ISJ 7: 165-180, 2010 ISSN 1824-307X

REVIEW

Role of α_2-macroglobulin in the immune responses of invertebrates

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Accepted June 30, 2010

Abstract

Although proteases play essential roles in the lives of all organisms, they are also important agents of disease and pathogenesis in metazoans. Most notably, proteases are essential virulence factors for a broad array of prokaryote and eukaryote parasites. One strategy used by the immune system of metazoans to defend against parasitic attack is to neutralize the toxins and essential virulence factors that allow the parasite to gain entry to the host and to survive and proliferate in the internal environment of the metazoan host. The particular defense system of interest to the present review is the system of endogenous protease inhibitors that operate to inactivate the secreted proteases utilized by invading parasites during the infection cycle within the host. Protease inhibitors are of two broad classes, active-site inhibitors that bind to and inactivate the active sites of target proteases and the α_2-macroglobulin class of inhibitors that operate as opsonins to bind and mark proteases in a manner that allows the subsequent endocytosis and intracellular proteolytic degradation of the α_2-macroglobulin-protease complex. Members of the α_2-macroglobulin class of inhibitors interact with target proteases by the novel process of enfolding the protease into a pocket within the interior of the α_2-macroglobulin molecule, which is followed by binding of the complex to the α_2-macroglobulin receptor at the surfaces of macrophages and other endocytotic cells and its endocytosis and degradation. In contrast to the active-site protease inhibitors, each of which is specialized to interact with a small subset of all endopeptidases, the α_2-macroglobulin inhibitors are remarkably promiscuous, binding proteases of all enzymatic classes and origins. This characteristic allows α_2-macroglobulin to play an important role in immune defense because this one protein is capable of binding and neutralizing the diverse array of proteases that function as virulence factors of the diverse array of parasites out there in the environment of metazoa.

Key Words: α_2-macroglobulin; thiol ester protein family; protease inhibitor; innate immunity

Introduction

An important barrier to the successful reproduction of metazoans is the disease and premature death that attends the attack by parasites. Barriers to parasitic attack are the integuments, which limit the range of parasites that can gain entry to the internal milieu, and the immune system, which defends against parasites and their toxic products both at the surface and in the internal spaces. Parasites may be unicellular or multicellular, prokaryote, eukaryote, or virus. In coelomate animals, the most important organ of the immune system is the blood, presumably because the blood has ready access to all parts of the body and is best prepared to concentrate defense effectors at sites of pathogen invasion. This review will be concerned with one of the important immune effector proteins found in the plasma of all major taxa of metazoans, α_2-macroglobulin.

α_2-Macroglobulin is an evolutionarily conserved element of the innate immune system whose best-characterized function is the clearance of active proteases from the tissue fluids. Proteases have a diverse set of essential roles in the lives of eukaryotes, including protein digestion, the activation of peptide hormones and other effector proteins that are secreted as zymogens, the physiological turnover of intracellular proteins, the removal of effete extracellular proteins, and

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remodeling of the extracellular matrix (Lopez-Otin and Bond, 2008). However, in addition, proteases, whether of endogenous or exogenous origin, are capable of considerable mischief when free in the blood and tissue spaces. They are important agents in a variety of connective tissue diseases (Perlmutter and Pierce, 1989), contribute to neoplastic invasion and metastasis (Deryugina and Quigley, 2006; Kessenbrock *et al.*, 2010), and are important virulence factors contributing to the pathogenicity of prokaryotic and eucaryotic parasites (Armstrong, 2006; McKerrow *et al.*, 2006). In response to protease challenge, animals have evolved a diverse array of protease inhibitors (Travis and Salvesen, 1983). In mammals, approximately 3-5 % of the plasma proteins are protease inhibitors (Laskowski and Kato, 1980) and in the American horseshoe crab, *Limulus polyphemus*, the hemolymph protease inhibitor, α_2-macroglobulin, is the third most abundant protein in the hemolymph (Enghild *et al.*, 1990). α_2-Macroglobulin is one of the most abundant proteins of human plasma, at a concentration of 2-4 mg/ml (Sottrup-Jensen, 1989) and is the second-most abundant protein of the hemolymph of the cephalopod, *Sepia* (Vanhoorelbeke *et al.*, 1993). α_2-Macroglobulin, the subject of this review, is present in representatives of several metazoan phyla, including coelenterates (Fujito *et al.*, 2010) and representative species of the Deuterostomia (Echinodermata, Tunicata, Vertebrata), Ecdysozoa (Arthropoda, Nematoda), and Lophotrochozoa (Mollusca) [for reviews see (Armstrong and Quigley, 1999; Armstrong, 2006)]. A diverse variety of Gram-negative eubacteria have acquired a gene encoding α_2-macroglobulin from eukaryote associates (Budd *et al.*, 2004; Doan and Gettins, 2008).

Mechanisms for attack on proteases

The protease inhibitors are of two fundamental classes, the active-site inhibitors, which bind to and inactivate the activate site of the targeted endopeptidase (Huntington *et al.*, 2000), and the α_2-macroglobulins, which react by a unique mechanism that involves the physical entrapment of the target protease within the folds of a molecule of α_2-macroglobulin (Table 1). By binding to the active site of the protease, the active-site inhibitors destroy both its proteolytic activity against proteins and its ability to cleave the ester and amide bonds of low molecular mass artificial substrates. Dedication for a particular active site means that the active-site inhibitors are restricted in their activity to one particular class, or even sub-class, of protease. By contrast, α_2-macroglobulin shows a unique mechanism for interaction with target proteases which is initiated by its proteolytic cleavage at a defined motif that is constructed as an exposed and highly flexible stretch of 30 - 40 amino acids that presents a suite of peptide bonds that are inviting targets for most extant proteases (Sottrup-Jensen *et al.*, 1989). This means that, whatever the protease, it will likely find a target for proteolytic attack at this "bait" region of the α_2-macroglobulin molecule. This is the basis for the broadly reactive ability of α_2-macroglobulin to bind proteases of diverse enzymatic reactivity and source. The forms of α_2-macroglobulin found in the tick (Saravanan *et al.*, 2003; Buresova *et al.*, 2009), horseshoe crab (Husted *et al.*, 2002), and carp (Mutsuro *et al.*, 2000) show multiple forms of the protein that differ by bait region sequence. Presumably this functions to expand the list of proteases recognized by the α_2-macroglobulins of these species. Proteolytic cleavage of the bait region is followed by the rapid re-folding of the α_2-macroglobulin molecule so as to produce an enclosed interior pocket that now contains the target protease, entrapped within the folds of the α_2-macroglobulin protein (Starkey and Barrett, 1973). Protease entrapment disables the enzyme's ability to proteolyze macromolecular substrates too large to penetrate the α_2-macroglobulin cage, but leaves intact the ability of the entrapped enzyme to hydrolyze low molecular mass substrates small enough to enter the α_2-macroglobulin cage and interact with the active site of the protease (Starkey and Barrett, 1973). One diagnostic feature to show that α_2-macroglobulin is responsible for a protease inhibitory activity of an uncharacterized sample is the demonstration that the sample inhibits proteolytic but not amidolytic activity of an exogenously added protease. I am not aware of any other enzyme inhibitor that operates by this unique "trap" mechanism.

