specific mannose 6-phosphate receptors in the trans-Golgi network. These receptor-enzyme complexes are packaged selectively into coated vesicles and are transported to the prelysosomal acidified compartment where the enzymes dissociate from the receptors. The enzymes are then transported in vesicles to lysosomes. Lysosomal enzymes are processed from the zymogen form to an intermediate form during the transport process. The final cleavage that generates the active form can occur during the transport process or within the lysosomes (Section IV.C.4).

Lysosomal enzymes such as cathepsin D also may be targeted to lysosomes by a mannose 6-phosphate receptor-independent mechanism.[207] This pathway has not been elucidated. It is known that the propeptide of cathepsin D is not sufficient to target the proenzyme to the lysosome by this mechanism.[208]

Lysosomal enzymes can be secreted from cells either from lysosomes or directly without prior incorporation into lysosomes. Cathepsin L, which is normally a lysosomal enzyme, is secreted from malignant cells and from normal cells after stimulation with growth factors or tumor promoters.[209–211] PDGF specifically stimulates the secretion of cathepsin L by a mechanism that is independent of synthesis.[209]

Direct secretion of cathepsin B is associated with metastasis of tumor cells and hormone stimulation of cells. Upon stimulation of exocrine pancreatic cells or rat duodenum with the cholecystokinin analog caerulein or untraditional liquid meals in rats, mature cathepsin B is secreted via the apical regulated exocytotic pathway.[211,212] Pancreatic duct obstruction increases secretion of cathepsin B.[212] Cathepsin B is also secreted by articular chondrocytes.[213] Glycosaminoglycans can inhibit the secretion of cathepsin B from these cells. Stimulation of calcium uptake in chick intestine by 1,25-dihydroxy vitamin D3 redistributes cathepsin B from the lysosomes to the basal and lateral membrane areas of epithelial cells and to the villus core within 3 min.[214]

Released lysosomal enzymes can then bind to mannose-6-phosphate receptors on cell membranes and be transported to the lysosomes by an endocytic mechanism.[82] This process can be modified by cytokines such as PDGF that causes a time-dependent increase in mannose 6-phosphate receptor-mediated endocytosis.

Nonlysosomal secretory proteinases are also cotranslationally glycosylated (but not with mannose-6-phosphate) at selected asparagine residues and transported to the Golgi apparatus.[47,204] Here, they are processed and then packaged into vesicles in the trans-Golgi network. Some secretory vesicles contain only the zymogen form of proteinases, as observed for pepsinogen in the stomach and for trypsinogen, chymotrypsinogen, and proelastase in pancreatic acinar cells.[7] These molecules are released in the zymogen form. Other secretory vesicles contain both proenzymes and active enzymes. In these vesicles, the zymogens are processed to active enzymes in the vesicles and are stored as the active enzymes. This processing is dependent on an ATP proton pump that regulates the pH of the granule and thus the proteolytic activity of the proteinases.[47] This mechanism is observed for the proteinases present in the granules in mast cells, eosinophils, neutrophils, monocytes/macrophages, natural killer cells, and tumor and leukemia cells. Active proteinases are present at high concentrations in the presence of proteoglycans, which stabilize these molecules (Section V.H).

Proteinase-containing granules can also be found in the extracellular matrix. Chondrocytes, which mineralize their matrix, release matrix vesicles to form hydroxyapatite crystals and initiate mineralization.[215] Within these vesicles are proteinases that mediate the loss of extracellular matrix proteoglycans, including metalloproteinases and plasminogen activator.

Not only do vesicles serve as a means of protecting cellular proteins during the storage of proteinases, they can also serve to isolate proteolytic reactions involved in protein processing and proteolytic degradation of proteins. Examples including prohormone[47] and proenzyme processing (Section IV.C.4) in immature secretory vesicles and digestion of cellular, membranous, and phagocytosed proteins in vesicles.[203] These prohormones are processed during the maturation process of secretory vesicles by furin and other proprotein conversion endopeptidase.[47] Prohormones, such as proinsulin, are mainly found in immature clathrin-coated granules, while mature hormones after processing are found in mature uncoated granules.[47]

Controlled degradation of cytoplasmic proteins occurs intracellularly through the formation of autophagic vacuoles (cytoplasmic and organelle proteins), endocytic vesicles, (membrane/receptor-bound proteins) and phagocytic vesicles (extracellular matrix proteins and microorganisms).[203] Maturation of autophagic vacuoles requires the acquisition of lysosomal membrane proteins. The vacuoles fuse with prelysosomes that are deficient in hydrolytic enzymes. They

are acidified and then fuse with lysosomes or Golgi apparatus-derived vesicles to acquire hydrolases.[203]

Cells that carry out specialized functions often contain proteinase granules (i.e., neutrophils, macrophages and natural killer cells). These enzymes can be released into membrane-bound vesicles, where proteolytic degradation occurs.

The neutrophil has three types of granules, azurophilic, specific, and tertiary, that contain proteinases.[216] Upon phagocytosis of microorganisms, these proteinase-containing granules fuse with the phagosome, forming a phagolysosome. The enzymes are active under the pH conditions of the phagosome. Neutrophil proteinases can be released by several other mechanisms into the extracellular matrix. This can occur in response to "frustrated phagocytosis", where the cell attempts to phagocytose an object that is too large. The neutrophil binds tightly to the extracellular matrix, thereby localizing the reaction to a target area. Neutrophils can also release stored proteinases by receptor-mediated stimulation. N-formylated peptides released from bacteria (i.e., formylated Met-Leu-Phe), complement C5A, leukotriene B_4, and protein degradation products (i.e., plasminogen fibronectin-derived peptides and fibrinopeptides A and B) bind to receptors on neutrophils and induce secretion.[217]

Natural killer cells and cytotoxic lymphocytes use vesicle membranes and the plasma membrane of the target cell to restrict proteolysis to the target cells.[39,40] The secretory granules of natural killer cells and cytotoxic lymphocytes are called granulosomes because they have properties of both lysosomes and secretion granules. Granulosomes have an acidic pH, mannose 6-phosphate receptors, proteoglycans, proteinases called granzymes (Section II.A), and other hydrolytic enzymes. These granules receive molecules through mannose 6-phosphate-dependent and -independent mechanisms.[218] These cells release their enzymes directly into target cells by a carefully regulated mechanism. Natural killer cells bind to target cells through surface receptors. Once bound to the target cells, the secretory vesicles bind through CD3 molecules to T cell receptors.[39] Perforin and granzymes are delivered to the target cells. Perforin can perforate membranes and form transmembrane pores through which granulosome enzymes can enter the target cell.

C. Membrane Localization

Active proteinases can be localized to membrane surfaces by binding via calcium ions to phospholipids (Section IV.C.6) or to receptors (Section IV.C.4). Lysosomal proteinases can bind to mannose 6-phosphate receptors on cell membranes and mediate surface proteolytic reactions as well as undergo endocytosis (Section V.B). When bound to membranes, lysosomal proteinase activity towards pericellular and extracellular proteoglycans is enhanced.[219] Neutrophil proteinases, cathepsin G, and elastase bind to neutrophil membranes, assisting in the invasion of these cells into tissues.[220] Bound to the membranes, these enzymes are partially protected from inhibition. Urokinase binds to urokinase receptors on cell membranes forming an active complex.[186]

Active proteinases such a signal peptidases can be integrated into membranes. These proteinases are localized in the endoplasmic reticulum membrane and the mitochondrial inner membrane, where they cleave the signal peptide of the newly synthesized proteins.[34] Other proteinases associated with membranes include the aspartic proteinase, cathepsin E[221] and the metalloproteinases meprin (rodent) and PABA peptidase (human).[69]

D. pH Effects

Proteinase activity can be altered by a change in pH. These changes can be reversible or irreversible. Many enzymes have simple or modified bell-shaped curves relating activity to pH. The effect of pH is due to the pK_a values of critical amino acid residues. Serine proteinase activity is controlled by the protonation state of the histidine residue in the catalytic triad Ser-His-Asp.[42] The deprotonation of a lysine residue is believed to be important in the loss of activity of these proteinases at high pH. The activity of an enzyme can be influenced by multiple residues with differing pK_a values. Some of these may be due to residues that bind specific substrate residues as well as catalytic residues. Three pK_a values (5.4, 6.1, and 9.5) control the activity of stromelysin, resulting in a bell-shaped curve with shoulders.[222] Seven pK_a values have been identified for cathepsin B, from pH 3.6 to 8.6. The pK_as were determined using substrates with Arg in the P_1 site and either Arg or Phe in the P_2 site.[223,224] Based on the three-dimensional structure of cathepsin B, residues were assigned to these pK_a values. The active-site residues Cys29 and His199 are responsible for the pK values at pH 3.6 and 8.6, respectively.[202] The ionization of the group with a pK_a of 5.1, Glu245, increases the activity of the enzyme eightfold toward substrates with an Arg in the P_2 site, while the activity toward substrates with a Phe in that site is not affected. Two additional binding-site groups with pK_a values of 6.9 and 7.7 (His 110 and His 111) are involved with the binding of the leaving group. The pK_as of 4.9 and 5.3 are involved with the binding of Arg at the P_1 site and are either Asp22 or Asp69, and Glu171 or Glu122.

