Receptor-Mediated Antigen Delivery into Macrophages

Complexing Antigen to α2-Macroglobulin Enhances Presentation to T Cells

Charleen T. Chu and Salvatore V. Pizzo

Department of Pathology, Duke University Medical Center, Durham, NC 27710

ABSTRACT. Macrophages secrete α2-macroglobulin (α2M), a protein that may facilitate early Ag handling. α2M is able to entrap and form covalent linkages with diverse proteins during a transient proteinase-activated state. The resulting complexes are rapidly endocytosed after binding to high affinity receptors. Such a system could be capable of efficiently delivering a multitude of proteins to macrophages. We have used T hybridoma clones that respond only to hen egg lysozyme, in a MHC-restricted manner, to probe the effect of complex formation on Ag uptake and processing by murine macrophages. Radiolabeled lysozyme was internalized more rapidly and to a greater extent when bound to α2M than when unbound. Macrophages pulsed with lysozyme-α2M-elastase complexes required 200 to 250 times less Ag than those pulsed with free lysozyme to achieve effective presentation to T cells. Adding equimolar amounts of α2M-elastase complexes, or of α2M-methylamine, to free lysozyme had no effect on basal lysozyme presentation. Receptor-recognized forms of α2M, but not lysozyme or BSA, competed effectively for both uptake and presentation of lysozyme-α2M-elastase complexes. These results indicate that proteinase-activated α2M can enhance Ag processing by carrying Ag into macrophages through a receptor-mediated process. Journal of Immunology, 1993, 150: 48.

The effective internalization and processing of diverse proteins forms a central issue in Ag presentation by macrophages. The immune system must balance the capacity for interacting with vast numbers of dissimilar molecules with the requirements for efficiently responding to very low amounts of Ag. Although macrophages are able to sample their environments through pinocytosis, a need for more efficient means of internalization, such as a receptor-mediated system, has been suggested (1). The targeting of Ag to surface receptors on macrophages or B cells, either by artificial cross-linking or by exploiting membrane Ig, enhances the efficiency of presentation (1–3); however, a naturally occurring system in macrophages has not yet been identified.

α2M is an abundant protein with the intrinsic capacity for irreversibly capturing diverse proteins for rapid delivery into cells. It consists of four identical subunits arranged to form a double sided molecular “trap” (4). This trap is sprung when proteolytic cleavage within a highly susceptible stretch of amino acids, the “bait region,” initiates an electrophoretically detectable conformational change that entraps the proteinase (5). In addition to being noncovalently trapped, lysine-containing proteinases can spontaneously form covalent linkages by nucleophilic substitution at a thiolester located on each of the α2M subunits.

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1 This work was supported by Grants HL-24066 and CA-29589 from the National Institutes of Health, Bethesda, MD.

2 Address correspondence and reprint requests to Charleen T. Chu, Department of Pathology, Box 3712, Duke University Medical Center, Durham, NC 27710.

3 Abbreviations used in this paper: α2M, α2-macroglobulin; s-α2M, the native conformational form of α2M; f-α2M, the receptor-recognized forms of α2M derived from treatment with proteinase or with methylamine; HEL, hen egg lysozyme; PPE, porcine pancreatic elastase.
This thiolester becomes highly reactive during the conformational transition from native, or "slow" $\alpha_2$M (s-$\alpha_2$M) to the more compact "fast" forms (f-$\alpha_2$M) (7, 8). The resulting receptor-recognized f-$\alpha_2$M is efficiently internalized by macrophages and other cells that express the $\alpha_2$M receptor (reviewed in Reference 9), which has recently been cloned and sequenced (10, 11). Receptor-recognized $\alpha$-macroglobulins from different animal species cross-react with similar affinities for the $\alpha_2$M receptor regardless of the proteinase used (see References 9, 12, and 13 for review). The additional binding of nonproteolytic proteins does not appear to affect the rate of internalization even when artificial cross-linking is employed (14–16). Therefore, regardless of the mechanism of binding, any proteins complexed with f-$\alpha_2$M can be effectively internalized.

