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Altered Internalization of *Cis*-Dichlorodiammine Platinum (II) Modified α_2Macroglobulin

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by

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I. Background and Significant Information

A. Introduction

Widely distributed throughout the animal kingdom are monomeric, homodimeric, and homotetrameric members of the α_2-macroglobulin family.[1,2] These proteins share the ability to inhibit the proteolytic activities of proteinases displaying different specificities and catalytic mechanisms. Human α_2-macroglobulin is one such high molecular weight (720kDa) proteinase inhibiting glycoprotein of the human plasma. It constitutes as much as 2-4 % of total serum protein and acts as an inhibitor with the unique ability to bind proteinases from all four mechanistic classes (serine, metallo, cystein, and acid).[3-7] α_2-macroglobulin is the major member of a novel class of structurally and functionally related plasma proteins which include complement proteins C3, C4, and C5.[4,9] Structurally α_2M may be described as a "dimer among dimers" for it exits as a homotetrameric molecule with subunits held together by disulfide bonds and as a tetramer by noncovalent forces.[7,5] Homologues of α_2M can be traced to arthropods, including the American horseshoe crab (*Limulus plyphemus*), thus leading to the conclusion that the protein emerged more than 550 million years ago when the arthropod and vertebrate lineages diverged.[10,11]

B. Biological Significance

Numerous cell lineages, including lung fibroblasts, monocytes-macrophages, hepatocytes, and astrocytes synthesize α_2M.[4,9,12] Some tumor cell lines are also capable of producing significant amounts of α_2M.[13] Yet, despite the knowledge of what cells produce and what cells bind this macroglobulin, its exact biological function is unknown. Although it is an effective inhibitor of many proteinases, more specific inhibitors exist for each of the studied proteinases in circulation and in tissues. α_2M may serve as a backup inhibitor in cases of other inhibitor deficiencies,[14] sequestration away,[15] or overwhelming

circumstances as during prancreatitis sepsis.[16] However, the fact remains that no cases of complete deficiency have ever been discovered and this suggests a more central role for α_2M. A few cases of partial deficiency have been reported, yet, the term 'partial' is not clearly defined. Patients with prostate cancer, arterial thrombosis, and a collagen vascular disorder called Ehler-Danlos are among subjects reported with deficiency of varying degrees.[17,19] Still, there are reports of patients having greatly reduced levels of α_2M who are devoid of any symptoms.[20]

By having the ability to bind proteins nonspecifically during its activation process and to be internalized efficiently by macrophages, α_2M is thought to have a role in antigen presentation. Chu and Pizzo[21] have demonstrated that antigens can be bound by α_2M during activation with proteinases, or methylamine, internalized and processed by macrophages, and effectively presented to T cells.[21] This could provide a means to survey the cellular environment in situations of increased proteolysis for antigenic proteins. This property was confirmed *in vivo* studies in which rabbit α-macroglobulin was coupled to antigen and shown to elicit antibody titers in rabbits equivalent to antigen suspended in Freund's adjuvant.[22]

Conclusively, α_2M* has recently been demonstrated to elicit cellular signaling cascades in macrophages.[23] Cells exposed to α_2M* result in a transient increase in 1,4,5-inositol triphosphate levels (IP_3), followed by a rise in intracellular calcium $[Ca^{2+}]_i$ levels. Moreover, α_2M* binding stimulates activities of adenylate cyclase, phospholipase A_2, and protein kinase C.[24] The possible role of this intracellular signaling upon the enhancement of the aforementioned antigen presentation has yet to be investigated.

C. Methods of Inhibition: Bait Region and Internal Thiol Ester

The methods by which the inhibitor works are unusual mechanisms involving steric entrapment and covalent binding.[4] This is in opposition to the classical motif of inhibition that employs binding directly to the proteinase's active site, thereby rendering

the enzyme inactive. In understanding the 'trap hypothesis' first described by Barret and Starkey[5], two domains of the α_2M monomer are important - the bait region and the thiol ester bond.