The conformational change in the protease-activated α_2-macroglobulin molecule exposes a domain at its carboxy terminus, the receptor-binding domain, that now targets the molecule, with its entrapped cargo of protease, for receptor-mediated endocytosis and proteolytic degradation by phagocytes and other cells that display the α_2-macroglobulin receptor at the cell surface (Van Leuven, 1984). In this fashion, α_2-macroglobulin operates as an opsonin that delivers proteases of every enzymatic class to endocytotic cells of the innate immune system for internalization and destruction.

Several strands of evidence support this unusual scheme for the removal of potentially destructive proteolytic enzymes. Active site protease inhibitors block both the proteolytic activity and the amidase and esterase activities against low molecular mass substrates. In contrast, proteases bound to α_2-macroglobulin remain active against low molecular mass substrates while losing the ability to cleave peptide bonds of proteins (Barrett and Starkey, 1973; Quigley and Armstrong, 1983). This was interpreted to mean that the active site of α_2-macroglobulin-bound proteases remained intact but was sterically blocked from reacting with macromolecular substrates. Protein sequencing of α_2-macroglobulins from mammals (Sottrup-Jensen *et al.*, 1989) and a variety of invertebrates (Iwaki *et al.*, 1996; Husted *et al.*, 2002; Saravanan *et al.*, 2003) has identified the bait region as a defined stretch of approximately 30 amino acids that contains the protease-sensitive peptide bonds whose cleavage initiates the refolding of α_2-macroglobulin around the target protease. Protein

Table 1 Comparison of α_2-macroglobulin with active site protease inhibitors

α_2-Macroglobulin	Active site inhibitors
Inhibits the proteolytic activity of proteases without inhibiting the hydrolysis of low molecular mass amide or ester substrates	Inhibits activity of target proteases against polypeptide and low molecular mass substrates
Reacts with endopeptidases of diverse catalytic mechanisms and substrate specificities	Reacts with a narrow spectrum of related proteases
Shields bound proteases from antibodies and high molecular mass active site inhibitors	Bound proteases remain reactive with antibodies
Presence of a unique internal reactive thiol ester group	Internal thiol ester is found only in proteins of the α_2-macroglobulin family and C3 families (the TEP superfamily)

sequencing has facilitated the molecular characterization of the receptor-recognition domain that is exposed by proteolytic cleavage (Sottrup-Jensen *et al.*, 1986; Van Leuven *et al.*, 1986; Enghild *et al.*, 1989b; Holtet *et al.*, 1994). The molecular compaction responsible for the physical entrapment of a protease molecule was initially shown by the retarded elution of the protease-reacted form during gel filtration chromatography and by the increased electrophoretic mobility of the reacted form during non-denaturing polyacrylamide gel electrophoresis. This latter observation generated the term for the reacted and more compact form of the molecule as the “fast form” (Barrett *et al.*, 1979). The molecular compaction has also been demonstrated by direct electron microscopic comparison of α_2-macroglobulin molecules before and after reaction with a protease (Armstrong *et al.*, 1991).

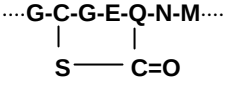
The spectrum of proteases that interact with α_2-macroglobulin is unusually broad and includes proteases of all major enzymatic classes. Although active-site protease inhibitors have been shown to function in immune defense (Kanost, 1999), the promiscuous reactivity of α_2-macroglobulin has adapted this single molecule to function as a particularly effective scavenger of the novel proteases introduced by parasites and pathogens. Indeed, the α_2-macroglobulins of vertebrates and invertebrates have been shown to react against the exoproteases of important parasites (Freedman, 1991; Araujo-Jorge *et al.*, 1992; Fryer *et al.*, 1996; Srimal and Armstrong, 1996; Scharfstein, 2006). The broad spectrum of reactivity of α_2-macroglobulin contrasts with the much more restricted reactivity of the naturally-occurring active-site protease inhibitors and is another feature that can be used to demonstrate that an uncharacterized protease inhibitor is, indeed, α_2-macroglobulin.

Interestingly, some microbial proteases are immune to certain versions of α_2-macroglobulin. In some instances, the protease is too large to fit into the interior hydrophilic pocket of the reacted form of α_2-macroglobulin [the collagenase of *Chlostridium perfringens* has a molecular mass greater than 100 kDa (Abe *et al.*, 1989)] but other non-reactive enzymes cleave peptide bonds not found in the bait region of certain versions of α_2-macroglobulin. Human α_2-macroglobulin lacks the lysine residues in the bait region targeted by the lysyl endopeptidase of *Acromobacter lyticus* and fails to react with that enzyme (Ikai *et al.*, 1999). A related example of this restriction is the relative insensitivity of the proteases of the *Limulus* clotting system to *Limulus* α_2-macroglobulin, but with a sensitivity of those same proteases to human α_2-macroglobulin (Armstrong *et al.*, 1984; Iwaki *et al.*, 1994). The *Limulus* clotting protease specifically cleaves the clottable protein, coagulogen, at the carboxyl sides of two Arg residues with the sequences Leu-Gly-Arg and Ser-Gly-Arg, which are conserved in coagulogens isolated from the four extant species of horseshoe crabs (Iwanaga *et al.*, 1992). *Limulus* α_2-macroglobulin lacks the target sequence in the bait domain, but Met-Gly-Arg is present in human α_2-macroglobulin, which may account for the differences in reactivity of these two forms of α_2-macroglobulin to *Limulus* clotting enzyme. Presumably, this is adaptive because blood clotting might be seriously compromised if the proteases necessary for the clotting reaction were rapidly inhibited by the principal protease inhibitor in the hemolymph. A similar situation may obtain in the hemolymph of the crayfish, *Pacifastacus*, in which the proteases involved in *activ*ation of the pro-phenol oxidase system, a component of the systems involved in humoral immunity in crustaceans, are unaffected by the α_2-macroglobulin

found in the hemolymph of that organism (Hergenhain *et al.*, 1987).