The biologic role of $\alpha_2$M is unknown. Although usually considered a protease inhibitor, $\alpha_2$M possesses several properties that suggest its function extends beyond simple proteinase inhibition. Proteinases representing each of the major mechanistic classes are capable of cleaving the $\alpha_2$M "bait region" and becoming entrapped. However, these proteinases are not so much inhibited as sterically shielded, retaining activity against small substrates (5, 17).

Many of these proteinases also possess more efficient and specific inhibitors in vivo (reviewed in Reference 18). Although an evolutionarily conserved thiolester efficiently forms covalent cross-links with proteinases, covalent linkage is not necessary for "inhibition" (7). Covalent linkage does comprise, however, a major mechanism by which $\alpha_2$M binds nonproteolytic proteins (7, 19, 20).

Many nonproteolytic proteins and peptides appear to bind to $\alpha_2$M (7, 15, 20), including growth factors (see Reference 21 for review). We have previously established the mechanism by which porcine insulin is bound by $\alpha_2$M (20). Insulin incorporation proceeds most rapidly and to the highest extent during proteinase-induced conformational change. Such binding is predominantly covalent and readily occurs at physiologic concentrations of $\alpha_2$M and insulin. Sequence analysis of the complex and the use of competing nucleophiles confirm that the covalent incorporation mechanism is of general applicability, extending beyond just proteinases or growth factors.

Although size and charge may affect the extent of binding, $\alpha_2$M possesses the intrinsic ability to nonselectively incorporate proteins and peptides bearing nucleophilic amino acid side chains. This covalent incorporation is, however, temporally specific, occurring only during the proteinase-initiated conformational change and before decay of the activated thiolester (20). Upon completion of conformational change, the complexes are rapidly cleared from the circulation by high affinity receptors ($K_d \sim 1$ nM at 4°C) (13). Thus, $\alpha_2$M is versatile enough to bind diverse proteins, yet displays highly conserved receptor recognition signals that allow targeting to macrophages. Such a system could effectively enhance delivery of potential Ag to macrophages.

We proposed that this thiolester-mediated covalent bond formation may reflect an important functional role. Using T hybridoma cells specific for HEL, in conjunction with various HEL and/or $\alpha_2$M derivatives, we probed the effect of HEL-$\alpha_2$M complex formation on Ag uptake and processing by murine macrophages. The results indicated that $\alpha_2$M was capable of mediating receptor-facilitated Ag delivery to macrophages, enhancing presentation of the Ag to specific T cells.

Materials and Methods

Materials

HEL was purchased from Boehringer Mannheim (Indianapolis, IN). $\alpha_2$M was purified as previously described (22, 23), except that buffers made from pyrogen-free sterile water (Abbott Laboratories, Chicago, IL) were used for extensive washing and elution. Fractions were analyzed by pore limit gel electrophoresis in a Tris/boric acid/EDTA-buffered system (24), (modified from 25), and those fractions containing any trace amounts of "fast" form were discarded, resulting in material that was >98% native, as determined by 5',5'-dithiobis(2-nitrobenzoic acid) titration (20, 24). PPE of the higher purity grade was purchased from Sigma (St. Louis, MO). Human neutrophil elastase was the gift of Drs. James Travis and Wieslaw Watorek, University of Georgia, Athens, GA. Carrier-free $^{125}$I[Na and methyl-$^{3}$H]TdR were obtained from New England Nuclear (Boston, MA).

Radiolabeling

HEL and PPE were radiolabeled using an Iodo-Bead (Pierce, Rockford, IL), employing conditions recommended by the manufacturer, and desalted on PD-10 columns (Pharmacia, Piscataway, NJ). All $^{125}$I-labeled materials were counted in a LKB-Wallac Clinigamma counter 1272 (LKB, Piscataway, NJ).

The concentrations of proteins in solution were determined in a Shimadzu UV 160 U spectrophotometer (Columbia, MD), using the following constants:

\[
A_{280 \text{ nm}} = 8.93 \text{ for } \alpha_2 \text{M} \quad (26),
\]

\[
A_{280 \text{ nm}} = 26.5 \text{ for } \text{HEL} \quad (27),
\]

and \[
A_{280 \text{ nm}} = 10 \quad \text{as determined for}
\]

$\alpha_2$M-HEL complexes using amino acid analysis (28).
Characterization of HEL incorporation

The binding of radiolabeled HEL to α2M was analyzed by systematically varying conditions as previously described for insulin (20). Incubation products were analyzed by gel electrophoresis and autoradiography using native 4 to 20% Tris/boric acid/EDTA gels (24) and denaturing 5 to 15% polyacrylamide gradient gels in an Ammediol-buffered SDS system (29). Radioactive bands were excised and counted after autoradiography.