Located with in the middle of the subunit, a consecutive peptide stretch consisting of residue 666-706 serves as the primary site of functional contact between α_2M and a prtoteinase.[25,26] This area, so termed the 'bait region', acts as the 'bait' that will ultimately 'catch' the prey. It is reported to constitute a flexible and highly exposed segment of the overall molecule making it easily adaptable to any conformation which may favor and account for its wide range of binding capabilities.[27] This view is further favored by the fact that restricting disulfide bridges and potentially blocking carbohydrate moieties are absent within the molecule due to the presence of one cysteine and no asparaginyl residues within the sequence.[28] Proteolytic cleavage of a peptide bond, acting as bait, triggers an immediate and distinct conformational change of α_2M that sterically hinders proteinase access to larger substrates, active site directed inhibitors[5], and antibodies.[29] Intimately linked with this conformational change is the cleavage of the internal, reactive β-cysteinyl-γ-glutamyl thiol ester.

As a result of this cleavage, a transient intermediate ("nascent",$<\alpha_2M>$)[33] is formed containing a reactive glutamyl and cysteinyl residue that is subject to nucleophilic attack in each of the four subunits. This linkage formed between a cysteine and glutamine is labile and readily cleaved by heat,[11] small nucleophile including primary amines, reductants, water, as well as proteinase activity.[4] Glutamyl residue reactivity decays rapidly by reacting with water, but it is also capable of forming a covalent bond with ε–lysine residues on an adjacent peptides,[4] including those of the attacking proteinase or nonproteolytic peptides present during the reaction. The cysteinyl residues remain active and are known to bind cytokines.[31](Figure 1) Due to this cleavage of the internal thiol ester, an electrophorectic shift from a slow/unactivated (α_2M) to a fast/activated (α_2M^*)

migrating form of the complex can be observed in native polyacrylamide gel electrophoresis.[41]

D. Methylamine Activation

An alternative route, and one frequently exploited in scientific study, is the direct nucleophilic attack upon the thiol ester through treatment of α_2M with small primary amines (namely, methylamine) to produce the structurally compact activated form (α_2M*) of the inhibitor. Because methylamine derivatives have similar compact conformations of the α_2M-proteinase complex and are recognized equally well by cellular receptors[37],α_2M* is often used to designate either receptor recognized form.

E. Receptor Recognition

In addition to the conformational change displaying four free thiols, α_2M* looses any further proteinase inhibitory capacity, and previously buried receptor recognition sites become exposed. These receptor sites lead to rapid clearance of the α_2M-proteinase complexes from human circulation with a half-life of approximately 10 minutes.[38,39] Due to the fact that the proteinase has not lost its proteolytic activity in consequence of its hindered entrapment, this clearance mechanism is critical for effective control of such activity. Consequently, much interest has been focused on the receptor responsible for clearance of surface bound α_2M*.

The receptor binding epitopes of α_2M* have been mapped to a ~20kDa carboxy terminal receptor binding fragment (RBF) that has been proteolytically derived from α_2M [40,41]and has now been cloned and expressed.[42,43] RBF's from several animal species are capable of binding cell surface receptors form other species with comparable affinities[43] indicating a high level of conservation form amphibians to mammals.[44] The proteolytically derived RBF of rat α_1M binds to purified LRP and to cells with K_d values[†] of 100nM and 100-300nM respectively.[40,41,43] Notably, the cloned and expressed rat

α_1M RBF binds to purified LRP/α_2MR and to cells with affinity values approximately 10 times greater (K_d values of 20nM and 8nM, respectively); however, these values are still 16-40 fold less than that for intact α_2M*.[42,45] Initial analysis may lead the investigator to postulate that the decreased affinity of RBF might be explained by its monovalent binding ability as compared to the multivalent binding capability of α_2M* Yet, studies examining the binding of the monomeric rat $\alpha_2$macroglobulin, α_1M , have shown its K_d value to be similar to α_2M*. (1.0nM vs. 0.5nM, respectively).[43] Therefore, ligand valency cannot explain the differences in K_d values obtained for RBF and α_2M*.