Entrapment in the α_2-macroglobulin cage both prevents the entrapped enzyme from accessing external proteins as substrates for proteolytic attack and protects the entrapped enzyme molecule from inactivation by macromolecular active site protease inhibitors. In other words, the protease molecule cannot get out of the α_2-macroglobulin cage, but neither can macromolecular protein inhibitors get in. This has allowed the development of a specific and semi-quantitative assay for α_2-macroglobulin, in which an uncharacterized sample is reacted first with trypsin, next with excess soybean trypsin inhibitor (STI, Mr 21.5 kDa), and then the residual trypsin activity is assayed with the low molecular mass amide substrate, BA*p*NA (Na-benzoyl-DL-arginine *p*-nitroanilide) (Ganrot, 1966; Armstrong *et al.*, 1985). Any trypsin not sequestered by α_2-macroglobulin is inactivated by STI and the only enzyme still able to hydrolyze BA*p*NA is that which is protected within the α_2-macroglobulin cage. In the absence of α_2-macroglobulin in the sample, degradation of BA*p*NA is zero. In the presence of increasing amounts of α_2-macroglobulin, the hydrolysis of BA*p*NA is increased in a dose-dependent fashion, indicative of increasing amounts of α_2-macroglobulin-sequestered, and thus protected, trypsin. A positive reaction in a properly controlled STI-protection assay is a sure indicator for the presence of α_2-macroglobulin in the sample, but the assay is subject to false negative responses that can occur if the sample also contains low molecular mass trypsin inhibitors small enough that they can enter the α_2-macroglobulin cage and inactivate bound enzyme or if the α_2-macroglobulin in the sample contacts proteases during sample collection and storage. Because α_2-macroglobulin is large, low molecular mass trypsin inhibitors can be removed by dialysis and premature reaction of α_2-macroglobulin can often be prevented by the inclusion of appropriate cocktails of low molecular mass protease inhibitors that can subsequently be removed by dialysis prior to assay with the STI-protection assay.

We have seen now three diagnostic features of the α_2-macroglobulin family of protease inhibitors: a broad reactive capacity against proteases of all classes, a unique molecular trap mechanism for interaction with target proteases, and a failure to inactivate the active site of the entrapped protease. A fourth diagnostic feature of the members of the α_2-macroglobulin family of proteins is the presence of a reactive internal thiol ester bond linking cysteinyl and glutamyl residues,



which in *Limulus* α_2-macroglobulin are Cys-999 and Glx-1002 (Iwaki *et al.*, 1996). The thiol ester bond is cleaved when α_2-macroglobulin experiences proteolytic attack on the bait domain, thereby exposing the cysteinyl thiol and the carbonyl of glutamic acid. This was seen by the exposure of a new titratable thiol group, the thiol of Cys-999 in *Limulus* α_2-macroglobulin (Armstrong and Quigley, 1987). The newly-exposed carbonyl group of the glutamyl residue is highly reactive and can engage in isopeptide bonding with ε-amino groups of lysines of protein targets in the reaction environment (Fig. 1). In human α_2-macroglobulin, crosslinking of the thiol esterified glutamic acid is preferentially with the entrapped protease (Sottrup-Jensen *et al.*, 1990b), whereas with α_2-macroglobulin from the American horseshoe crab, *Limulus*, the isopeptide bonds crosslink chains of α_2-macroglobulin itself (Dolmer *et al.*, 1996). Although the internal thiol ester of α_2-macroglobulin is stable in the intact protein, it will react with small primary amines such as ammonium and methylamine in the absence of proteolysis (Fig. 1). An important diagnostic feature of α_2-macroglobulin is its sensitivity to methylamine. The contribution of α_2-macroglobulin to the protease inhibitory activities of a biological sample is inactivated following methylamine treatment.

Protease clearance

Although α_2-macroglobulin is usually thought of as a protease inhibitor, it might better be considered a protease-binding molecule whose principal function is to deliver its protease cargo to an endocytotic protease clearance pathway. In this context, unreacted α_2-macroglobulin serves the recognition function and the protease-reacted fast-form α_2-macroglobulin serves the delivery function. The role of α_2-macroglobulin in protease clearance is especially well illustrated in *Limulus* because α_2-macroglobulin is the only protease inhibitor in the hemolymph (Quigley and Armstrong, 1983). Clearance was studied by injecting fluorescein-labeled proteins into the lumen of the heart and using a fluorometer to assay their concentration in blood drawn from the peripheral circulation at various times after injection. Native proteins such as hemocyanin or native α_2-macroglobulin (Fig. 2B) require approximately 10 min to exit the heart and mix with the blood of the peripheral circulation, and then remain at constant concentration. In contrast, trypsin (Fig. 2A) or the α_2-macroglobulin-trypsin complex (Fig. 2B) show rapid clearance from the hemolymph with a half-clearance time of 10 - 15 min. and maximal clearance at 20 - 25 min after dispersal from the injection site. Clearance of trypsin depends on its catalytic activity because trypsin that has been inactivated by treatment with phenylmethylsulfonylfluoride (PMSF) is not cleared (Fig. 2A). During the clearance stage, fluorescent trypsin is associated with a protein of high molecular mass. Later, fluorescence reappears in the circulation as a form with Mr < 10 kDa (Melchior *et al.*, 1995). Our interpretation of these observations is that the clearance of trypsin is mediated by binding to α_2-macroglobulin and the protease-α_2-macroglobulin complex is then degraded to low molecular mass components that later reappear in the hemolymph. The blood cell appears to be involved in clearance and degradation because