The specificity of some enzymes such as cathepsin E is controlled by pH. At higher pH values, 7.4 and above, cleavage by cathepsin E is much more specific than at pH 3.0 and 5.5.[225] Cathepsin B possesses pH-dependent dipeptidylcarboxypeptidase and endopeptidase activities. The pH optimum for the dipeptidylcarboxypeptidase activity is lower (pH 4 to 6) than the endopeptidase activity (pH 6).[226] Elastase and cathepsin G in azurophilic granules are inactive because of the low pH of these granules.[216] Upon release into the neutral pH environment of the extracellular matrix, these enzymes become active. Some proteinases are irreversibly denaturated by an adverse pH environment. Pepsin is irreversibly denatured at pH 7 and above.[83] Cathepsin B in the low molecular weight mature form is inactivated under alkaline conditions.[165]

Cells can create an acidic environment to provide the protons required for activity. The pH of granules and lysosomes is controlled by ATP-dependent proton pumps.[47] Murine B16F10 melanoma cells catalyze the hydrolysis of substrate incorporated into the substratum region.[227] Aspartic and metalloproteinase activities are observed in this region, where the pH is 5.5.

E. Calcium Regulation

Many proteinases have a requirement for calcium for membrane localization, enzyme stability, or the expression of activity. Calcium is required for the binding of coagulation enzyme precursors to membranes via N-terminal γ-glutamic acid residues on the prepeptides.[13,228] Calcium is needed also for zymogen activation of matrix metalloproteinases.[229] Matrix metalloproteinases have calcium bound to specific sites on the surface of the molecules. The X-ray structure of the catalytic domain of fibroblast collagenase shows that one calcium is bound outside the active site.[74] Matrix metalloproteinases,[74,76] and serine proteinases[230] re-

quire calcium for stability. Calpains are a major class of enzymes that requires calcium for activity.[54–56] Furin, and the prohormone convertases PC-1 and -2 are activated by micromolar levels of calcium.[231]

Calpains are a subset of cysteine proteinases whose activity is regulated by the level of calcium available[54–56] and the presence of phospholipids. In the absence of Ca^{2+}, the active site of the mature form of either calpain I or II is unavailable. Addition of Ca^{2+} (0.6 μ*M* calpain 1 and 180 μ*M* calpain II[193]) induces a conformational change, exposing the active site of the calpains. The calcium requirement can be further modified by cellular components. In the nucleus, formation of complexes between calpain II, DNA, Ca^{2+}, and nuclear protein substrates decreases the calcium required for calpain cleavage from 1 m*M* to 3 μ*M*.[232] Isovaleryl carnitine is an intermediate in the degradation of leucine and acts as a specific activator of muscle calpain II.[233] This activator also decreases calpain II's Ca^{2+} requirement.

Binding of ATP to the P_2 receptor activates a phosphatidylinositol signal transduction pathway.[2] This pathway increases intracellular calcium levels and activates calpains. Physiological conditions known to increase calcium levels in cells resulting in the activation of calpains include ischemia and reperfusion.[234,235]

F. ATP Regulation

ATP regulates proteolysis by a number of mechanisms. It can interact directly with proteinases (cathepsin E) and phosphorylate proteinases (Section V.A). It is required for ubiquination of proteins (Section VIII.C) and the assembly of the 26 S proteasome (Section V.G). ATP stabilizes cathepsin E at pH 5.8 and above.[236] This stabilization is not dependent on hydrolysis because the nonhydrolyzable methylene-ATP analog is also able to stabilize the activity.[236]

G. Complex Formation

Proteinases can be activated, stabilized, or inhibited by forming homo- or heteroproteinase multimer complexes[237] or by forming complexes with proteoglycans (Section V.H), specific inhibitors (Section V.I), or other proteins. Dimerization provides a way to stabilize proteinases against inactivation. Procathepsin E and its activated form, cathepsin E, are dimers with the monomers joined by a disulfide bridge.[237] Activated cathepsin E in the dimer form is stable at alkaline pH, while the monomer form is unstable, leading to inactivation.

High molecular weight cathespin B, is a non-covalent complex between mature cathespin B and its propeptide found in sputum of purulent bronchiectasis and culture medium from human mammary tumor explants.[238] In contrast to free mature cathepsin B, this form is stable at alkaline pH. It is proposed this is a means of protecting the enzyme until regional acidification activates the enzyme.

Proteasomes are found in most cells and composed of proteinases and other regulatory molecules.[3,35,239,240] There are two types of proteasomes: the 20 S (multicatalytic proteinase complex) and 26 S proteasome. The associated proteinases are active only when present in the proteasome complexes. Proteasomes are located in the nuclear matrix, nucleoli, and cytoplasmic matrix and are associated with the endoplasmic reticulum.[241] The proteasome distribution among these locations depends upon cell type and stage of the cell cycle. Rapidly proliferating cells contain more proteasomes than resting cells.[242,243] In rapidly proliferating cells, most of the proteasomes are found in the nuclei.[3]

In PtK2 cells and L132 cells, the number of nuclear proteasomes increases with progression from early S phase to G1.[243] Cytoplasmic proteasomes are localized in the perinuclear region at the start of S phase and move to the periphery as the cell moves to G2 phase.

The 20 S proteasome has a molecular weight of ~720 kDa and is composed of more than 20 different homologous polypeptides ranging from 20 to 36 kDa.[244] In addition, one or more small RNAs (70 to 90 nucleotides) can associate with the complexes.[245] The polypeptide subunits are arranged in a hollow, cylindrical structure composed of four rows with a pseudo helical arrangement containing six to seven subunits per row.[246] This complex possesses numerous independent proteinases, two chymotryptic-like and one tryptic-like proteinases, two peptidyl glutamylpeptidases, a caseinolytic enzyme with specificity for cleavage between branch-chained amino acids and small amino acids, and a proteinase that cleaves between two small amino acid residues.[246,247] The amino acid sequences of the polypeptides present are homologous with each other but not to other known proteins.[3] Candidate aspartic acid and serine residues have been identified as possible active-site residues for the proteinases inhibitable by serine proteinase inhibitors.[35] However, no histidine residues has been identified as a candidate for the third member of the traditional serine proteinase catalytic triad. The complex does not generate substrate degradation intermediates. This suggests that proteasome proteinases act in concert to degrade proteins.[248]

Proteasome subunit synthesis is regulated independently. The relative proteolytic activities of the 20 S proteasome change during development in a tissue-specific manner.[249] The pattern of proteasome proteolytic activities differs in the brain, liver, and muscle. Interferon-γ and 1,25-$(OH)_2$ vitamin D_3 regulate the synthesis of several components of the proteasome.[242,250]

The activity of the 20 S proteasome is controlled using multiple means. The components present in the proteasome probably determine the function of the individual complex. Distinct forms result from the presence or absence of the major histocompatibility complex molecules LMP 2 and 7.[251,252] LMP 7 modifies the specificity of the complex by increasing its cleavage of substrates containing hydrophobic, basic, or asparagine residues.[252] Proteasome subunits that contain the nuclear translocation consensus sequence X-X-K-K(R)-X-K(R) and a potential tyrosine phosphorylation site probably regulate nuclear translocation of a population of proteasomes.[253]

Latent and active forms of the 20 S proteasome can be isolated. The latent form can be activated by proteolytic cleavage of a 32-kDa "latency" subunit.[254] Rat liver, bovine heart, and red blood cells contain an 180-kDa protein composed of 28-kDa subunits. This protein binds to the proteasome and serves as an activator of the peptidase activities but not the cleavage of large proteins.[255] C-terminal cleavage by a carboxypeptidase inactivates this activator.[256]

Mg^{2+} stimulates caseinolytic and peptidylglutamyl peptidase activities of the multicatalytic proteinase complex while inhibiting the trypsin-like and chymotrypsin-like activities.[256] Cardiolipin (diphosphatidylglycerol) activates the peptidase and proteolytic activities of the proteosome.[257] Substrates also can activate the 20 S complex. Positive cooperativity is observed with a substrate for the peptidylglutamyl-peptide hydrolase.[246] Phosphorylation may also be involved in the regulation of this complex.[258] Casein kinase II copurifies with the 20 S proteasome. This enzyme phosphorylates a 30-kDa proteasome subunit.

Noncompetitive inhibitors have been identified that control the active form of the

20 S proteasome. These include a 250-kDa hexamer composed of 40-kDa subunits,[35] a 90 kDa protein identical to heat shock 90[259] and a 200-kDa tetramer composed of 50-kDa subunits.[35]

The 26 S proteasome complex preferentially degrades proteins that have been modified by the addition of multiubiquitin molecules.[254,260] (See Section VII.C) This proteasome also can degrade nonubiquinated proteins, including ornithine decarboxylase.[261] There are probably several forms of the 26 S proteasome, all composed of similar subunits.[262,263] It is generally accepted that the 20 S proteasome is the core of at least most of the 26 S proteasomes.[3,35] However, some reports dispute the presence of the 20 S proteasome as the core of the 26 S proteasome.[264]

In addition to the components of the 20 S proteasome, the 26 S proteasome contains about ten additional 40- to 62-kDa subunits, two 100-kDa subunits, and two 110-kDa subunits.[35] The 26 S complex, when visualized by electron microscopy, has the shape of a dumbbell, where the 20 S proteasome is the central portion with two large structures attached to both ends.[265,266] The outer portions, the 100- and 110-kDa subunits, bind ubiquitin-conjugated proteins. Assembly of the 26 S proteasome requires Mg^{2+} ATP and ATPase activity.[267,268] KEKE sequences (alternating lysine and glutamic acid residues) are found on the 26 S proteasome subunits which may promote associated between subunits.[269] This proteasome possesses a 51-kDa ATPase[268] and an ubiquitin C-terminal hydrolase.[4] The proteolytic activity of the 26 S complex is ATP dependent, in contrast to the 20 S complex. A 250-kDa inhibitor, which is a component of the 26 S complex but not the 20 S complex functions, is responsible for the ATP requirement.[270] This is identical to δ-aminolevulinic acid dehydratase.[271]

The properties of the proteasomes can be altered by interferon-γ. This cytokine increases the mRNAs for LMP 2 and 7 and down regulates the expression of the homologous subunits X and Y.[272] The peptidase activities of the 26 S proteasome, but not the ATP-ubiquitin dependent activities, are altered by interferon γ.[273,274]

H. Proteoglycans Control

Proteoglycans are important in modifying the activities of proteinases. The activation of protein C by thrombin is controlled through the binding of protein C and thrombin to thrombomodulin. One form of thrombomodulin is a chondroitin sulfate proteoglycan and is present on the surface of endothelial, smooth muscle, trophoblasts, and tumor cells.[275,276] The binding of thrombin to thrombomodulin inhibits the ability of thrombin to cleave large molecules such as the coagulation factors. Thrombin, when bound to thrombomodulin, efficiently activates proprotein C. Thrombomodulin-bound thrombin is not inhibited by antithrombin III. Both the glycosaminoglycan chain and the protein portion of thrombomodulin is required for direct anticoagulant activity and antithrombin-dependent anticoagulant activity.[275] Protein C activation does not require the glycosaminoglycan portion of thrombomodulin.