† K_d is a binding constant defined as one half of the concentration at which 50 percent of the added ligand remains bound to cells. ($K_d = \frac{1}{2}IC_{50}$)

F. The α_2M* Binding Receptor

In recent years, the endocytic α_2M* receptor has been identified as a single transmembrane spanning protein of the LDL receptor family termed the low-density lipoprotein receptor-related protein (LRP/α_2MR).[46,47,48] LRP/α_2MR is a classical scavenger receptor that binds and internalizes a variety of unrelated ligands through clathrin coated pits including *Pseudomonas* exotoxin A, lipoprotein lipase, apolipoprotein-E enriched lipoproteins, urokinase-and tissue-type plasminogen activators alone or in complexes with plasminogen activator inhibior1, tissue factor pathway inhibitor, lactoferrin, and lipoprotein(a).[49-55] The affinity of LRP/α_2MR for ligands of diverse biological functions could imply that this receptor has various biological roles. The LRP/α_2MR is a 600-kDa glycoprotein that undergoes proteolysis in the trans-Golgi being expressed as a noncovalently associated heterodimer of ~515 and ~85 kDa, respectively.[3] Expression of this receptor has been found in glial cells,[56] trophoblast[57], fibroblasts, macrophages, dendritic cells, hepatocytes, adipocytes, astrocytes, neurons, and fetal mesenchyme.[58] LRP/α_2MR expression and activity may be regulated by

lipopolysaccharides, interferon–γ, and insulin.[59,60] A 39kDa associated protein (RAP) copurifies with the receptor and is capable of inhibiting the binding of all known ligands to LRP/α_2MR.[61] Even though RAP and its fragments are themselves endocytosed,[62] the role of RAP is still unclear since it appears to be localized predominantly in the endoplasmic reticulum.[63]

G. The α_2M* Signaling Receptor

Recently studies within our laboratory have demonstrated evidence for a second cellular receptor for α_2M* responsible for signaling cascades that produce a transient 1.5-2.0 fold increase in inositol 1,4,5-triphosphate [23] as well as increases in intracellular calcium, and cyclic AMP. [24] This receptor is distinct from the LRP/α_2MR in a two fold manner. Through studies using murine macrophages, it has been proven to be coupled to a pertussis toxin-insensitive G protein and possibly to a cholera toxin-sensitive G protein.[64] This is in direct contrast to the LRP/α_2MR which is pertussis- toxin sensitive. Moreover, though RAP has been demonstrated to be an antagonist for LRP signal transduction, no effect upon signaling via the α_2M* signaling receptor (α_2MSR) is observed.[64]

II. Abstract

Human α_2-macroglobulin (α_2M) is a 720kDa glycoprotein present in the serum in micromolar quantities retaining the ability to inhibit proteinases from all four mechanistic classes.[†] More than 200,000,000 years ago, a cellular receptor evolved which recognizes proteinase-treated (α_2M*) but not native α_2M. Many cell types have been shown to express a receptor for α_2M*, including fibroblasts, macrophages, hepatocytes, adipocytes, and dermal dendritic cells. Heightened interest concerning the receptor responsible for clearance has led scientist to search out interesting qualities inherent of this unique inhibitor. Cloning and sequencing have identified the receptor for surface bound α_2M* to be identical to the low-density-lipoprotein receptor-related protein(LRP/α_2MR). Recently studies within this laboratory have shown evidence for a second α_2M receptor- the α_2M signaling receptor (α_2MSR). The epitopes of binding to LRP/α_2MR have been localized to a 20kDa carboxy-terminal fragment (RBF) within each subunit of the activated molecule.