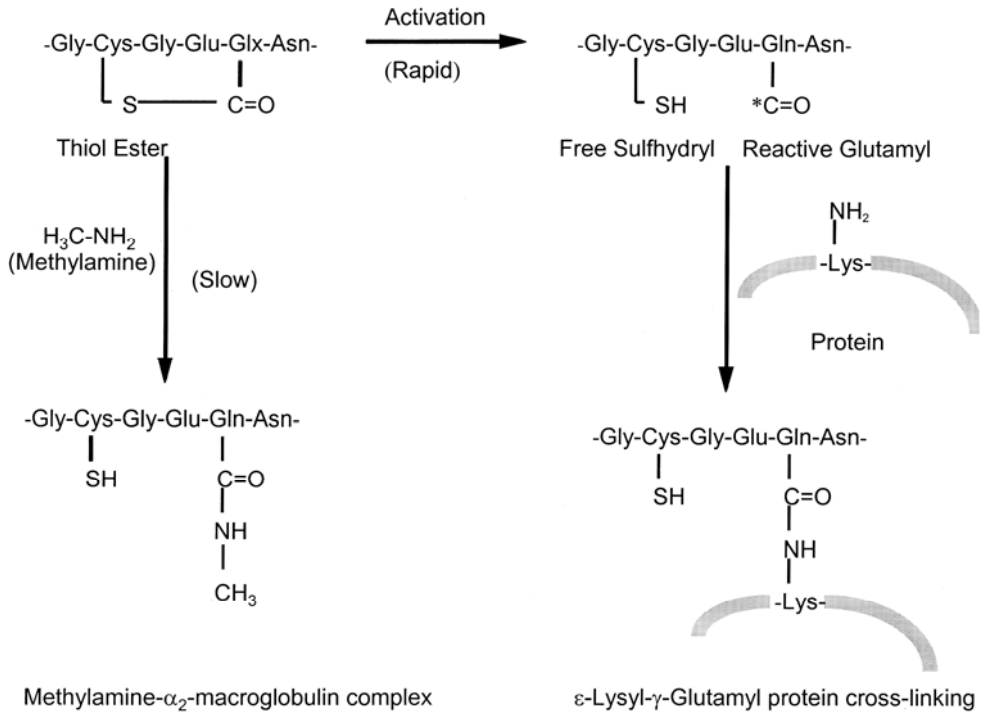


Fig. 1 Activation and cleavage of the internal thiol ester of α_2-macroglobulin exposes a new thiol group on the cysteine and a reactive (-carbonyl on the glutamyl residue, which in *Limulus* α_2-macroglobulin are Cys^{999} and Glx^{1002} . The reactive internal thiol ester of members of the α_2-macroglobulin protein family is cleaved following proteolysis at the distantly-located protease bait region of the protein. Thiol ester cleavage generates an activated (-carbonyl at the glutamyl residue and a free thiol at the cystenyl residue (top line of the diagram). The reactive glutamyl can form amide linkages with proteins (right arm of the diagram). The thiol ester can also react slowly with small primary amines, such as methylamine (left arm of diagram), even in the absence of proteolytic cleavage at the bait region. Methylamine treatment eliminates many of the functional activities of α_2-macroglobulin in parallel with its inactivation of the thiol ester. In general, sensitivity of a molecular function such as protease inhibition to treatment with methylamine is a useful test for the possibility that that function is dependent on the activity of a protein of the thiol ester protein family.

fluoresceinated trypsin and fast-form α_2-macroglobulin associate with the blood cells at the very stages that these proteins are being cleared from the hemolymph (Fig. 2B).

The conformational change of protease-reacted α_2-macroglobulin serves both to entrap the protease and to expose a previously cryptic domain at the carboxyl terminus, the receptor-binding domain (Fig. 3) (Sottrup-Jensen *et al.*, 1986; Van Leuven *et al.*, 1986; Enghild *et al.*, 1989b; Holtet *et al.*, 1994), that is recognized by cell surface receptors, leading to the binding and endocytosis of the protease-α_2-macroglobulin complex (Van Leuven, 1984). This removes the protease from the circulation, thereby neutralizing its potentially damaging actions. The three major steps in protease recognition, capture, and delivery are mediated in part by three identifiable domains of the α_2-macroglobulin molecule, the bait region, the thiol ester domain, and the receptor-recognition domain (Fig. 3). Presumably, there are additional domains that are under molecular strain in the unreacted form of α_2-macroglobulin and that are most actively involved in the active change in molecular shape following protease reaction and hydrolysis of the thiol ester, putative “hinge-domains”. In the absence of high resolution information of the structure of the native and fast-forms of the molecule, these have not been characterized.

The best-characterized mammalian receptor for protease-reacted α_2-macroglobulin (Jensen *et al.*, 1989; Moestrup and Gliemann, 1989; Ashcom *et al.*, 1990) has been identified as low density lipoprotein receptor-related protein-1 (LRP/α_2M-R, a.k.a. LRP1, CD91) (Kristensen *et al.*, 1990; Strickland *et al.*, 1990). CD91 is synthesized as a 600 kDa protein with a single transmembrane domain and is a member of the low density lipoprotein receptor family (for review see Lillis *et al.*, 2008). CD91, in addition to binding the α_2-macroglobulin-protease complex, is also active as a receptor for a diverse variety of other exogenous ligands and plays an important regulatory role in a diverse array of important cellular processes (Gonias *et al.*, 2004;

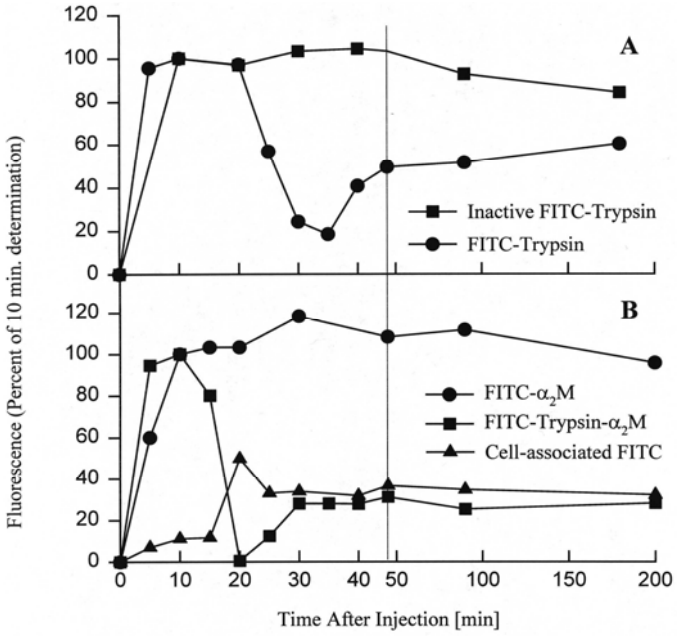


Fig. 2 Clearance of proteases from the hemolymph of *Limulus* is mediated by α_2-macroglobulin. Fluoresceinated proteins were injected into the lumen of the heart and their concentration in the peripheral hemolymph drawn from the leg joints was measured with a fluorometer. Fluoresceinated protein reached the periphery by 5 - 10 min after injection. Enzymatically active trypsin, but not trypsin inactivated by treatment with PMSF, was substantially cleared from the hemolymph by 30 min (Fig. 1A). The fluorescence that appeared at later times was of low molecular mass, and presumably consisted of peptide digest products of the injected protein. Fast-form, but not slow-form *Limulus* α_2-macroglobulin showed a similar pattern of clearance from the hemolymph. During the period of clearance of fast-form α_2-macroglobulin from the hemolymph, fluorescence accumulated in the blood cells. This is interpreted to indicate that clearance of the introduced protease is mediated by its binding to α_2-macroglobulin and that the blood cells participate in the uptake and clearance of the α_2-macroglobulin-protease complex.