Ag preparations

HEL-α2M-PPE complexes were prepared in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HEPES buffer) by incubating 1.4 μM α2M with a twofold molar excess of PPE for 15 min at room temperature in the presence of a 100-fold molar excess of HEL. α2M-PPE complexes were formed in similar incubations omitting the HEL. After addition of 3,4-dichloroisocoumarin (100 μM) to inhibit bound and unbound proteinase, complexes were separated from unbound HEL and PPE by gel filtration utilizing a Sephacryl S-300 HR column (Pharmacia/LKB), as previously described (20). Proteinases were active-site standardized as previously described (30). α2M-methylethylamine was formed as previously described (31). All buffers used were prepared with pyrogen-free water and filtered through 0.22-μm filters.

Solutions of HEL and of the complexes were passed through a 2-ml Detoxi-Gel column (Pierce) several times. Final endotoxin levels in the presentation assays were <0.1 ng endotoxin/ml, as assessed by Pyrotell Limulus amebocyte lysate clotting times (Associates of Cape Cod, Woods Hole, MA) using a THERMOMax microplate reader (Molecular Devices, Menlo Park, CA) according to the manufacturer’s recommendations. Native α2M was inhibitory in this assay, probably because of its proteinase inhibitory properties; therefore, those samples were heat inactivated before assay. Ag preparations were concentrated in sterilized Centriprep-30 concentrators (Amicon, Beverly, MA) and filtered through 0.22-μm filters (Millipore, Bedford, MA). Ag dilutions were prepared immediately before use in 96-well polypropylene plates (Costar, Cambridge, MA).

Cell lines

Peptide-induced peritoneal macrophages were harvested from female CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) and allowed to adhere to 96-well tissue culture plates (Costar) for 2 h before use (32). The 3A9 T cell hybridoma line specific for residues 52-61 of HEL (33, 34), and the IL-2-dependent CTL line (CTLL-2) (35) were kindly provided by Peter Cresswell, Yale University, New Haven, CT. Both cell lines were grown in RPMI 1640 (GIBCO, Gaithersburg, MD), supplemented with 10% heat-inactivated newborn calf serum (Hyclone Laboratories, Logan, UT), 1% (v/v) l-glutamine (200 mM, GIBCO), and 0.5% (v/v) gentamicin solution (10 mg/ml, GIBCO). The CTLL cell media was further supplemented with 20% rat spleen cell Con A supernatant, prepared as previously described (36).

Uptake studies

Internalization of [125I]HEL-α2M-PPE complexes and of free [125I]HEL were studied at 37°C using monolayers of macrophages plated on Dynatech (Chantilly, VA) Removacell plates. The respective Ag were diluted in HEPES buffer containing 10 mg/ml BSA to obtain the desired concentrations of radiolabeled Ag and of unlabeled competitors. This was added to macrophages in RPMI 1640. After specified time intervals, the incubation was terminated by aspiration, and the cells underwent extensive ice-cold washes. Wells were allowed to air dry before counting in a gamma counter.

Ag presentation assay

A well characterized in vitro Ag presentation system was used (33, 34, 37). Macrophages (105) pulsed with dilutions of various HEL- and/or α2M-derivatives in serum-free media were tested for their ability to induce IL-2 secretion by 3A9 T hybridoma cells (106). Extensive washing with RPMI 1640 over a 30-min period was employed to remove uninternalized Ag from the 96-well plates before addition of 3A9 cells in serum-containing media. After 24 h of incubation at 37°C, 100 μl of culture supernatant was removed and frozen at −70°C to lyse any transferred cells. In some experiments, Ag, 3A9 cells, and macrophages were coincubated for 24 h followed by harvesting of the supernatant. The 3A9 T hybridoma response was quantified by measuring the amount of IL-2 released into the supernatants using 3H]TdR incorporation in the CTLL-2 line (36). A Skatron automated combi-harvester (Sterling, VA) was used, followed by counting in a Packard Minaxiβ Tri-Carb 4000 liquid scintillation counter (San Fernando, CA). The values from control incubations that lacked Ag, which were <3.5% of the total, were defined as the base line and subtracted.