In order to determine if the receptor recognition sites in α_2M governing signaling and internalization were the same, we turned to a chemical modification of α_2M known to dramatically affect clearance *in vivo*. Previous studies examining treatment of α_2M with *cis*-dichlorodiammine platinum (II) (*cis*-DDP) have demonstrated an increase in the binding constant on murine macrophages (~11nM) and a significant lengthening of the half-life in murine circulation from 2-4 min for unmodified α_2M* to 74 min for *cis*-DDP-α_2M*. *Cis*-DDP is a bidentate crosslinker which forms intramolecular cross-links among two reactive amino acid side-chains within α_2M*. It reacts readily with histidines and methionine residues.

abbreviations: α_2M, α_2-macroglobulin; α_2M*, the receptor recognized form of α_2M- either α_2M-methylamine or α_2M-protease; LRP/α_2MR, the low-density-lipoprotein receptor-related protein/ α_2M receptor; α_2MSR, the α_2M signaling receptor; RAP, receptor-associated protein; α_1I_3, α_1-inhibitor$_3$; *cis*-DDP, *cis*-dichlordiammineplantinum(II); $[Ca^{2+}]_i$, intracellular free Ca^{2+}, IP_3, inositol 1,4,5-triphosphate; Fura-2/AM, 1-[2-(5-carboxyoxazol-1-yl)-6-aminobenzofuran-5oxyl-2-(2'-amino-5'methylphenoxy) ethane-NNN'N'-tetra-acetic acid acetoxymethyl ester; Pt, platinum.

In this present study, the *in vitro* binding and internalization properties by murine macrophages of this modified form of α_2M^* , as well as the internalization of RBF have been investigated. Based on the data collected from this work and that previously published, we postulate that RBF contains sites governing internalization by LRP/α_2MR as well as signaling via α_2MSR. In addition, there exists a cis-DDP-sensitive site outside of RBF which is important for internalization of α_2M via LRP/α_2M which is not involved in the interaction of α_2M with α_2MSR.

III. Materials and Methods

Reagents and Proteins- Culture media was purchased from Life Technologies (Grand Island, NY). Bovine serum albumin (BSA), papain and HEPES were purchased from Sigma (St. Louis, MO). *Cis*-DDP was purchased form Aldrich (St. Louis, MO) ^{125}I for protein iodination was obtained form New England Nuclear (Boston, MA), and Iodobead were purchased form Pierce (Rockford, IL). Gel filtration materials were purchased form Pharmacia Biotech (Piscataway, NJ). All other reagents were of the best commercial grade available.

Preparation of α_2M^* and cis-DDP-α_2M^* - Human α_2M was purified as previously described.[39] α_2M was activated with 200mM methylamine for 16-18 hours at room temperature in the dark and dialyzed extensively against 20mM HEPES, 150 mM NaCl, pH 7.4 to remove unreacted methylamine. The following method for *cis*-DDP treatment of α_2M^* is in modification of that described by Gonias and Pizzo.[65] Crystalline *cis*-DDP was dissolved in 20mM Tris-HCl, 150mM NaCl, pH 8.0 at $\sim 60\,^\circ$C and allowed to cool to $37\,^\circ$C. α_2M^* was then incubated with 1.7mM *cis*-DDP in the dark for 3-4 hours at $37\,^\circ$C. The reaction mixture was then dialyzed extensively against 20mM HEPES, 150mM NaCl, pH 7.4 to remove unreacted *cis*-DDP. α_2M and *cis*-DDP were radiolabeled with ^{125}I

using the Iodobead method and applied to a gel filtration PD-10 column for separation form free ^{125}I.

Preparation of RBF-The preparation of RBF was performed in the laboratory of Dr. Dianne Decamp, Duke University Medical Center, Pathology Department. The correct nucleotide sequence of the cloned RBF product was confirmed by DNA sequencing. (Methods currently pending publication.)