Secretory granules of mast cells, eosinophils, neutrophils, monocytes/macrophages, natural killer cells, and several types of tumor and leukemia cells contain heparin and/or chondroitin sulfate proteoglycans in addition to the proteinases.[201,277–279] Proteinases, which have high isoelectric points, are bound by electrostatic interaction to the negatively charged glycosaminoglycan portion of proteoglycans both inside the secretory granules at pH 5.5 and outside the cell. The

negative-charge density is critical in this interaction rather than the carbohydrate composition.[219] These proteoglycan-proteinase complexes in secretory granules are inactive. In contrast, the complex between lung tryptase (as a tetramer) and heparin is active.[280] Heparin is required to retain the tetrameric structure and the activity of the enzyme. Proteoglycan complexes, from bone marrow-derived mast cells, composed of an ~200-kDa chondroitin sulfate E proteoglycan, an ~200-kDa chondroitin sulfate proteoglycan, and proteinase molecules also are active.[201] The proteoglycans decrease the ability of the proteinases to degrade large molecular weight substrates and alter the specificity of cleavage by the proteinases.

I. Protein Inhibitor Control

Proteinase inhibitors are found in cells, the extracellular matrix, blood, and secreted fluids. Many inhibitors are competitive inhibitors that are specific for a given class of proteinases. These inhibitors bind tightly to proteinases to form covalent complexes.[281] The formation of some complexes is reversible. In other complexes, the inhibitors are cleaved by the proteinases. The cleaved inhibitors often are released very slowly from proteinases. The simplified reaction equation is the following: $E + I \rightleftharpoons E \cdot I \rightarrow E + I^*$. An exception is α2-macroglobulin, which inhibits the activity of most proteinases toward protein substrates. This involves a trapping mechanism rather than a competitive mechanism[282] (Section V.I.4).

Inhibitor levels as well as proteinase levels are controlled at the levels of transcription, translation, and degradation. In some cases, the synthesis of a proteinase and its inhibitor is controlled coordinately. Synthesis of tissue inhibitors of metalloproteinases and many of the matrix metalloproteinases are coordinately controlled.[283] In other cases, the inhibitor and its target enzyme are controlled in opposite manners.[121] Some inhibitor-proteinase complexes bind to specific cell receptors, where they are endocytosed and degraded.[282,284–286] Proteinase inhibitors also are degraded by proteinases.[287–289]

1. Serine Proteinase Inhibitors

Serine proteinases are prohibited by several groups of inhibitors (Table 5). These groups include the serpins, bikunins (Kunitz-type inhibitors), mucus proteinase inhibitors, and the specialized modulator of thrombin, thrombomodulin. (Section V.H). Serine proteinases are also inhibited by the general inhibitor α2-macroglobulin (Section V.I.4).

a. Serpins

Serpins, *ser*ine *p*roteinase *in*hibitors, constitute a family of proteins with molecular weights in the range of 40,000 to 100,000 (see reviews in References 291 and 292). This family includes the inhibitors α1-antitrypsin, α1-antichymotrypsin, antithrombin III, heparin cofactor II, C1-inhibitor, α2-antiplasmin, plasminogen activator inhibitor I and II, protein C inhibitor, and protease nexin-1. The noninhibitory proteins, cortisol- and thyroxine binding globulins, ovalalbumin, and angiotensinogen, are also in this class of proteins based on the homology of their amino acid sequences to the inhibitors.[293] Most of the inhibitory serpins are found in an active form under physiological conditions. Plasminogen activator inhibitor-1 is the only serpin that can be reversibly converted from an active form into an inactive latent inhibitor without enzymatic cleavage

TABLE 5
Proteinase Inhibitors

Inhibitor type	Inhibitor	Major proteinases inhibited	Refs.
Serpin	α1-Proteinase inhibitor	Neutrophil elastase	292
	α1-Antichymotrypsin	Cathepsin G	463
	Plasminogen activator inhibitors-1 and -2	Urokinase-type and tissue-type plasminogen activator	14, 317, 464
	α2-Antiplasmin	Plasmin	464
	Antithrombin	Thrombin, factors Xa and VIIa	13, 394, 466
	C-1 inhibitor	C1r, C1s	15
	Heparin cofactor II	Thrombin, chymotrypsin	467
	Proteinase nexin-1	Thrombin, urokinase-type plasminogen inhibitor	320, 468
Bikunin-Kunitz-type inhibitors	Inter-α-trypsin inhibitor and bikunin	Trypsin, chymotrypsin, neutrophil elastase, plasmin, acrosin	334
	β-Amyloid precursor protein 751	Plasmin, tryptase	338
Mucus proteinase inhibitors	Secretory leukocyte proteinase inhibitor	Cathepsin G, neutrophil elastase	340
	Elfin	Neutrophil and pancreatic elastase, proteinase-3	342, 343
Cystatins	Stefins A and B	Cathepsins H and L	346
	Cystatins C, DS, SN, SA, sarcocystatin A	Cathepsins B, H, L	346
	H-, L-, and T-kinnogens	Cathepsin L, Calpains I and II	346, 350
	Cathelin	Cathepsin L	346
Calpastatins	Calpastatin I	Calpain I	233
	Calpastatin II	Calpain II	233
Tissue inhibitor of metalloproteinases (TIMPs)	TIMP-1 and TIMP-2	Interstitial collagenase, neutrophil collagenase, stromelysin, gelatinases A and B, matrilysin	355, 356
Macroglobulin	α2-Macroglobulin	Most proteinases of all classes	282
	Pregnancy zone protein	Chymotrypsin, elastase	373

under physiological conditions.[294] Plasminogen activator inhibitor-1 interconverts from a latent inactive form to an active form by a mechanism involving an intermediate denaturation step.[295] *In vivo*, plasminogen activator inhibitor-1 is kept in the active conformation by binding to vitronectin.[294] Vitronectin is found in plasma and in extracellular matrices such as the subendothelial matrix.[294]

Inhibitory serpins possess a reactive-site loop region. This loop is the most variable region of the serpins.[292] Serine proteinases react at a 1:1 stoichiometry with serpins on the reactive-site loop of the inhibitory members of the serpin family. The exact site on the reactive loop where the proteinase reacts is determined by the sequence of the reactive loop for a given inhibitor and the specificity of the enzyme inhibited. The proteinase initially may form a reversible tetrahedral complex with the inhibitor.[296] Reversibility is demonstrated by the transfer of plasmin from α2-plasmin inhibitor to α2-macroglobulin and the subsequent formation of a complex between the inhibitor and chymotrypsin.[297] Chymotrypsin and plasmin react at different but overlapping sites on α2-antiplasmin.[297] In contrast, all proteinases react at the same site on α1-proteinase inhibitor. Many proteinase react with serpins and form acylated complexes that are stable to SDS. Exceptions to this include the complexes formed between α1-proteinase inhibitor and elastase and between plasminogen activator inhibitor-1 and tissue plasminogen activator, which are labile to SDS.[298]

The inhibitor molecules in other serpin-proteinase complexes are cleaved. In the cleaved form, the complexes are not reversible. Cleavage can occur very slowly, with a half-life of hours to days, or rapidly, with a half-life of seconds or minutes.[291] An example is the reaction between α1-antichymotrypsin and chymase.[299] Some complexes are stable over hours, while others have a very short half-life. In this case, α1-antichymotrypsin is cleaved, and the chymase molecule is released.

Cleaved serpins are conformationally more stable than uncleaved serpins.[293] The three-dimensional structures are known for the cleaved forms of α1-proteinase inhibitor,[293] α1-antichymotrypsin,[300] and antithrombin,[301] and the inactive conformation of native plasminogen activator inhibitor.[294] In the cleaved structures, the two residues of the reactive bond are found on opposite sides of the molecule, with one cleaved strand inserted as the middle strand into a β-sheet. In the inactive conformation of plasminogen activator inhibitor, a portion of the reactive loop is found inserted into the corresponding β-sheet. The remaining reactive loop is wrapped around the molecule. In the X-ray structure of ovalalbumin, a noninhibitory serpin, the residues corresponding to the active-site loop forms a mobile α-helix above the molecule.[293] The structure of a hybrid serpin, α1-antichymotrypsin containing the reactive loop of α1-proteinase inhibitor reveals the active site loop as a distorted helix which is not inserted into the beta sheet.[302]

Cleavage of the reactive loop of serpins is believed to induce a major conformational change.[303] The insertion of the cleaved part of the reactive loop into the β-sheet is part of this conformational change. A major conformational change is supported by the appearance of new epitopes when antithrombin III interacts with and binds to factor Xa or thrombin but not with heparin.[303] These new epitopes are also present in complexes formed between antithrombin III and a synthetic reactive loop tetradecapeptide. In this case, it is known that the tetradecapeptide inserts into the β-strand. The exact form of serpin-proteinase complexes is not known.