Results

Characterization of HEL binding to α2M

The interaction between HEL and α2M was characterized, and the predominant mechanism of binding was found to parallel that of insulin (20). Covalent binding of HEL occurred only if HEL was present during proteinase-induced α2M conformational change (Fig. 1B, lanes b). Native gels demonstrated that there was more adher-
FIGURE 1. Binding of [125I]HEL to different conformational forms of α2M. A twofold molar excess of [125I]HEL was incubated for 30 min at room temperature with 1.4 μM amounts of native α2M, α2M undergoing PPE induced conformational change, or preformed f-α2M. The PPE was inhibited by addition of 100 μM dichloroisocoumarin after 10 min. Samples were analyzed by SDS-PAGE under reducing (R) and nonreducing (NR) conditions (A and B) and by native pore limit gel electrophoresis (C and D). Coomassie blue-stained gels (A and C) and their corresponding autoradiograms (B and D) are shown. Four reactions were analyzed as indicated: (a) s-α2M + [125I]HEL, (b) s-α2M + [125I]HEL + subsaturating PPE (~ equimolar with α2M), (c) α2M pre-treated for 10 min with PPE, which was inhibited with dichloroisocoumarin, before addition of [125I]HEL, (d) [125I]HEL + methylamine-treated α2M. The arrow represents the position of migration for HEL that is covalently complexed to the C-terminal half of the "bait-region"-cleaved α2M, when analyzed after reduction. Molecular weight standards are indicated on the left: denatured but nonreduced α2M (360 kDa), reduced α2M (180 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), OVA (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Positions of migration for α2M conformational forms were determined using native α2M (s-α2M) and methylamine-treated α2M (f-α2M).

ence to f-α2M than seen with insulin (D, lane c), probably because of the basic nature of HEL, but total binding was greatest upon coincubation of HEL, α2M, and proteinase (D, lane b).

The Coomassie-stained SDS gel (A) reveals a typical fragmentation pattern for α-macroglobulins (38). Under nonreducing conditions, the 720-kDa protein dissociates into disulfide-linked dimers (360 kDa). Under reducing conditions, α2M migrates as its constituent 180-kDa subunits. Denaturation and boiling promotes autolytic cleavage at the thioesters, resulting in the characteristic 120-kDa and 60-kDa bands (39). Proteinase-treated α2M migrates as a doublet (~90 kDa) (40) when examined by reducing SDS-PAGE (A, lane c); the binding of HEL to the thioester-containing fragment resulted in the appearance of a new band whose position is marked by the arrow (A, lane b). All of the bound [125I]HEL migrated at this position. Some uncleaved α2M subunits (180 kDa), and a small amount of material that underwent autolytic cleavage (120 kDa and 60 kDa), can be seen in the reduced proteinase-treated samples (A, lanes b and c) because a subsaturating amount of PPE relative to α2M was used. The band just below the intact subunits in lanes b and c represent PPE-bound to the thioester-containing fragment (7). All of the α2M-PPE and HEL-α2M-PPE complexes used in the cellular assays were formed using saturating levels of PPE, and were repurified by gel filtration. When analyzed by reducing SDS-PAGE, there were no extraneous bands other than the expected doublet or triplet, respectively.

To further verify that covalent HEL incorporation into α2M reflected the general mechanism of nucleophilic displacement at the activated thioester, an α-effect nucleophile β-aminopropionitrile, which has previously been shown to compete for incorporation into the Glix of the activated α2M thioester, was employed (7, 20). This nucleophile effectively competed away all covalent binding (Fig. 2A), confirming the mechanism. In contrast to proteinase binding to α2M, where β-aminopropionitrile competes away covalent binding, but does not affect noncovalent "trapping" (not shown) or "inhibition" (7), the loss of covalent HEL binding resulted in a loss of >85% of total binding. This may indicate that HEL, being smaller than most proteinases, is not effectively trapped and requires covalent bond formation to remain associated with α2M.