Preparation of Rap- The pGEX-39kDa expression construct was the kind gift of Dr. Joachim Herz (University of Texas at Southwestern). The protein was expressed and purified as described in Herz et al. with the following modifications.[66] Before passing the bacterial lysate through needles, DNAse and $MgCl_2$ were added to a final concentration of 20mg/L and 10mM, respectively. After a 30 min incubation on ice, the cell debris were removed by centrifugation at 14,000 x g for 40 min. During the affinity chromatography step, glutathione-Sepharose 4B(Pharmacia Biotech, Piscataway, NJ) was substituted for glutatione-agarose. Protein-containing fractions were pooled and dialyzed extensively against 20mM HEPES, pH 7.3, containing 150mM NaCl. Protein samples were sterile-filtered and stored in aliquots at -80°C.

Macrophage Harvesting - This procedure may be found in detail within the published work of Misra et.al.[67], and will only be described briefly here. C57B1/6 mice were obtained form Charles River Laboratories (Raleigh, NC). Thioglycollated-elicted macrophages were routinely obtained via peritoneal lavage with a solution of 20mM HEPES, 150mM NaCl, pH7.4. Cells were then pelleted by centrifugation at ~800 x g for 5 min and resuspended in RPMI 1640 medium containing 12.5 units/ml penicillin, 6.5μg/ml streptomycin, and 10% fetal bovine serum.

Binding Assay - Macrophages were plated in 24 well plates at 2 x 10^6 cells/well or 48 well plates at 1 x 10^6 cells/well and incubated for >3h at 37°C in a humidified 5% CO_2 incubator. Plates were then moved to 4°C and allowed to equilibrate for ~ 30min. The monolayers were rinsed three times with ice-cold Hank's balanced salt solution (HBSS) containing 25 mM HEPES, 12.5units/ml penicillin, 6.5μg/ml streptomycin, and 5% BSA. (From this point forward this solution will be referred to as Buffer A.) In an effort to assess nonspecific binding some wells were rinsed three times with ice-cold HBSS without Ca^{2+} and Mg^{2+} containing 25 mM HEPES, 12.5units/ml penicillin, 6.5μg/ml streptomycin, and 5% BSA. (From this point forward this solution will be referred to as Buffer B.) Various concentrations of unlabeled competitors along with $Na^{125}I$-α2M* (1nM) were added to each well and allowed to incubate at 4°C for 16-18 h. Radioligand solutions were removed from the wells, and the wells were rinsed two times with ice-cold Buffer A or B. Solubalization solution (1.0M NaOH, 0.1%SDS) was added to each well and allowed to incubate at room temperature for ~5h before transferring the solution to tubes to be counted in a γ -counter (LKB-Wallac Clini Gamma 1272). Nonspecific binding was determined by using Buffer B in some wells and averaged ≤ 10% of total binding over several assays. Specific binding is reported as total binding minus nonspecific binding.

Uptake Assay - Macrophages were plated in 24 well plates at 2 x 10^6 cells/well or 48 well plates at 1 x 10^6 cells/well and incubated for >3h at 37°C in a humidified 5% CO_2 incubator. Monolayers were rinsed three times with Buffer A. To assess nonspecific binding, some wells were rinsed three times with Buffer B. ^{125}I-ligand was added to each well with or without various concentrations of unlabeled competitor and allowed to incubate at 37°C for 90min. Radioligand solutions were removed form the wells, and the wells were rinsed two times with ice-cold Buffer A or B. Solubalization solution (1.0 M NaOH. 0.1% SDS) was added to the wells and allowed to incubate at room temperature for ~ 5 h before transferring the solution to tubes to be counted in a γ -counter (LKB-

Wallac Clini Gamma 1272). Nonspecific uptake was determined by using Buffer B in some wells and averaged $\leq 10\%$ of total uptake over several assays. Specific uptake is reported as total uptake minus nonspecific uptake.