May *et al.*, 2007). Of practical interest is its ability to bind two or more mols/mol of a 39 kDa endogenous ligand, LRP receptor-associated protein (RAP) (Ashcom *et al.*, 1990; Herz *et al.*, 1991). RAP functions as a dedicated chaperone and regulator of members of the LDL receptor family of proteins (Bu, 1998) and its high affinity binding makes RAP-conjugated Sepharose an appropriate affinity matrix for purification of these proteins.

To date, a receptor for protease-reacted α_2-macroglobulin has not been identified in an invertebrate. The family of proteins that includes vertebrate CD91 is, like α_2-macroglobulin itself, an ancient family that was present prior to the great evolutionary radiation that established the divergent deuterostome and protostome invertebrate superphyla. Representatives of the CD91 family have been found in modern representatives of lineages that diverged at the time of the Precambrian radiation, notably in vertebrates, the nematode, *Caenorhabditis elegans* (Yochem and Greenwald, 1993), and the arthropod, *Drosophila melanogaster* (Locus tag Dmel CG33087). Whether invertebrate orthologs of vertebrate CD91 operate as α_2-macroglobulin receptors has not been decided. The *Limulus* blood cell does contain a protein of very high molecular mass that, like CD91, binds RAP (Aimes *et al.*, 1995) and human CD91 binds to protease-reacted *Limulus* α_2-macroglobulin (Iwaki *et al.*, 1996). Although these observations hint at a possibility that the clearance pathway for protease-reacted *Limulus* α_2-macroglobulin involves an ortholog of CD91, they fall well short of any convincing demonstration of that prospect.

Phylogenetic distribution of the α_2-macroglobulins

Application of the criteria cited above has permitted the protein-based demonstration of α_2-macroglobulin in the plasma of lower vertebrates (Starkey and Barrett, 1982), arthropods, and molluscs (Armstrong and Quigley, 1999). The initial demonstration of α_2-macroglobulin in an invertebrate occurred when James Quigley and I stumbled upon the molecule while looking for fibrinolytic proteases active in the dissolution of blood clots in the American horseshoe crab, *Limulus polyphemus*. We failed to find a fibrinolytic system, but we did find a hemolymph protease inhibitor that

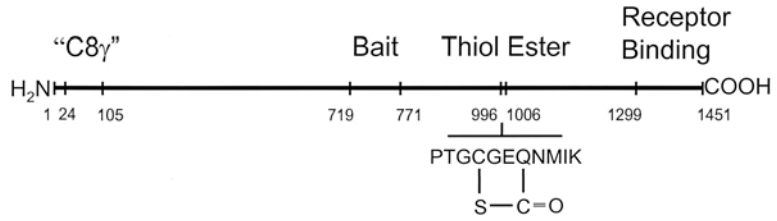


Fig. 3 Domain structure of *Limulus* α_2-macroglobulin. Based on the amino acid sequence of *Limulus* α_2-macroglobulin (Iwaki *et al.*, 1996), it is possible to identify important functional domains by comparison with the already established domain structure of human α_2-macroglobulin. The amino terminal end of *Limulus* α_2-macroglobulin has a unique stretch identified as the C8 (domain, based on modest sequence identity with the human defense protein C8 (of the mammalian complement pathway. The bait region contains a sequence of amino acids that present target sites for nearly all proteases. Proteolysis of the bait region initiates the compaction of α_2-macroglobulin that is responsible for entrapment of the molecule of reacting protease. The thiol ester domain contains the unique thiol ester bond linking cysteine-999 and glutamic acid-1002. The receptor-binding domain at the carboxyl terminus of the polypeptide chain is exposed following molecular compaction and makes the protease-reacted form of α_2-macroglobulin a target for binding to the cell surface α_2-macroglobulin receptor.

suppressed the action of serine, thiol, and metalloproteases against [^{14}C]-casein, a protein substrate, but that failed to suppress hydrolysis of the amide substate BA*p*NA by trypsin. The antiprotease activity of hemolymph was eliminated if *Limulus* hemolymph was treated with methylamine. This suggested the presence of α_2-macroglobulin in the hemolymph of this invertebrate (Quigley *et al.*, 1982). Our early analysis was facilitated by the absence of other trypsin inhibitors in *Limulus* hemolymph (Quigley and Armstrong, 1983). Subsequently, we showed that *Limulus* hemolymph protected trypsin from inactivation by the macromolecular active-site inhibitor, soybean trypsin inhibitor (Armstrong *et al.*, 1985), that purified *Limulus* α_2-macroglobulin did indeed contain an internal thiol ester domain (Armstrong and Quigley, 1987), and that the protein from *Limulus* shows significant sequence identity in key functional domains with human α_2-macroglobulin (Sottrup-Jensen *et al.*, 1990a; Iwaki *et al.*, 1996). Although overall sequence identity between human and *Limulus* α_2-macroglobulin is relatively low, the thiol ester domain shows significant sequence conservation, the receptor binding domains shows conservation of key basic amino acids, and the bait regions shows no sequence similarity.

The strict conservation of the thiol ester domain of the various α_2-macroglobulins has facilitated the molecular cloning of the genes for a variety of invertebrates from c-DNA libraries. With the advent of complete genomic sequences for an ever-increasing variety of species, data mining has revealed additional examples of genes encoding α_2-macroglobulin from these species. For example, the presence of genes for α_2-macroglobulin in Gram-negative bacteria was first demonstrated using the data-mining strategy (Budd *et al.*, 2004). These methods have identified both representatives of α_2-macroglobulin that, upon further investigation, have shown the unique suites of functional and structural characteristics of canonical α_2-macroglobulin, and have also revealed novel related proteins, several of which await functional characterization.