In order to fully characterize the composition of HEL-α2M-PPE complexes for use in the cellular assays, dose response studies were conducted to determine the stoichiometry of the bound components. Under saturating conditions, about 1 mol HEL (Fig. 2B) and 1 mol of PPE (not shown) were bound to each mole of α2M. About 85% of HEL binding was covalent and resistant to reduction, again supporting the existence of a γ-glutamyl linkage. The use of human neutrophil elastase as the activating proteinase also resulted in a maximum of 1 mol HEL bound/mol α2M, but in this case, binding was 100% covalent. These results differ from those of insulin, which demonstrated a maximum of 3.7 mol bound/mol α2M (20), probably reflecting the greater size of HEL relative to insulin. Proteinase trapping, which was usually ~1.5/1 (mol PPE/mol α2M) in the absence of HEL, decreased to 1/1 as HEL binding increased, lending further evidence that HEL was inside the trap.
Internalization of HEL derivatives by macrophages

After characterizing the composition of the various complexes, their relative rates of uptake by macrophage monolayers were compared. In these uptake assays, as well as in the subsequent Ag-presentation assays, concentrations of the HEL-α2M-PPE complexes were adjusted relative to that of free HEL so that the corresponding incubations contained the same molar amount of HEL, whether bound or free. Because f-α2M tended to precipitate during concentration, and because the calcium and magnesium requirements of α2M receptor-mediated endocytosis (9, 41) necessitated diluting Ag preparations into media, concentrations of HEL-α2M-PPE greater than about 1 μM could not be examined. Small primary amines, such as methlylamine, can directly substitute into the thiolester, resulting in f-α2M complexes that lack proteinase (42). Methlylamine-treated α2M interacts with the receptor in a manner indistinguishable from α2M-proteinase complexes (23). Thus, it can be used either as a specific competitor for α2M-proteinase complexes or as a method to investigate the effects of α2M receptor-binding in a context free of proteinases.

Specific uptake of the [125I]HEL-α2M-PPE complex proceeded rapidly in a manner consistent with published literature on f-α2M uptake (15, 41, 43) (Fig. 3). This uptake was sensitive to competition by α2M-methylamine, but not by unlabeled HEL (Fig. 4A). The specific uptake of free [125I]HEL was much less, and displayed a 50- to 90-min time lag before values above the base line were detected (Fig. 3). It is not clear what mechanism accounts for the uptake of free [125I]HEL, because neither unlabeled HEL nor α2M were able to compete with it effectively (Fig. 4A). Nevertheless, these studies demonstrate that
α2M is capable of delivering Ag into macrophages effectively, resulting in a higher level of internalization than observed with free Ag.

Presentation to HEL-specific T hybridoma clones

An Ag presentation assay that measured the activation of HEL-specific T hybridoma cells was then employed to study the effects of enhanced internalization upon Ag processing. Complexing HEL to α2M lowered the threshold for achieving detectable T cell responses by 2.2 to 2.7 log units, allowing 2-nM amounts to be effectively presented (Fig. 5). By contrast, more than 400 nM was required for the presentation of free HEL. Because the 3A9 T hybridomas were added after extensive washing to remove uninternalized Ag, the observed enhancement was not caused by an effect of the complex on the T hybridoma. To control for the possibility that f-α2M was affecting macrophage metabolism directly through receptor-binding events, complexes of α2M-PPE or of α2M-methylamine were added to free HEL, exposing the macrophages to the same molar amounts of f-α2M and HEL as when the HEL-α2M-PPE complexes were given. This had no effect on the presentation of free HEL (Fig. 5), confirming that the enhancement observed with the HEL-α2M-PPE complexes required direct attachment of the HEL to α2M.