Digestion of α_2M and Cis-DDP-α_2M* with Papain* - This procedure was described according to previously published methods.[68] α_2M* and cis-DDP-α_2M* (3.5μM) were reacted with papain (20:1 w/w) in a 100mM NaCl, 1mm EDTA, 50mM Ch_3COONa, 1mM cystein buffer, pH 4.5. After incubation for 24h at 37°C, the pH was raised to 8.0 using concentrated HEPES buffer to a final 50mM HEPES concentration and 5mM iodoacetamide was added. Following a 30min incubation, the mixture was applied to a Sephacryl S-200 column (2.5 x 100cm) and eluted in 25mM HEPES, 150mM NaCl. pH 7.4 buffer at a flow rate of 20ml/h. The material in Peaks I and II represent the 600kDa and 55kDa fragments previously described.[68]

IV. Results

α_2M, cis-DDP-α_2M*, and RAP Binding to Macrophages* - The binding constant (K_d) of α_2M* to murine macrophages was determined by displacing 1.0nM radiolabeled α_2M* bound to the cells with varying concentrations of unlabeled α_2M*. (Figure 2) Using the Sysstat program from Sysstat, Inc.(Evanston, IL), the K_d was determined to be 0.6nM with a program fit of $r^2 = 0.979$. Binding constant values were also determined for RAP and *cis*-DDP-α_2M* based upon their ability to displace bound α_2M* from murine macrophages. The K_d for RAP binding was determined to be 4.4nM with a program fit of $r^2 = 0.992$ and the K_d for *cis*-DDP-α_2M* was 14.5nM with a program fit of $r^2 = 0.987$.

α_2M, cis-DDP-α_2M*, and RBF Internalization by Macrophages*- In comparing the internalization of increasing concentration of α_2M* and *cis*-DDP-α_2M*, 48 well

plates containing 1 x 10^6 cells were used. At each concentration, the internalization of the modified form of α_2M^* is approximately 90% less than that of the unmodified α_2M^*.(figure 3A) In comparing the effects of 2μM RAP on the internalization of 25nM α_2M^* , RBF, and *cis*-DDP-α_2M^*, 24 well plates containing 2 x 10^6 cells per well were used. (figure 3B) α_2M^* and RBF demonstrated comparable levels of internalization (51.7 ± 0.1 vs. 67.3 ± 1.9 fmols, respectively) under these conditions. Internalization levels of *cis*-DDP-α_2M^* was approximately 18% of the level for α_2M^*. (12.0 ± 0.4 vs. 67.3 ±1.9 fmols, respectively) In the presence of 80-fold molar excess RAP, the internalization of α_2M^* is decreased by 74%. This finding is consistent with the findings of Hertz, et al.[66] which demonstrate a 75% inhibition of α_2M^* internalization in the presence of a 20-fold molar excess of RAP. The internalization levels of RBF and *cis* -DDP-α_2M^* are also decreased in the presence of an 80-fold molar excess of RAP by 50% and 46%, respectively.

Papain Digest of α_2M^* and cis-DDP-α_2M - We examined the % yield of RBF obtained via papain digest of α_2M^* and *cis*-DDP–α_2M^* followed by gel filtration. A typical elution profile for papain digested *cis*-DDP-α_2M^* is shown in figure 3. This profile is similar to that of α_2M * previously published.[68] Based upon previous studies, the M_r of 20,000 and A(1%, 1cm) = 1.0 were used in calculation of % yield of RBF.[68] For three independent experiments the yield of RBF from α_2M^* and *cis*-DDP-α_2M^* was 1.7 ±1.3% and 3.7± 2.3% respectively. This difference in yield was not found to be significant using the ANOVA in the Excell 4.0 program from Microsoft Corporation (Seattle, WA). If *cis*-DDP had involved crosslinking between a residue within the RBF and a residue of an adjacent domain, the yield of RBF from papain digest of the modified α_2M * would have been decreased.