Serpin synthesis can be either constitutive or controlled by cytokines and hormones. α1-Proteinase inhibitor gene transcripts synthesized constitutively by the liver and intestine differ from those synthesized by monocytes.[304] In the liver, the reaction of LF-A1 to the A domain and LF-B1 to the B domain of the gene are required for constitutive expression.[305] The macrophage-specific promoter is about 2000 bp upstream of the hepatocyte-specific promoter.[306] There are three macrophage-specific transcription start sites[304] and the possibility of alternative splic-

ing[306] for constitutive synthesis. A total of six mRNA transcripts is possible. The α1-proteinase inhibitor translation products from these macrophage transcripts are identical and are identical to that made by the liver.

IL-6 induces synthesis by α1-proteinase and α1-antichymotrypsin in human hepatocytes[307] and monocytes.[308] This induction is at the level of mRNA transcription. In hepatic and intestinal cells, IL-6 increases the amount of the short, constitutively synthesized α1-proteinase inhibitor mRNA transcripts and induces the synthesis of the longer monocyte forms.[304] In monocytes, IL-6 increases the synthesis of all forms of the longer mRNA transcripts. These cells do not synthesize the shorter liver transcript.

Steroids also control serpin synthesis. Androgens stimulate synthesis of the inhibitor protease nexin I in seminal vesicles.[309] Estrogens stimulate the synthesis of α1-antichymotrypsin and α1-proteinase inhibitor in breast cancer cells.[310]

Other molecules can also control the synthesis of serpins. Proteinases-inhibitor complexes,[285,311] degraded inhibitor peptides,[312] and bacterial components[312,313] can induce the synthesis of inhibitors. The reaction of neutrophil elastase-α1-proteinase inhibitor complexes with monocyte receptors for these complexes induces the synthesis of α1-proteinase inhibitor by monocytes.[285] The binding of α1-antichymotrypsin-cathepsin G complexes to membrane receptors stimulates α1-antichymotrypsin synthesis.[311] This is probably through the induction of IL-6 synthesis. Bacterial components can also modulate serpin synthesis. *Pseudomonas* elastase-generated cleavage products of α1-proteinase inhibitors induce the synthesis of α1-proteinase inhibitor by binding to the α1-proteinase inhibitor-proteinase receptor on monocytes.[312] Bacterial LPS induces α1-proteinase inhibitor and plasminogen activator inhibitor-2 synthesis in monocytes.[312,313] LPS-stimulated α1-proteinase inhibitor synthesis is through the enhancement of mRNA translation into protein rather than an increase in mRNA.[274] Plasminogen activator inhibitor-2 production is stimulated by LPS in human peripheral blood monocytes by both the induction of gene transcription and the stabilization of the mRNA.[313]

When a tissue expresses both an inhibitor and its target proteinase(s), the synthesis of the two types of molecules can be regulated noncoordinately or coordinately. Plasmin induces the synthesis of tissue-type plasminogen activator but not plasminogen activator inhibitor-1 in human endothelial cells, while thrombin induces the synthesis of both tissue-type plasminogen activator and plasminogen activator-1.[122] Similar inhibitors, such as plasminogen activator inhibitor-1 and -2 can be coordinately or differentially regulated. For example, in endothelial cells, IL-1α induces the synthesis of both of these inhibitors in endothelial cells, while TGF-β increases plasminogen activator inhibitor-1 but not plasminogen activator inhibitor-2 synthesis.[314]

Serpins can be posttranslationally modified by glycosylation and cross-linking into complexes. Glycosylation of serpins does not affect inhibitory activity, but does play a role in the secretion of the inhibitor, the determination of the half-life of the active form, and the recognition of the inhibitor-proteinase complex by membrane-bound receptors.[292] Serpin glycosylation differs from one tissue to another. For example, the molecular weight of α1-proteinase inhibitor and α1-antichymotrypsin synthesized by trophoblasts is 50 and 49 kDa, respectively, while that synthesized by hepatoma and breast cancer cells is 54 and 68 kDa, respectively.[315] The N-glycosylation pattern for α1-proteinase inhibitor is controlled by IL-6, TGF-β, TNF, and IL-1 in human hepatoma cells.[316] Serpins can be modified by cross-linking to other proteins. Active plasminogen activator inhibitor-2 is present in large covalent com-

plexes in trophoblast membranes.[317] This process may be involved in focally regulating fibrinolysis.

Serpins can react *in vitro* with type of many serine proteinases, yet only a few are the physiological target of the inhibitor. For example, although α1-proteinase inhibitor reacts with most serine proteinases, the K_i for many of the complexes is not adequate for α1-proteinase inhibitor to play a physiological role in the regulation of the enzyme. α1-Proteinase inhibitor inhibits neutrophil elastase (the physiological target) with a K_i of 10^{-14}, while inhibition of cathepsin G has a K_i of 10^{-8}.[318] In general, serpins only inhibit serine proteinases. There are several initial reports of serpin inhibition of thiol proteinases. An α1-proteinase inhibitor-like molecule regulates a prohormone cysteine proteinase that processes proenkephalin.[319]

Some serpins can bind to membranes and retain inhibitory activity. Membrane-bound proteinase nexin-1 can inhibit thrombin.[320] This complex can stay bound to the membrane and can be endocytosed.

The activity of some serpins can be modified by forming noncovalent complexes with sulfated glycosaminoglycans. Formation of these complexes converts some serpins from low-affinity inhibitors to high-affinity inhibitors. There are two mechanisms for interaction of the glycosaminoglycans with serpins.[291] Heparin reacts with antithrombin III by a mechanism that involves a conformational change in the inhibitor.[321] Four sulfate groups on a pentasaccharide of heparin interact with aligned, conserved lysine and arginine residues (47, 125, 129, 132, and 133) in antithrombin III. Heparin cofactor II, protein C inhibitor, and protease nexin I react with heparin by a mechanism in which the glycosaminoglycan reacts with both the inhibitor and the proteinase.[291] No conformational change is involved. The specificity of an inhibitor can be changed by glycosaminoglycans. Plasminogen activator inhibitor-1 alone inhibits urokinase-type and tissue-type plasminogen activator but not thrombin. In the presence of heparin, plasminogen activator inhibitor-1 is converted into an effective inhibitor of thrombin.[322]

Glycosaminoglycans can also interact with serine proteinases, altering their ability to be inhibited by serpins. Heparin decreases the rate of inhibition of neutrophil elastase by α1-proteinase inhibitor through the formation of a strong interaction with neutrophil elastase.[323]

b. Bikunins: Kunitz-Type Inhibitor Family

The mammalian Kunitz-type inhibitors inhibit serine proteinases and are found in both tissues and body fluids.[324,325] The members of this family include the basic pancreatic trypsin inhibitor bikunin (also called urinary trypsin inhibitor, HI30, acid-stable proteinase inhibitor, the inhibitory subunit of inter-α-trypsin inhibitor), pre-α-trypsin inhibitor, lipoprotein-associated coagulation inhibitor, the α3 chain of type VI collagen, and one form of the amyloid β-precursor protein.[324,326] All of these inhibitors contain either bikunin, the inhibitor subunit, or a homologous inhibitory subunit. Bikunin has two tandem repeats of Kunitz-type domains.[327] The distribution of bikunin is wider than those of other members of this class. It is found in the brain, liver, kidney, and gastrointestinal tract.[325]

Inter-α-trypsin inhibitor is composed of a 30-kDa inhibitory subunit (bikunin) and two homologous noninhibitory subunits of 65 and 70 kDa.[324] The genes for these three subunits are found on three separate chromosomes.[327,328] The inhibitory subunit HI30 (bikunin) is synthesized as a fusion protein with α1-microglobulin on its N-terminal end.[329,330] The two proteins are proteolytically separated during posttranslational pro-

cessing in the Golgi apparatus.[331] Additionally, an O-linked chondroitin sulfate chain has been identified on the Ser10 of this inhibitory bikunin subunit.[332] The second end is bound to the C-terminal Asp of the 70-kDa heavy chain via an α-carbon linkage to an internal N-acetylgalactosamine.[332] The chondroitin sulfate chain is not involved in inhibition.[333] Inter-α-trypsin inhibitor is unusual in that the noninhibitory subunits can be degraded yet retain inhibitory activity.[332] A smaller inhibitor, pre-α-trypsin inhibitor, contains two polypeptides, bikunin and a 90-kDa polypeptide linked by a chondroitin-4-sulfate chain in the same manner as inter-α-trypsin inhibitor.[332]

The physiological target of either bikunin or inter-α-trypsin inhibitor is not known. These molecules inhibit serine proteinases, including trypsin, chymotrypsin, neutrophil elastase, cathepsin G, plasmin, and acrosin with K_i values of 10^{-9} to 10^{-7} *M*.[334] Proteinases bound to inter-α-trypsin inhibitor and bikunin quickly dissociate in the presence of α1-proteinase inhibitor, α2-antiplasmin, or α2-macroglobulin to form complexes with the latter inhibitors.[335] Based on the K_i values and the ability of proteinases to transfer from inter-α-trypsin inhibitor or bikunin to other inhibitors, these inhibitors may serve as "shuttle inhibitors".[336]