To verify that receptor-mediated events were critical for the enhanced ability of the macrophages to present α2M-complexed HEL, competition studies were performed using receptor-recognized f-α2M or BSA. The presence of f-α2M as a competitor during the Ag pulse prevented the macrophages from being able to process and present HEL-α2M-PPE complexes to the T cells, whereas 100-fold excesses of BSA had no effect (Fig. 4B). These results substantiated the hypothesis that receptor-enhanced uptake of
**Discussion**

$\alpha_2M$ displays a unique capacity, upon proteolytic activation, for rapidly forming essentially irreversible complexes with proteins possessing dissimilar structures. The interrelated mechanisms of trapping and of covalent cross-linking allow it to complex with a wide variety of proteinases and other proteins, including large proteinases up to 90 to 110 kDa (5, 12, 44). The capture of nonproteolytic proteins such as insulin is very efficient, occurring readily at physiologic concentrations of reactants (20). The resulting complexes bind the $\alpha_2M$ receptor and are effectively internalized. These properties have been exploited to deliver enzymes and hormones into cells for experimental purposes (14, 15). We now present evidence that the immune system may also exploit these properties to enhance the early stages of nonspecific Ag processing by macrophages.
When complexed to α2M, HEL was processed and presented much more efficiently than when it was unbound (Figs. 3 and 5–7). Ag-pulsed macrophages were able to present nM amounts of HEL-α2M-PPE complexes in contrast to the μM amounts necessary for presentation of free HEL, and of free HEL in the presence of f-α2M. Most previous studies of HEL presentation utilized more than 7 μM (100 μg/ml) concentrations of Ag (34, 37, 45). The ability of α2M to spontaneously incorporate HEL during its activation by proteinases, allowed examination of macrophage Ag processing at much lower concentrations of Ag, as might be found in vivo. Receptor-enhanced uptake of HEL-α2M-PPE complexes also appeared to result in more rapid processing. Although 20 nM of uncomplexed HEL could be presented after 24 h of coincubation with macrophages and T cells, macrophages could present a comparable level of α2M-complexed HEL after only a 15 min pulse (Fig. 6).

In the past, there have been numerous studies suggesting a role for α2M in immune modulation (reviewed in Reference 46). Many studies reporting an apparently suppressive effect on mitogen-induced proliferation or MLC may require reexamination on the basis of current knowledge. Factors complicating interpretation include failure to distinguish between functionally distinct forms of α2M (47, 48), the presence of uninhibited porcine trypsin that can degrade IL-2 (49, 50), potential contamination with endotoxin (46, 48), which has been shown to suppress Ia expression (51), and the binding of growth-regulating substances or lectins to α2M, which may also contribute to its apparent effects in assays (21, 46). Different concentrations of α2M often yielded paradoxical results (21, 46), and some effects attributed to f-α2M required concentrations several hundred-fold higher than the Kd for receptor binding. It has been claimed that f-α2M could oppose the IFN-γ-induced Ia up-regulation (48); however, it was later demonstrated that this observation was an artifact of the technique used, which emphasized differences in localization of Ia rather than their numbers (52). It has also been proposed that s-α2M functions in host defense against pathogen-derived proteinases, but this has not yet been experimentally confirmed (5). Thus, the role of α2M in immune regulation remains undetermined.

The results presented here represent the first evidence for a specific effect of α2M on the processing of a particular Ag and its presentation to a homogeneous population of T hybridoma clones. This study extends and is supported by earlier studies using less specific presentation assays. Macrophages pulsed with α-galactosidase-α2M-trypsin complexes were 16 times better at stimulating proliferation in T cells compared with those pulsed with free α-galactosidase (53). In addition, incubating α2M-complexed viral proteins with macrophages and spleen-derived cells resulted in increased anti-viral antibody secre-


tion (54). The levels of covalent and noncovalent binding of Ag to α2M were not fully characterized, so it is difficult to know whether the compared incubations possessed equivalent molar amounts of α-galactosidase. Although the observed effects could have been the result of nonspecific stimulation of the T cell population, when considered alongside the results presented here, these earlier studies attest to the potential generality of the ability of α2M to enhance uptake and processing of Ag by macrophages.

In addition to HEL, α-galactosidase, and viral proteins, α2M has been shown to covalently bind proteins as diverse as lysozyme, aprotinin, inactive forms of trypsin (7), insulin (8, 20), luteinizing hormone, and possibly streptokinase (C. T. Chu and S. V. Pizzo, unpublished observations). All these appear to interact with α2M by nucleophilic attack at the thioester Glx, as was originally described for the covalent attachment of lysine-containing proteinases (7). This is an efficient process because cross-linking occurs during a reactive intermediate state that decays in seconds (8, 20). Growth factors that appear to bind α2M include platelet-derived growth factor, transforming growth factor-β, IL-1β, IL-6, and basic fibroblast growth factor (reviewed in Reference 21). The association of these different proteins attests to the versatility of the α2M "trap."