The α_2-macroglobulin family includes the canonical α_2-macroglobulins, a variety of similar protease-binding proteins, including pregnancy-zone protein of humans, α_1I_3 of the rat, ovomacroglobulin of avian and reptile eggs, and proteins to be discussed below that were initially identified from the vertebrate complement system (Sottrup-Jensen, 1987). Additionally, humans have two newly identified and incompletely characterized members of the family, CD109, which is linked to the cell surface by GPI-bonding (Lin *et al.*, 2002), and CPAMD8, an acute-phase protein expressed by kidney, brain, and testis (Li *et al.*, 2004), and the settlement-inducing protein of the barnacle *Balanus amphitrite* is an α_2-macroglobulin (Dreanno *et al.*, 2006). The different α_2-macroglobulins show differing orders of multimerization. Human (Jones *et al.*, 1972; Swenson and Howard, 1979) and snail (Bender and Bayne, 1996) α_2-macroglobulins are homotetramers, whereas many forms of the α_2-macroglobulins from a variety of lower vertebrates and arthropods are homodimers (Starkey and Barrett, 1982; Feldman and Pizzo, 1984; Sand *et al.*, 1985; Feldman and Pizzo, 1986; Spycher *et al.*, 1987; Enghild *et al.*, 1990; Armstrong *et al.*, 1991), and a few monomeric forms of α_2-macroglobulin have been identified (Enghild *et al.*, 1989a). As indicated above, the molecular homologues of the canonical α_2-macroglobulins have a wide phylogenetic distribution and share a defined suite of structural and functional characters (Table 2).

Thiol ester protein family

α_2-Macroglobulin is one of the founding members of the larger protein family, the thiol ester protein (TEP) super-family (Dodds and Law, 1998). The thiol ester proteins share a similar domain

Table 2 Molecular and functional conservation of the α_2-macroglobulins

Character	Specific attribute	Ref.
1. Genetic identity		
Overall peptide sequence identity	28-29 % identity of *Limulus* and mammalian α_2-macroglobulins	(Sottrup-Jensen *et al.*, 1984; Kan *et al.*, 1985; Gehring *et al.*, 1987; Van Leuven *et al.*, 1992; Iwaki *et al.*, 1996)
Bait region	Bounded by PXXC and EXXR consensus sequences in *Limulus* and mammalian α_2-macroglobulin	(Sottrup-Jensen *et al.*, 1989; Iwaki *et al.*, 1996)
Thiol ester domain	>50 % identity for α_2-macroglobulins of mammals, crustaceans, chelicerates, cephalopods, and gastropods	(Sottrup-Jensen *et al.*, 1984; Spycher *et al.*, 1987; Hall *et al.*, 1989; Enghild *et al.*, 1990; Sottrup-Jensen *et al.*, 1990a; Stocker *et al.*, 1991; Thogersen *et al.*, 1992; Bender and Bayne, 1996; Iwaki *et al.*, 1996)
Receptor recognition domain	Conservation of two of the key Lys residues of human α_2-macroglobulin in *Limulus* α_2-macroglobulin; of 73 residues conserved amongst the mammals, 45 are conserved in *Limulus* α_2-macroglobulin	(Nielsen *et al.*, 1996; Iwaki *et al.*, 1996)
2. Biochemical homology		
Susceptibility of bait region to proteolysis by proteases of all catalytic mechanisms	Reactivity of purified α_2-macroglobulins of mammals, arthropods and molluscs to serine, cysteine, and metalloproteases	(Starkey and Barrett, 1977; Quigley and Armstrong, 1985; Hergenhahn and Soderhall, 1985; Enghild *et al.*, 1990; Thogersen *et al.*, 1992; Bender and Bayne, 1996)
Molecular compaction; non-covalent trapping of proteases	Molecular compaction of protease-reacted α_2-macroglobulin of mammals and *Limulus*	(Barrett and Starkey, 1973; Barrett *et al.*, 1979; Quigley *et al.*, 1991; Armstrong *et al.*, 1991)
Covalent isopeptide bonding of thiol esterified glutamyl residue	Covalent bonding to trapped protease in mammalian α_2-macroglobulin; covalent cross-linking to Lys-254 of partner chain of α_2-macroglobulin dimer in *Limulus*	(Sottrup-Jensen *et al.*, 1980; Sottrup-Jensen *et al.*, 1990b; Jacobsen and Sottrup-Jensen, 1993; Dolmer *et al.*, 1996)
Protease treatment exposes the recognition stretch for receptor-binding	Thiol ester-reacted *Limulus* α_2-macroglobulin binds the human α_2-macroglobulin receptor	(Sottrup-Jensen *et al.*, 1986; Van Leuven *et al.*, 1986; Enghild *et al.*, 1989b; Iwaki *et al.*, 1996)
3. Functional similarity		
Protease clearance	Mammals and *Limulus* utilize α_2-macroglobulin for the clearance of proteases from the internal milieu	(Feldman *et al.*, 1985; Davidsen *et al.*, 1985; Melchior *et al.*, 1995)

architecture (Janssen *et al*., 2005; Doan and Gettins, 2007), sequence similarity in key functional domains, and most, but not all, contain a stable internal thiol ester bond linking the cysteinyl and glutamyl residues of the thiol ester motif. Members of this family that lack the internal thiol ester include complement component C5 (Tack, 1983) and ovostatin from the albumin of the chicken egg (Nagase *et al.*, 1983) and *Drosophila* Mcr (NCBI accession number Y11116). In all three proteins, the Cys has has been substituted with another residue (Nielsen and Sottrup-Jensen, 1993). But these proteins retain the other signature features that validate their assignment to the thiol ester protein family and ovostatin retains the ability to capture proteases by the molecular trap mechanism (Nagase and Harris, 1983).