An increase in an amyloid β-precursor containing a domain with bikunin activity (β-APP751) has been observed in amyloid deposits of the brains of Alzheimer patients.[337,338] Increased deposits of amyloid precursor are observed in transgenic mice containing the human β-APP751 gene.[337] The APP751 protein strongly inhibits plasmin and tryptase.[338] It also inhibits trypsin, chymotrypsin, and kallikrein.

c. Mucus Proteinase Inhibitors

Serine proteinases are also inhibited by mucus proteinase inhibitors, which are small proteins of less than 25 kDa in size. These inhibitors are secreted by exocrine cells, neutrophils, and other types of cells and are found in mucous and cartilage.[339] The major inhibitor in this class is secretory leukocyte proteinase inhibitor (SLPI); it is also called mucous proteinase inhibitor and anti-leukoproteinase. It contains 107 amino acids in two homologous domains and is acid stable and cysteine rich with four disulfide bonds.[340] The C-terminal domain inhibits trypsin, chymotrypsin, cathepsin G, and neutrophil elastase at nanomolar proteinase concentrations.[340] The C-terminal domain can bind a second molecule of chymotrypsin, trypsin, and cathepsin G, but not a second elastase molecule when the enzymes are at micromolar concentrations. Neutrophil elastase, but not other neutrophil products (cathepsin G, myeloperoxidase, and lysosome), induces the synthesis of secretory leukocyte proteinase inhibitor mRNA by airway epithelial cells.[341]

A second member of the mucus proteinase inhibitor group is elfin. Elfin is a smaller inhibitor, yet homologous to secretory leukocyte inhibitor (38% homology).[342] It also has four disulfide bonds. The active precursor is 14 kDa and the mature inhibitor is 6 kDa.[343] This inhibitor inhibits pancreatic and neutrophil elastase and proteinase-3, but not trypsin, plasmin, chymotrypsin, or cathepsin G. Inhibition of neutrophil elastase by elfin is pH dependent.[342] The K_i changes from 6.7×10^{-9} at pH 5.4 to 2.0×10^{-6} at pH 9.0. This inhibitor is found in bronchial secretions[313] and the skin of psoriasis patients.[345] In these patients, elfin is found free and as an immobilized 9.9-kDa form cross-linked to cornified envelopes by transglutaminase.

2. Cysteine Proteinase Inhibitors

Cysteine proteinases are inhibited by three major types of specific inhibitors (Table 5) and the general inhibitor α2-macroglobulin (Section V.I.4). These include

the cystatin family that inhibits cathepsins B, H, L, and S,[346] and the calpastatins that inhibit calpains.[233] There are four sub-families of cysteine proteinase inhibitors in the cystatin family: the stefin, cystatin, kininogen, and cathelin families.[346] The members of this family are homologous proteins.

a. Cystatin Family

The stefin family consists of single-chain molecules of about 11 kDa with no carbohydrates or disulfide bonds.[346] The known stefins are human stefins A and B and rat cystatin-α and -β. These molecules are found distributed in tissues and extracellular fluids. Stefins are stable at neutral and alkaline pH and are heat stable. They are reversible competitive inhibitors with K_i values in the range of 10^{-8} *M* for cathepsin B to 10^{-11} *M* for cathepsins H and L. Stefins can be inactivated by formation of a disulfide-linked dimer.

The cystatins are slightly larger than the stefins, with molecular weights around 13 kDa, and have two disulfide bonds in the C-terminal portion of the molecules.[346,347] Included in this family are the human cystatins C, D, S, SN, and SA, sarcocystatin A, chicken cystatin, rat cystatins C and S, and bovine colostrum cystatin.[315,317] Of this group, only rat cystatin C is glycosylated. Chicken cystatin consists of two isoforms, one of which is phosphorylated. These inhibitors bind very tightly to cysteine proteinases with K_i values of 10^{-12} *M*.[346]

Cystatins may play a role in controlling kidney tissue damage. Cathepsins B, H, and L are present in the glomeruli and are capable of degradation of the glomerular basement membrane. Normally, cystatins balance these enzymes. Under hypertensive conditions, the level of these inhibitors decreases, allowing degradation of kidney tissues.[348]

Cystatins may play a role in the regulation of the maturation of virus in tissues. Human cystatin C and chicken cystatin inhibit the proteases of poliovirus and rhino viruses. These proteinases are involved in the cleavage of viral polypeptides required for the formation of their capsids.[349] This inhibition is a steric inhibition and does not involve the active site cysteine residues.

The kininogens are multipurpose molecules that are precursors of the vasoactive kinins and act as high molecular cofactors for the intrinsic coagulation system and cysteine proteinase inhibitors.[346] There are three forms: a high molecular weight (H)-kininogen with an M_r of about 120 kDa, a low molecular weight (L)-kininogen with an M_r of about 68 kDa, and a T-kininogen (thiostatin) with an M_r of about 68 kDa. L- and H-kininogens are found in many species, but T-kininogen is found only in the rat. L- and H-kininogens are coded by the same gene can form oligomers, and are potent inhibitors of calpain.[350]

The last group of cystatin inhibitors is the cathelin family. Cathelin is the only characterized member of this family. It inhibits cathepsin L but not other cysteine proteinases.[346]

b. Calpastatin

Two specific inhibitors of calpains, calpastatin I and II, are produced in many cells. Calpastatin I is a better inhibitor of calpain I than calpain II, while calpastatin II is a better inhibitor of calpain II than calpain I.[233] Like the proteolytic activity of calpains, their inhibition is calcium dependent. Calpain I inhibition requires micromolar levels of Ca^{2+}, and calpain II inhibition requires millimolar levels of Ca^{2+}. Calpastatins inhibit the autolytic activation of calpains as well as the proteolytic activity.[351] Inhibition of activation occurs by blocking calpain binding to cell membranes. Calpastatins have two do-

mains that interact with calpains. One domain is a competitive inhibitor.[324] The second domain interacts with the regulatory site on calpains.[351]

Calpastatin I and II activity is controlled by phosphorylation. Both forms of calpastatin can be phosphorylated at multiple serine residues and occasionally at threonine residues.[353] Most of the membrane-bound calpastatin molecules are phosphorylated, while only about 20% of the cytosolic calpastatin molecules are modified, which indicates that phosphorylation may regulate the subcellular distribution of the inhibitors.[353] The dephosphorylated form of calpastatin I is a better inhibitor of calpain I than the phosphorylated form of this inhibitor. However, the phosphorylated form of calpastatin II is a better inhibitor of calpain II than the dephosphorylated form of this inhibitor.[354]

3. Matrix Metalloproteinase Inhibitors

Matrix metalloproteinases are inhibited by two types of proteinases (Table 5). Tissue inhibitors of metalloproteinases (TIMPs) and inhibitors of metalloproteinases (IMPs) specifically inhibit this class of enzymes. The general proteinase inhibitor α2-macroglobulin also inhibits matrix metalloproteinases.

All active forms of matrix metalloproteinases are inhibited by TIMP-1 and TIMP-2.[355,356] TIMP-1 is a 28.5-kDa glycoprotein that is synthesized by most connective tissue cells and macrophages.[355] It forms reversible complexes with the matrix metalloproteinases, with K_d values in the range of $10^{-10.6}$. TIMP-2 is a 23-kDa unglycosylated protein and is found at lower concentrations than TIMP-1 in tissues.[355] The homology of the amino acid sequences for the two inhibitors is only 40%, but the key 12 cysteines that form disulfide bonds are conserved. Degradation products of these inhibitors are not inhibitory.

TIMP-1 and TIMP-2 rapidly inhibit active gelatinase A. These inhibitors interact with the active site plus a site in the carboxyl terminal hemopexin-like region.[357,358] The C-terminal region of the TIMPs interacts with the C-terminal region of the enzyme, increasing the rate of association by a factor of about 100. Recombinant carboxyl terminal truncated forms of this enzyme require a tenfold excess of the inhibitor for inhibition in comparison to the 1:1 ratio needed for the intact molecule.

The C-terminal hemopexin-like region contributes stability to complexes of TIMP-1, with other matrix metalloproteinases. Stromelysin binds to intact TIMP-1 with a K_i of 6×10^{-10} *M*.[356] Stromelysin mutants, missing the hemopexin-like region, bind TIMP-1 with a K_i of 5×10^{-9} *M*. Matrilysin, which does not have a hemopexin-like region, binds this inhibitor with a similar K_i value. The binding of TIMP-1 to collagenase is noncompetitive, but inhibition with the active site inhibitor 1,10-phenanthroline prevents binding of TIMP-1. Active collagenase and stromelysin bind to TIMP-1 very slowly. These enzymes bind at a faster rate to α2-macroglobulin.[355]

In addition to the inhibition of active matrix metalloproteinases, TIMP-1 can regulate the activation of progelatinase B. TIMP-2 can regulate the activation of progelatinase A[158] (Section IV.C.1).

Two other types of matrix metalloproteinase inhibitors have been identified. These are the smaller inhibitors of metalloproteinase (IMPs) and the large inhibitor of metalloproteinases (LIMP). IMPs are smaller than TIMPs.[359] IMP-1 is 26 kDa, IMP-2 is 21 kDa, and IMP-3 is 18 kDa. They do not cross-react with TIMPs and are found in many cells from multiple species. LIMP is a complex composed of TIMP-2 and progelatinase A.[360] This complex inhibits colla-

genase, gelatinase A, and stromelysin. The ability of the TIMP-2-progelatinase complex to inhibit these enzymes indicates that the inhibitory site is exposed in the TIMP-2 molecule.