Besides being noncovalently trapped or forming covalent e,L-lysyl-γ-glutamyl amide bonds with α2M, proteins may be captured by additional mechanisms. Basic proteins can adhere to α2M in a manner distinct from trapping (18). About 15% of the total binding seen with HEL was noncovalent, was not lost with β-aminopropionitrile, and could bind to preformed α2M-PPE, supporting the possibility of adherence to α2M or PPE (Figs. 1, B and D and 2, A and B). Additional forms of covalent bonds are also possible. The free thiols released by thiolerase decay have been suggested as potential sites for thiol-disulfide interchange (19). In addition, we have recently reported evidence supporting the existence of γ-glutamyl ester bonds (20). Both ester and amide linkages are observed in other members of the α-macroglobulin superfamily, specifically, the thiolester containing C components C3 and C4 (55), and possibly limac, the horseshoe crab macroglobulin (56). Interestingly, the covalent bonds formed by the thiolestes of limac allow it to participate in a hemolytic system but have no relation to its proteinase inhibitory activity (56).

Just as the thiolestes of the C components mediate their biologic activity (reviewed in References 57 and 58), the reactions of the conserved α-macroglobulin thiolester may reflect potential roles of these proteins in immune processes. Although thiolester incorporation is critical to the proteinase inhibitory role of the monomeric α-macroglobulins (17), the role of the α2M thiolester is a mystery (18). It is not needed for proteinase inhibition by tetra-
meric and most dimeric α-macroglobulins (6, 7, 56, 59, 60), but may perhaps be essential to the ability of α2M to capture smaller proteins. The 14 kDa HEL appears to slip out of the closed trap if covalent binding is abolished by β-aminopropionitrile (Fig. 2A). This is consistent with earlier observations that proteins smaller than 20 kDa appear capable of diffusing through the arms of the “sprung” trap (5, 50, 60, 61). Thus, thiolester-mediated covalent linkage may be essential to the efficient capture of potential Ag.

We propose that α2M may act to target potential Ag to macrophages within areas of inflammation. That is, by forming covalent complexes with differing proteins, α2M may be acting as a carrier or adaptor molecule that mediates rapid internalization of these proteins. The receptor recognition site at the C terminus of each α2M subunit is masked until after proteolytic activation (13), during which complex formation with a variety of proteins can occur. Because complex formation depends upon proteolytic activity, which is usually tightly controlled in vivo, the proteins carried by α2M into the macrophages would be limited to those that are present in areas of enhanced proteolytic activity, as might be expected in areas of inflammation. Since α2M can be activated by completely unrelated proteinases, both pathogen- and host-derived proteinases could serve this purpose. This proteolytic activity may also serve to cleave large Ag into smaller fragments that can enter the trap, whereas the covalent binding mechanism ensures capture of even the smaller peptides.

Human α2M is actively secreted by fibroblasts (62) and macrophages (63), which also secrete increased amounts of proteinases under inflammatory conditions (64). The native form is present in plasma at levels greater than 3 μM (18, 48), as well as in extravascular fluids (48). Changes in vascular permeability may result in leakage of serum protein into sites of inflammation. Bovine α2M is present in serum typically used to supplement culture media. Thus, native α2M would likely be present in areas of inflammation, as well as in many in vitro presentation systems. There are no known examples of complete α2M deficiency in mammals (57), lending further support to the importance of this conserved family of thiolester-containing proteins.

In conclusion, α2M represents a naturally occurring protein capable of complexing with diverse proteins for rapid delivery into cells. It is abundant in extracellular fluids in which a wide range of proteinases can activate it to complex with potential Ag. Its high affinity receptor will recognize only the “sprung trap” conformation (F-α2M), allowing internalization of proteins prevalent in areas of increased proteolysis or inflammation. In this study we demonstrated that complexing Ag with α2M, using its intrinsic binding capacity, does indeed enhance both the rate and efficiency of Ag uptake and presentation by macrophages. On the basis of these considerations, we propose that one possible function of the α2M receptor system may be enhancement of Ag capture, and therefore presentation, by reticuloendothelial APC.

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