The taxonomy of the TEP family is still a work in progress. One classification identifies two subfamilies, C3 and A2M (Sottrup-Jensen *et al.*, 1985; Fujito *et al.*, 2010) named for their founding members, human complement component C3 and human α_2-macroglobulin, respectively. A second scheme posits three distinct subfamilies, C3, A2M, and insect Tep. Members of the insect Teps show sequence relatedness intermediate between the C3 and A2M subfamilies and functional similarity to members of the C3 subfamily. They also, like C3, the canonical member of the C3 subfamily, have a Histidine residue (His 951 in *Drosophila* Tep1r; His 1,106 in human C3) in position to convert the reactive Glx of the activated thiol ester to an intermediate that favors its subsequent covalent linkage to hydroxyl groups rather than showing the default reactivity with amines (Dodds *et al*. 1966; Law and Dodds 1997). This residue is Asp, Asn, or Ala in thiol ester proteins where the covalent reactivity of the thiol ester is with amines. Subfamily membership is based on amino acid sequence and functional domain relatedness and is reflective of important functional differences between the members of the subfamilies. The best-characterized function of the A2M family members is protease binding, whereas the best-characterized function of the members of the C3 and insect Tep proteins is covalent binding to the surfaces of foreign cells to mark them for immune destruction (Dodds and Sim, 1997; Blandin *et al*., 2008). As will be described below, some members of the A2M subfamily, with well established protease-binding characters also, like members of the C3 and insect Tep subfamilies, bind to the surfaces of foreign cells and facilitate their phagocytotic uptake. I am unaware of any reports of members of the C3 or insect Tep proteins showing the unique protease capture abilities of the members of the a2M subfamily. The common ancestor of the TEP family is presumably ancient because representatives of both the C3 and A2M subfamilies are found in cnidarians, which are basal metazoans. The sea anemone, *Haliplanella* has two members of the C3 subfamily and two of the A2M subfamily (Fujito *et al.*, 2010). Representatives of both families have been identified in vertebrates (Sottrup-Jensen *et al.*, 1985), non-vertebrate chordates (Nonaka *et al.*, 1999), echinoderms (Al-Sharif *et al.*, 1998), and representatives of the Ecdysozoans, the horseshoe crab (Zhu *et al.*, 2005; Ariki *et al.*, 2008), and the Lophotrochozoans, a squid (Castillo *et al.*, 2009) and a clam (Prado-Alvarez *et al.*, 2009). The insect Tep subfamily has been reported only from insects and possibly nematodes (Blandin and Levashina, 2004), but not from other arthropods. It is important to note that the subdivision of the thiol ester proteins into just two or just three major subfamilies is not yet definitive. There are several TEP proteins that may not fit within a two- or three-subfamily classification schemes (Lin *et al.*, 2002; Li *et al.*, 2004; Dreanno *et al.*, 2006).

Although the best-characterized function of the proteins of the A2M subfamily, both from vertebrates and invertebrates, is protease binding, biochemical pathways other than protease clearance have been identified. The α_2-macroglobulins also modulate cell proliferation and cell survival pathways (LaMarre *et al.*, 1991; Shi *et al.*, 2008), protease-independent pathways of the innate immune system (Swarnakar *et al.*, 2000; Arnold *et al.*, 2006; Craig-Barnes *et al.*, 2010), antigen delivery in the operation of the adaptive immune system (Bowers *et al.*, 2009), and can operate as molecular chaparones (Yerbury *et al.*, 2009). As mentioned above, CD91, the canonical cell-surface receptor for protease-reacted α_2-macroglobulin recognizes and binds a diverse suite of ligands and is an essential agent for a diverse array of important biological processes.

The best-characterized function for members of the C3 subfamily is their covalent binding to the surfaces of foreign cells to target them for immune destruction, a function shared with members of the insect Tep subfamily. An appealing example of this is the protein TEP1, the mediator of an important pathway for the immune defense of the mosquito vector against the protozoan parasite, *Plasmodium falciparum*, the agent of human malaria (reviewed in Blandin *et al*., 2008). TEP1 is a member of the thiol ester protein family from the *Anopheles* mosquito. TEP1 binds to bacteria and *Plasmodium* cells and targets the cells for phagocytosis. The experimental elevation of the concentration of TEP1 in the hemolymph of the mosquito reduces infection rates and experimental reduction increases susceptibility of the mosquito to infection (reviewed in Volohonsky *et al.*, 2010). Different members of the insect Teps show specialization of their targeting to the surfaces of different classes of microbes, for example, with *Drosophila* Mcr targeting *Candida albicans* cells, *Drosophila* TepIII targeting *S. aureus,* and *Drosophila* TepII targeting *E. coli* (Stroschein-Stevenson *et al.*, 2006).

Prospects for future research

It is always presumptuous to predict the direction of research in any field of biology; the natural world holds many unexpected surprises and the ingenuity of biologists to identify and investigate those surprises seems without limit. That being said, some prospects for the future study of α_2-macroglobulin and of other members of the thiol ester protein superfamily are appropriate for a

review of this topic. A full characterization of the function(s) of the α_2-macroglobulins in a diverse array of species will involve both the functional characterization of the molecule *in vitro*, which has been the principal topic of this review, and the elucidation of its function *in vivo*. What functions in the animal require α_2-macroglobulin? The gene knock-out mouse has been available for a decade and a half and shows a surprisingly mild phenotype (Umans *et al.*, 1995; Umans *et al.*, 1999). Interpretation is complicated by the artificially sanitary conditions of the life of the laboratory mouse. This mouse model will be particularly interesting when its sensitivity to an extended suite of protease-wielding pathogens is investigated (Coutinho *et al.*, 1999). In appropriate model invertebrates, RNAi knock-down trials are possible and reduction of expression of various TEPs have been shown to significantly diminish host resistance to infection by some, but not all pathogens (Buresova *et al.*, 2009; Volohonsky *et al.*, 2010). One imagines refinements on this strategy where modified versions of α_2-macroglobulin are used to reconstitute function in α_2-macroglobulin-depleted animals. In this context, the modified α_2-macroglobulin constructs might feature bait regions with enhanced or restricted sensitivity to the various proteases of an appropriate pathogen (Ikai *et al.*, 1999). These could provide information on the roles of different proteases of the pathogen in the infection cycle and on the precise roles for α_2-macroglobulin in immune defense. For example, might forms of α_2-macroglobulin modified to include the appropriate novel cleavage sites for previously unrecognized microbial proteases now offer protection to the host from those bacteria?

A related challenge to the functional characterization of the diverse members of the thiol ester protein family is the high resolution structural characterization of representative thiol ester protein family members for a detailed understanding of the relation of protein structure to the several functions of different members of this protein family. The goal of establishing a detailed understanding of the relations between protein structure and function is best exemplified by the characterization of human C3 (Janssen *et al.*, 2005) and an insect Tep (Baxter *et al*., 2007). The establishment of a high resolution structural characterization of α_2-macroglobulin has proven elusive (Jenner *et al*.,1998) but the insights provided by the characterization of the domain structure of C3 have been used to develop a model for human α_2-macroglobulin (Doan and Gettins, 2007). One additional bonus of this line of research will be the provision of information for the refinement of the still-unsettled molecular taxonomy of the thiol ester protein superfamily.