The expression of TIMP-1 and metalloproteinases upon stimulation with some growth factors and cytokines is coordinated in some cells and is noncoordinated in others. Retinol in osteopetrotic bone inhibits TIMP-1 synthesis and stimulates collagenase synthesis.[283] TIMP-1 decreases to essentially zero in this tissue. In human skin and uterine cervical fibroblasts, IL-6 induces the synthesis of TIMP-1 while having no effect on matrix metalloproteinases.[361] Conversely, IL-1 stimulates the synthesis of both TIMP-1 and matrix metalloproteinases.

Expression of TIMP-1 and TIMP-2 is controlled separately.[355] TIMP-2 is downregulated by LPS, gelatin, and zymosan, while TIMP-1 and collagenase are stimulated by these agents.[362] In human dermal fibroblasts, TIMP-2 levels are unaffected by IL-1 but are downregulated by TGF-β.[362,363]

TIMPs are important in the control of numerous physiological processes. These include tumor cell invasion, angiogenesis, degradation of joint cartilage, trophoblast implantation, mammary gland involution, and wound healing.[72,355]

4. α-2-Macroglobulin

α2-Macroglobulin is an unusual inhibitor of proteinases in that this molecule reacts with most proteinases of all four major classes.[282] The rate of reaction can vary by several orders of magnitude. For example, the α2-macroglobulin reaction with plasmin is 100 times faster than with urokinase-type plasminogen activator.[364] The only known mammalian inhibitor of aspartic proteinases is α2-macroglobulin.[367] (The propeptide of aspartic proteinases does inhibit these proteinases.[365,366] Whether this is a physiological mechanism, it is not known.) The activity of chymosin and cathepsins D and E toward protein and peptide substrates at pH 6.2 is blocked by this inhibitor.

α2-Macroglobulin is composed of four identical subunits with a total molecular weight of 718 kDa.[282,368] Dimers are formed by intersubunit disulfide bridges. Two of these dimers associate by noncovalent interactions to form a tetrameric complex.

Proteinases initially cleave α2-macroglobulin on a 25-amino acid-exposed loop present on each subunit (residues 675 to 700) called the "bait region." Interestingly, the "bait region" of α2-macroglobulin is the best substrate for interstitial collagenase, with a k_{cat}/K_m value of 20 to 2000 times that for various collagens.[6] Cleavage of the inhibitor in the bait region of any subunit rapidly alters the conformation of two or four of the α2-macroglobulin subunits.[369] The proteinase is trapped by the inhibitor, and the thiol ester between cysteine 972 and glutamine 975 is activated in the altered α2-macroglobulin subunits. The resulting reactive glutamyl residue(s) can react with lysine ε-amino groups on the exposed surface of the trapped proteinase[370] or other trapped proteins or can react with water.[282] In addition, the resulting cysteinyl residue(s) can form disulfide bonds with cysteinyl residues on trapped proteinases or other proteins.

Each trapped molecule can bind through multiple cross-links to more than one α2-macroglobulin subunit.[364] One large proteinase (i.e., plasmin and thrombin) or two smaller proteinases (i.e., trypsin or chymotrypsin) are bound per α2-macroglobulin molecule.[364,371] Some proteinases, such as neutrophil elastase, do not have accessible lysine residues on their surface. These are only trapped by this inhibitor. Because proteinases are trapped or trapped and bound through their surface residues rather than to their active-site residues, most bound pro-

teinases retain activity toward small substrates but not toward large substrates.[372] Access of large substrates to the active site of the bound proteinases is sterically inhibited. This may be a mechanism to convert proteinases from endopeptidases to oligopeptidases.

Proteinase-activated α2-macroglobulin can bind other molecules in addition to proteinases. This activated inhibitor can bind growth factors and cytokines (i.e., TGF-β, TNF-α, b-FGF, nerve growth factor, PDGF, IL-1β and IL-6).[373]

There are homologs of α2-macroglobulin that are active proteinase inhibitors but have fewer than four subunits.[374] Examples include α_1-inhibitor-3, which occurs as a monomer in rat and hamster, and pregnancy zone protein, which is found in most mammalians as a dimer. Normal female human serum contains low levels of the pregnancy zone protein.[374] The sera of pregnant women contain significantly elevated levels of this protein. Unlike α2-macroglobulin, this molecule inhibits a narrow range of proteinases. Many species also have a monomeric α-macroglobulin.[374] Cross-linking of this inhibitor to proteinases is required for inhibition.

α2-Macroglobulin is synthesized not only by the liver, but also by numerous other tissues such as the ovary, testis, uterus, placenta,[375] lung fibroblasts, astrocytes, monocytes-macrophages,[282] and cornea.[376] α2-Macroglobulin is a single-copy gene in the human genome. It is found in a cluster with an α2-macroglobulin pseudogene and pregnancy zone protein.[377] Three transcription initiation sites for the α2-macroglobulin gene are utilized in a tissue-specific fashion. Liver produces all three transcripts, while uterus and lung fibroblasts synthesize only two transcripts.

α2-Macroglobulin gene regulation in rat and human liver is different. In rat, this inhibitor is an acute-phase protein, while in the human it is not. In the human, serum levels are significantly increased during embryogenesis, pregnancy, and childhood[282] but not by infections. In the rat liver, the acute phase-associated cytokine IL-6 induces transcription via an IL-6 response element.[378] This response element is also found on the human gene, but IL-6 does not induce the synthesis of this inhibitor in the human liver. This cytokine does induce synthesis of α2-macroglobulin in human neuroblastoma cells.[379] TGF-β selectively increases α2-macroglobulin synthesis in bovine adrenocortical cells.

In specialized tissues such as the ovary, thecal cells of developing follicles synthesize α2-macroglobulin when stimulated by low levels of luteinizing hormone.[375] Ovarian granulosa cells produce this inhibitor when stimulated to undergo luteinization by luteinizing hormone plus prolactin. Stimulated granulosa cells package and secrete this inhibitor.

5. Proteinase Inhibitor Degradation

Free proteinases can be inactivated by oxidation and direct enzymatic hydrolysis by proteinases. One or both of these methods are used by invading cells and microorganisms to gain access to tissues. Proteinase inhibitors bound to proteinases can bind to membrane receptors, be endocytosed, and be degraded (Section VI).

Activated neutrophils and macrophages release oxidants that can inactivate inhibitors such as α1-proteinase inhibitor,[380] secretory leukocyte proteinase inhibitor,[381] and plasminogen activator inhibitor.[382] α1-Proteinase inhibitor contains a methionine residue in the P_1 position of its active site. Oxidation of this methionine residue by the myeloperoxidase product HOCl[383] and the oxidants in cigarette smoke[292] causes loss of activity. Secretory leukocyte proteinase in-

hibitor is also susceptible to inactivation by oxidation of the methionine residue in the P_1' position.[381] Plasminogen activator inhibitor-1 does not contain methionine in its reactive-site loop, yet oxidation also inactivates this inhibitor.[382] Site-directed mutagenesis studies replacing the methionine residues with leucine residues show that oxidation of the two methionine residues of plasminogen activator inhibitor-1 outside the reactive-site loop induces a conformation change, leading to loss of activity.[382]

Proteinase inhibitors are also inactivated by proteolytic cleavage. Proteinases that are not inhibited by a given inhibitor often use the inhibitor as a substrate. The matrix metalloproteinases neutrophil collagenase,[384] stromelysin,[384] and the 92-kDa gelatinase[385] cleave α1-proteinase inhibitor within the reactive loop, thereby inactivating the inhibitor. Neutrophil collagenase, but not 92-kD gelatinase, cleaves and inactivates α1-antichymotrypsin.[385] Based on a kinetic model, stromelysin is probably the most important proteinase involved in the inactivation of α1-antichymotrypsin and α1-proteinase inhibitor *in vivo*.[386]

Serine and aspartic proteinases cleave cystatins. Neutrophil elastase cleaves cystatin C and inactivates the inhibitor.[287] The aspartic proteinase cathepsin D cleaves and inactivates cystatin C and kininogen.[387]

The reaction of some proteinases with inhibitors results in the formation of both stable inhibitory complexes and cleaved inactive inhibitors. For example, the reaction of human chymase with α1-antichymotrypsin produces 3.5 mol of cleaved inhibitor for every mole of inhibited chymase.[299]

Proteolytic cleavage can destroy an inhibitor's inhibitory activity toward all enzymes or only a few. The serine proteinases neutrophil elastase, cathepsin G, and proteinase-3 remove the N-terminal decapeptide of cystatin C.[287] The inhibitory activity of cystatin C changes very little toward cathepsins L and H, but there is a 250-fold reduction in activity toward cathepsin B. This probably is important in sepsis, tumor cell metastasis, active bone resorption, rheumatoid arthritis, and purulent bronchiectasis.[287]

Calpastatins can be degraded by one of the enzymes that they inhibit. Calpain II, but not calpain I, degrades both calpastatin I and II in the presence of high Ca^{2+} concentrations.[233] The same activators that enhance autocatalytic activation of calpain II enhance the degradation of calpastatins. Binding of the calpain activator to the 80-kDa subunit of calpain II increases the rate of calpastatin degradation as well as autolytic activation of calpain II.[197] The rate of calpastatin degradation by calpain II is also enhanced by isovaleryl carnitine.[233] In addition, the Ca^{2+} requirement is lowered in the presence of isovaleryl carnitine. One of the major roles postulated for calpain II is the control of calpastatin levels.[197]

Bacterial proteinases can cleave mammalian proteinase inhibitors. α1-Proteinase inhibitor is cleaved and inactivated by *Pseudomonas aeruginosa* elastase and *Staphylococcus aureus* V8 proteinase.[388] α1-Antichymotrypsin and C1-inhibitor are also cleaved by *P. aeruginosa* elastase.[389] Secretory leukocyte proteinase inhibitor is cleaved by catalytic amounts of *P. aeruginosa* elastase but not by *S. aureus* V8 proteinase.[388] A *Serratia marcescens* 56-kDa protease and *Pseudomonas* elastase degrade the serpins and α2-macroglobulin.[390]

6. Importance of Proteinase Inhibitors

The importance of proteinase inhibitors in controlling proteolysis has been established in pathological conditions where there is a deficiency or excess of proteinases inhibitors and by the use of inhibitors for treatment of disease. One of the best studied pathological conditions is α1-proteinase in-

hibitor (α1-antitrypsin) deficiency. Emphysema in the adult and liver disease in children are the major clinical manifestations.[391] The immune-related diseases, rheumatoid arthritis, asthma, and panniculitis are also associated with α1-antitrypsin deficiency. The most severely affected patients synthesize the Z variant of α1-proteinase inhibitor. In this variant, Glu342 is substituted by a Lys residue. A critical salt bridge is disrupted, altering the conformation of the protein.[293] This leads to precipitation of the inhibitor in liver cells of the affected patients. α1-Antichymotrypsin also is important for lung function. A Leu-to-Pro substitution in this inhibitor is associated with familial obstructive lung disease.[392] Children with cystic fibrosis have normal levels of α1-proteinase inhibitor but have an excess of leukocyte elastase in their lungs.[393] An excess of inhibitors is associated with neural diseases characterized by amyloid deposits. The bikunin-related inhibitory form of amyloid precursor protein[338] and serpins, including antithrombin III[394] and α1-antichymotrypsin[395] as well as other proteinase inhibitors, are present in amyloid deposits observed in Alzheimer's disease and Downs syndrome.

Proteinase inhibitors have been used to successfully treat diseases characterized by excessive proteolysis of tissues. Aprotinin (bovine pancreatic trypsin inhibitor) has been used to treat acute pancreatitis[396] and corneal ulcers[397] and to prevent blood loss following cardiac surgery.[398] Administration of human secretory leukoproteinase inhibitor and tissue inhibitor of metalloproteinases-2 suppresses immune complex-induced alveolitis.[399,400]

VI. CONTROL OF PROTEINASE DEGRADATION

The half-life of a proteinase can be altered by posttranscriptional glycosylation. The two major isoenzymes of plasminogen differ only by their degree of glycosylation.[401] The form that is glycosylated at both Asn288 and Thr345 has a longer half-life than the second enzyme, which is glycosylated only at Asn288. The first form is a better fibrinolytic enzyme upon activation. Each isozyme has subforms that differ in pI due to differing degrees of sialylation.

Proteinases are irreversibly inactivated by several pathways. The first is direct degradation by either autolysis or degradation by a different proteinase. The second pathway involves receptor-mediated endocytosis of either the proteinase alone or in complex with an inhibitor.

Many active proteinases in the absence of inhibitors or alternative substrates can undergo autolysis. Examples include cathepsin B,[102] inhibitor-free 72-kDa gelatinase,[357] collagenase,[402] calpains I and II,[194] and n-calpain-1.[403] Autolysis of n-calpain-1 occurs under normal conditions within the muscle very shortly after synthesis.

One proteinase can degrade other proteinases. Membrane-bound thrombomodulin accelerates thrombin inactivation of single-chain urokinase-type plasminogen activator.[404] The glycosaminoglycan chain plays a role in the formation of the thrombomodulin-thrombin-urokinase-type plasminogen activator complex and the inactivation of the enzyme.

Proteinase-inhibitor complexes bind to cell membrane receptors, undergo endocytosis, dissociate from the receptor, and then are degraded. Serine proteinases complexed to serpins bind to specific receptors on various cells, including phagocytic cells, macrophages, or Kupffer cells.[292] A conformational change in the inhibitor upon reaction with proteinases exposes a domain that is recognized by the receptor.[285] This domain is present in the C-terminal region of serpins (amino acids 359 to 374 in α1-proteinase inhibitor) and is highly conserved. Synthetic

peptides in this region specifically bind to the receptor and inhibit proteinase-serpin binding to the receptor. Astrocytes have a receptor for α1-antichymotrypsin-cathepsin G complexes that binds the complex but not the individual components.[286]

Some proteinases bound to other inhibitors can be transferred to α2-macroglobulin. α2-Macroglobulin-proteinase complexes bind to membrane receptors, are endocytosed, and degraded. Examples include proteinases bound to some serpins[297] and bikunins.[336]

α2-Macroglobulin-proteinase complexes are rapidly cleared upon binding to specific cell receptors found on many cells, including hepatocytes, astrocytes, adipocytes, synctiotrophoblasts, monocytes, fibroblasts, and macrophages.[405–407] The receptors bind to a 138-amino acid C-terminal site on α2-macroglobulin that is exposed upon reaction with proteinases.[408]

The α2-macroglobulin receptor is identical to the low density lipoprotein (LDL) receptor-related protein.[409] This receptor is synthesized as a 600-kDa protein. It is cleaved in the trans-Golgi to two polypeptides, 515 and 85 kDa. The 515-kDa polypeptide resembles a complex composed of four LDL receptor molecules. The 85-kDa polypeptide anchors the receptor to the cell membrane. The binding of α2-macroglobulin-proteinase complexes to this receptor is controlled by several competing molecules: the α2-macroglobulin receptor-associated protein,[405,410] pregnancy zone protein,[410] very low density lipoproteins enriched with apolipoprotein E,[410] and urokinase-type or tissue type plasminogen activator bound to plasminogen activator inhibitor-1.[411]

Synthesis of the α2-macroglobulin receptor is regulated. The receptor has a differentiation-dependent pattern of expression in trophoblasts that is negatively regulated by cAMP.[410] In macrophages, this receptor is markedly decreased by LPS and interferon-γ but not by TNFα, TGF-β1, or IL-6.[412] In adipocytes, it is controlled by insulin, and in bone marrow macrophages by CSF-1.[282]

α2-Macroglobulin-proteinase-receptor complexes are endocytosed. Within the endosome, the complex dissociates in a pH-dependent manner.[282] The receptors are recycled to the membrane. Processing of the proteinase-α2-macroglobulin complex differs based on the cell type, the proteinase, and the type of linkage between the proteinase and α2-macroglobulin.[282] In most cases, both the proteinase and the modified inhibitor are degraded. However, the *Serratia marcescens* proteinase-α2-macroglobulin complex is more cytotoxic than the proteinase alone.[390] Receptor-mediated uptake of the α2-macroglobulin-Serratia proteinase complex is the means by which the proteinase enters cells. Upon acidification, the proteinase is reactivated and kills the cell.

VII. CONTROL OF INFLUX OF MICROORGANISMS AND CELLS CONTAINING PROTEINASES

Tissue degradation can be controlled by inhibiting the influx of microorganisms and cells that release proteinases. Bacteria can release proteinases, which degrades host proteins, facilitating invasion by the organisms.[390,413] The amount of direct damage depends upon the amount of proteinases released by the organisms. *P. aeruginosa* strains that release high levels of proteinases cause the formation of corneal descemetoceles independent of host inflammatory cells.[414]

Polymorphonuclear leukocytes are the first line of defense against infections. Often, these cells are very destructive due to the release of active oxidants and proteinases.[413,415,416] Control of the influx of these cells has been recognized as a way to decrease the severity of certain pathological

conditions. These include cystic fibrosis,[413] corneal ulceration,[415] and rheumatoid arthritis.[416] Control of the influx of these cells into tissues involves minimizing the production of chemotactic factors. Many of these factors are peptides generated by proteolysis of proteinases in tissues (i.e., complement factor C5a, fibronectin, and fibrin peptides). Prevention of infections is also important because many small exoproducts of bacteria are recognized as chemotactic agents by these cells (i.e. formylated-Met-Lea-Phe).

VIII. CONTROL OF SUSCEPTIBILITY OF CELLULAR AND EXTRACELLULAR PROTEINS

Not only is proteolysis regulated by controlling the activity of the enzymes, but it is also controlled by the specificity of the enzymes for their substrate and the substrate properties (Table 6). Cleavage of a protein requires the availability of amino acid residues that are complementary to the active site of the degrading proteinase. The presence of amino acid sequences such as PEST[417] or KFERQ[418] in a protein can potentially mark proteins for degradation. Posttranslational protein modifications can also increase or decrease the lifetime of a protein. Multiubiquitination,[4] deamination, and oxidation[419] can decrease the half-life of a protein, while glycosylation,[420] phosphorylation,[420] acetylation, and complex formation[421] can increase the half-life of a protein.

A. Proteinase Specificity

The specificity of a proteinase depends upon the amino acids present and the size and shape of the active site of the enzyme. The proteinase also has a range of requirements a protein must fulfill in order for it to be an acceptable substrate. Some proteinases have modest requirements for cleavage, e.g., trypsin cleaves after exposed arginine and lysine residues on the acyl side of the susceptible bond. Other proteinases have more extensive substrate specificities that extend in both directions of the cleavage site. (The residues at the cleavage site are defined as $P_3,P_2,P_1,P_1',P_2',P_3'$. Cleavage occurs at the P_1P_1' peptide bond.) Matrix metalloproteinases prefer hydrophobic groups in the P_2 to P_2' positions (Table 7).[422] The subtilisin-like serine proteinases, which cleave prohormones and neuroendocrine precursors, require two basis residues in the P_1 and P_2 positions, with a preference for arginine in the P_1 site.[48] One member of this family, furin, also requires an arginine residue in the P_4 position.[423]

TABLE 6
Control of Proteolysis at the Substrate Level

Mechanism	Type	Effect on proteolysis
Substrate motif	KFERQ	Increase
	PEST	Increase
Substrate modification	Phosphorylation	Decrease
	Glycosylation	Decrease
	Oxidation	Increase
	Ubiquitination	Increase

Note: See text for references.