It will be interesting and illuminating to identify and characterize the clearance pathways for protease-reacted α_2-macroglobulin in taxa other than mammals. To date, the sole experimental study is the investigation of the protease clearance pathway for the horseshoe crab (Melchior *et al.*, 1995) and this has implicated a cell-based clearance pathway that is selective for fast-form α_2-macroglobulin. Initial evidence described above hints at a role for a role for a cell surface receptor with properties similar to mammalian CD91 in this pathway. Although CD91 is the best-characterized cell surface receptor for mammalian α_2-macroglobulin, a second cell surface protein, GRP78, has been shown to bind protease-reacted α_2-macroglobulin with nM affinity (Quinones *et al.*, 2008; Gonzalez-Gronow *et al.*, 2009). GRP78 is a protein found in diverse eukaryotes and it will be interesting to discover if this or other undiscovered receptors operate to bind α_2-macroglobulin in different metazoans.

In mammals, both α_2-macroglobulin and its receptor, CD91, have been shown to contribute to the operation of a number of physiological processes that are independent of protease clearance. It will be interesting to identify and characterize the possible diversity of functions of α_2-macroglobulin in invertebrates. A scattering of examples have already been identified. In invertebrates, α_2-macroglobulin also regulates the activities of other immune effector proteins. In the shrimp, *Penaeusmonodon*, α_2-macroblobulin binds syntenin (Tonganunt *et al.*, 2005). Syntenin is an acute-phase protein that is dramatically up-regulated in shrimp infected with the white spot syndrome virus. In the horseshoe crab, *Limulus*, protease-activated, but not native α_2-macroglobulin binds and inactivates the cytolytic actions of limulin (Armstrong *et al.*, 1998; Swarnakar *et al.*, 2000). Cytolytic destruction of foreign cells is a ubiquitous strategy of metazoan immune systems (Canicatti, 1990; Gabay, 1994). In mammals, cytolytic destruction of foreign cells is mediated in part by the multi-protein complement system (Law and Reid, 1995), whereas the hemolymph cytolytic pathway of *Limulus* is mediated by the single protein limulin, a sialic acid-binding member of the pentraxin gene family (Armstrong *et al.*, 1996; Harrington *et al.*, 2008). The adaptive significance of the α_2-macroglobulin-limulin binding reaction has not been identified, but the pentraxins of *Limulus* are multi-functional mediators of immunity, with potentially important roles in the inactivation of bacteral lipopolysaccharide (Ng *et al.*, 2004) and in the formation of hydrophilic pores across the lipopolysaccharide-rich outer membrane of Gram-negative bacteria (Harrington *et al.*, 2009). It will be interesting to discover if the binding to protease-reacted α_2-macroglobulin affects other functions of the *Limulus* pentraxins or the pentraxins of other invertebrates. Perhaps the reduced cytolytic activity of limulin complexed with α_2-macroglobulin is correlated with augmentation of other immune functions of limulin.

The recent identification of members of the C3 subfamily of TEP proteins in diverse invertebrate taxa (Zhu *et al.*, 2005; Fujito *et al.*, 2010) and the insect Tep subfamily in insects (Blandin and Levashina, 2004; Blandin *et al*., 2008) have opened fertile fields for investigation. Functional characterization of proteins of this class indicates that they operate much as C3 of the vertebrate complement system by their covalent binding to the surfaces of foreign cells to mark them for

subsequent immune destruction. It will be interesting to determine if this functional characterization is universal amongst metazoans or whether there is functional diversity for members of the C3 family from different taxa. For example are there instances where proteins of the C3 or insect Tep sub-families function in a manner similar to the A2Ms as protease binding proteins in addition to their canonical cell-binding functions or are there members of the A2M family that, in addition to the display of an ability to bind proteases, also function as opsonins that promote the phagocytosis of foreign cells that have become decorated with surface-associated α_2-macroglobulin? For example, murine α_2-macroglobulin binds to the surfaces of the pathogen, *Trypanosome cruzi* (Coutinho *et al.*, 1997), and a form of α_2-macroglobulin from a hard tick binds target bacteria and promotes their ingestion by phagocytes (Buresova *et al.*, 2009).

Conclusion

It is the solemn duty of every animal to live to adulthood and to reproduce the species. Survival requires efficient means to thwart the myriad invading pathogens that would compromise that survival. Since many invertebrates regularly live to a considerable age, at least 20 years for *Limulus*, 80 years for lobster, and 375 years for the ocean quahog, a mollusc (Finch, 1990; Philipp and Abeke, 2010), while inhabiting highly septic environments, they have to possess efficient immune processes. These are largely of the innate class of immune systems, because invertebrates lack lymphocytes and the traditional RAG-mediated adaptive immune system, which are restricted to the vertebrate lineage (Marchalonis and Schluter, 1990; Agrawal *et al.,* 1998). As we and others have shown, certain of these innate immune systems arose early in evolution and have been faithfully preserved in species of diverse lineage, and inhabiting very different environments and displaying very different life styles.

For example both vertebrates and arthropods utilize the pentraxin (a.k.a., the C-reactive protein) and the TEP families of proteins to protect from invading parasites. Interest in immunity in invertebrates is driven by a basic curiosity in how species other than our own survives pathogenic attack and may have application to the development of rational veterinary care for aquacultured invertebrates of commercial importance and the development of improvements in the application of disease organisms for the biological control of invertebrates that are agricultural pests or disease vectors.

List of abbreviations:

A2M, the α_2-macroglobulin family of the TEP protein superfamily; BApNA, Na-benzoyl-DL-arginine p-nitroanilide; C3, the complement component 3 family of the TEP protein superfamily; CD91, the canonical cell-surface receptor for fast-form α_2-macroglobulin and also known as LRP1, LDL-receptor-related protein/α_2-macroglobulin-receptor; MA, methylamine, a small primary amine that reacts with the internal thiol ester of the TEPs; PMSF, phenylmethylsulfonylfluoride; RAP, CD91-associated protein, a natural ligand of CD91; STI, soybean trypsin inhibitor; TEP, the superfamily of proteins characterized by the presence of a stable internal thiol ester motif and other conserved motifs.

Acknowledgments

Supported by grant 0344360 from the National Science Foundation.

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