B. Substrate Motifs

Stretches rich in proline, glutamic acid, serine, and threonine residues (PEST sequences) have been postulated to be signals for rapid degradation.[55,424,425] Proteins that contain these sequences tend to be extremely short-lived. Regulation may occur at the level of the conformational availability of these residues on the proteins and at the level of the attacking proteinases.[53] Calpain and the 26 S proteasomes may be involved in the degradation of PEST-containing proteins. The PEST-containing proteins, c-Fos, c-Jun, microtubule-associated protein 2, and insulin receptor substrate-1, are degraded by calpain.[55,424] The tumor-suppressing protein p53 and the oncoproteins c-myc and c-myb contain PEST regions and are degraded by ubiquitin-dependent degradation by the 26 S proteasome.[425] Factors other than PEST sequences may be involved

TABLE 7
Comparison of Substrate Specificity of Matrix Metalloproteinases

		Cleavage site residues ↓ cleavage site				
Enzyme	**Native substrates**	**P_2**	**P_1**	**P_1'**	**P_2'**	**P_3'**
Fibroblast collagenase	Collagens I, II, III, and VII	Gln **Leu** **Met** Tyr	**Ala** Met Pro	Ile Leu Met	Phe **Trp**	Ala Ser
Neutrophil collagenase	Collagens I, II, III, and VII	Gln **Leu** **Met** Tyr	**Ala** **Gln** Met Pro	Ile Leu Met **Tyr**	Leu Phe **Trp**	Ala Ser
Gelatinase A (72 kDa)	Collagens IV and V and gelatin	Arg Gln **Leu** Val Met Tyr	**Ala** **Gly**	**Met** Ile Leu	Leu Phe Trp	Ala **Ser**
Gelatinase B (92 kDa)	Collagens IV and V and gelatin	**Leu** Gln Arg Val Met Tyr	**Ala** **Gly**	Ile Leu **Met**	Arg Leu Phe Trp	Ala Ser
Matrilysin	Proteoglycans, fibronectin, laminin, and gelatin	Gln **Met** **Leu** Tyr	**Ala** Gly Gln Glu Met Pro	Ile **Leu** Met	Arg Leu Trp	**Ala** Arg **Met** Ser

Note: The specificity of the matrix metalloproteinases was determined using small polypeptides of five to eight amino acid residues.[422] Preferred amino acid residue is given in bold. Peptide bond cleavage occurs between P_1 and P_1'.

in determining the rate of degradation of this group of proteins. Removal, by site-directed mutagenesis, of the F domain containing PEST domains of the estrogen receptor does not alter the rate of degradation.[417]

The lysine-phenylalanine-glutamic acid-arginine-glutamine (KFERQ) sequence and other related sequences in proteins are a second putative motif for marking protein for enhanced degradation.[418] These sequences are recognized by a 73-kDa heat-shock cognate protein.[418] This heat-shock protein is induced in mammalian cells upon serum deprivation. Binding of this protein results in translocation of the proteins to lysosomes. Uptake and degradation of these proteins in lysosomes is stimulated by ATP.[426]

C. Substrate Posttranslational Modifications

Phosphorylation and glycosylation of proteinases can alter the susceptibility of proteins to proteolysis. The position of phosphate residues on a protein molecule is important in the regulation of the half-life of the protein. Phosphorylation of the microtubule-associated protein-2 by cAMP-dependent protein kinase decreases the degradation of this protein by calpain.[63] Phosphorylation of this protein by Ca^{2+} calmodulin-dependent protein kinase II does not protect against calpain degradation.[63] Phosphorylation of connexin-32 by protein kinase C protects against degradation, while phosphorylation by protein kinase A does not protect this protein.[427]

Oligosaccharides coupled to asparagine, threonine, and serine residues of proteins protect proteins from degradation. These residues increase the conformational stability of the proteins and protect the susceptible sites from proteolysis.[420]

Oxidized proteins are usually, but not always, more susceptible than unoxidized proteins to degradation by proteinases.[419] Many proteases degrade oxidized proteins more efficiently than native unoxidized proteins. For example, oxidized but not native Na^+/K^+ ATPase is digested by calpains I and II.[428] This oxidized protein is more efficiently degraded by cathepsin D than the native form.[428] Conformation changes are due to oxidations exposing hydrophobic groups that target proteins to proteasomes.[429] Dityrosine residues can be formed in the presence of H_2O_2 on some proteins.[430] This modification also targets proteins to proteasomes for degradation.

Covalent conjugation of proteins with multiple ubiquitin molecules can target proteins for degradation by the 26 S proteasome[3,4] or possibly by lysosomal enzymes.[5] Ubiquitin is a highly conserved 76-amino acid eukaryotic intracellular protein. This molecule is conjugated to abnormal and short-lived regulatory proteins by a series of regulated enzymatic reactions requiring ATP.[4] In the first step of the ubiquitin conjugation reaction, the ubiquitin-activating enzyme E_1 binds ubiquitin in an ATP-dependent step, forming an E_1-thiol ester between a cysteine residue on the enzyme and the C-terminal residue of ubiquitin. In the second step, activated ubiquitin is transesterified to a cysteine residue on one of a family of E_2-ubiquitin carrier proteins. These proteins mediate the transfer of ubiquitin to target proteins, including ubiquitin. Some proteins are bound to a carrier protein, E_3, during ubiquitination. Isopeptide bonds are formed between the C-terminal carboxyl group of ubiquitin and the ε-amino group of a specific lysine residue on the target protein. Additional ubiquitin molecules can then be added by the formation of isopeptide bonds between the ε-amino group of the Lys48 of one ubiquitin molecule and the C-terminal Gly residue of a second ubiquitin molecule.[431] Alternatively, multiubiquitin chains are directly conjugated to proteins.[432] Proteins con-

jugated with multiple ubiquitin chains are hydrolyzed readily by the 26 S ATP-stimulated proteinase complex[4] (Section V.G).

Many roles for ubiquitinated proteins have been proposed. These include involvement in DNA repair, cell cycle control, stress response, ribosome and peroxisome biogenesis, transcription, viral infection, and cellular differentiation.[5] Some of these systems may be related to the degradative mechanism of the ubiquitin system, while others may be independent of protein degradation.

IX. SUMMARY

Proteolytic activity in tissues is controlled at multiple levels. The importance of the various mechanisms depends on the given proteinase (Table 8). Not all mecha-

TABLE 8
Major Control Mechanisms of the Four Classes of Proteinases

Control mechanism	Proteinase class			
	Serine	Cysteine	Metallo	Aspartate
Synthesis	Some are constitutively synthesized. Synthesis can be stimulated.	Some are constitutively synthesized. Synthesis can be stimulated.	Can be constitutively synthesized. Stimulated synthesis is a major control mechanism.	Most are constitutively synthesized. Synthesis can be stimulated.
Zymogen activation	Major control mechanism except for cathepsin G and neutrophil elastase	Most are activated during processing except for some secreted enzymes.	Most secreted as the zymogen form; major means of control of activity	Major control mechanism for pepsin and renin; cathepsin D activated during processing.
Post-translational modifications	Urokinase-type plasminogen activator is phosphorylated. Glycosylation targets proteinases to secretory vesicles.	Glycosylation targets proteinases to the lysosome.	Glycosylation targets proteinases to secretory granules.	Glycosylation targets proteinases to the lysosome and secretory vesicles.
Sequestration	Important for secretory members of this family	Important for lysosomal members of this family	Important for neutrophil and macrophage forms of these enzymes	Important for secreted and lysosomal members of this family

TABLE 8 (continued)
Major Control Mechanisms of the Four Classes of Proteinases

Control mechanism	Proteinase class			
	Serine	Cysteine	Metallo	Aspartate
Membrane localization	Important for urokinase-type and tissue-type plasminogen activator and coagulation enzymes	Major control mechanism for calpains	Can be important in metastasis meprin and PABA peptidase are membrane localized.	Cathepsin E can be localized to membranes.
pH	Used to control activity of lysosomal members	Control of specificity; used to control lysosomal members	Controls level of activity	Control of cathepsin D in lysosomes; major control of pepsin.
Calcium	Stabilizes most; activates furin and prohormone-converting enzymes	Stabilizes most; activates calpains	Stabilizes most	Not a major factor
Proteoglycans	Important in lysosomes and secretory vesicles; major means of stimulating inhibition	Important in lysosomes and secretory vesicles	Not a major factor	Important in lysosomes and secretory vesicles
Inhibitors	Important	Important	Important	α2-Macroglobulin is the only known mammalian inhibitor.
Degradation	Important	Important	Important	Important

Note: See text for references.

nisms control all proteinases. The major means of control of proteinases are at the level of synthesis, zymogen activation, the active form of the enzyme, and proteinase degradation. The active form can be controlled by pH, calcium ions, ATP, and the formation of complexes with other proteinases, proteoglycans, and inhibitors. Proteinase activity toward a given protein depends upon its primary and tertiary struc-

tures and posttranscriptional modifications.

The importance of control mechanisms is realized when an imbalance occurs between these mechanisms and active proteinases levels. Examples of conditions that are associated with excessive amounts of proteolytic activity include emphysema,[391] arthritis,[97,416,433,434] malignancy,[32,72,99,435–437] pemphigus vulgaris (type Neumann),[438] and bullous pemphigoid.[439] Excessive proteinase inhibitory activity is associated with the formation of amyloid deposits associated with Alzheimer's disease[394,395,440] and Downs syndrome.[441]

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