V. Discussion

α_2M^* is known to bind to both the LRP/α_2MR and α_2MSR . Yet, only the later is capable of eliciting classical signaling cascades. The two receptors are further differentiated by the fact that α_2M^* binding to LRP/α^2MR is inhibitable by RAP, whereas α_2M^* binding to α_2MSR is not affected.[64] The previously documented effects of this signaling cascades do not occur with exposure of macrophages to native α_2M. Thus, *in vivo*, these results would only be activated during situations involving proteolysis. Though the region of α_2M^* which binds to LRP/α_2MR has been localized to the RBF[40,41], there is evidence which suggests that there are necessary additional sites of binding outside the carboxy terminal fragment located 20kDa upstream of the RBF. These additional sites may possibly account for the ~16-40 fold greater K_d values observed of RBF binding to purified LRP in comparison to intact α_2M^* binding under the same circumstances.[2,43,69] Supporting studies have examined the binding of the 40kDa carboxy-terminal fragment of rat α_1M and indicate that its K_d of binding to macrophages (5.0nM) is similar to the K_d of intact α_1M *(1.0nM). Yet, the RBF of rat α_1M binds with much less affinity, K_d(125nM).[43] One known property for sufficient ligand binding to LRP/α_2MR is the presence of a cluster of positive charges.[70,71] Presumably, this property is also true of α_2M^*. The enhanced recognition of the 40kDa carboxy-terminal fragment may involve the presence of such charges within the additional 20kDa region upstream of the RBF. In consequence, it is possible that residues in this upstream region are also important for influencing the three dimensional structure of RBF and therefore affecting its binding affinity for LRP/α_2MR.[2,43,69] The region of α_2M^* needed for binding to α_2MSR has been localized to the RBF.[64] Thus, binding sites inherent for both cellular receptors are present in the RBF and an additional upstream site important for LRP binding exists outside of the RBF.

Our results support this hypothesis by demonstrating that a site outside of the RBF is involved in the interaction between α_2M^* and LRP and is susceptible to chemical

modification with the anti-tumor agent, *cis*-dicholordiammineplatinum (*cis*-DDP).[72] Prior studies have demonstrated a moderate shift in the K_d of binding to macrophages from 0.5nM for untreated α_2M^* to 11.0nM following *cis*-DDP treatment. We also have findings of similar values revealing a shift in K_d form 0.6 to 14.5nM with *cis*-DDP treatment of α_2M^*. Gonias and Pizzo revealed a significant decrease in the rate of clearance of α_2M^* in mice following chemical modification.[65] Likewise, we presently show a significant effect of *cis*-DDP treatment upon the internalization of α_2M^* via LRP/α_2MR. The internalization of α_2M^* is decreased by more than 80% following treatment with *cis*-DDP. This internalization of *cis*-DDP-α_2M^* is shown to still be mediated by LRP/α_2MR due to its 46% inhibition in the presence of 80-fold molar excess RAP. Therefore, cis-DDP modification of α_2M^* yields a ligand that binds to LRP/α_2MR with decreased affinity and is internalized poorly.

Interestingly, however, *cis*-DDP modification of α_2M^* has no effect upon signaling. (Howard et al pending publication.) Signaling cascades within murine macrophages are initiated rapidly with *cis*-DDP–α_2M^* revealing elevated IP_3 levels (figure 4) followed by an increase in $[Ca^{2+}]_i$ (figure 5) similar to α_2M^*. Prior studies have demonstrated a lack of incorporation of *cis*-DDP into RBF with *cis*-DDP treatment of α_2M^*[68], and since RBF is capable of eliciting signaling responses comparable to that of α_2M^*[64], it would serve to reason that *cis*-DDP modification would indeed have no effect upon the ability of α_2M^* to generate cellular signaling cascades. Consistent with this hypothesis, our studies clearly demonstrate no effect of *cis*-DDP modification on signaling.(figure 6)

Although our data supports assigning the *cis*-DDP sensitive site to a region outside the RBF, an alternative explanation for our results could be that a site within RBF was modified by *cis*-DDP, but this site was unimportant for binding to LRP/ α_2MR. Possibly, *cis*-DDP incorporation into α_2M^* could involve a cross-link between a residue within the RBF and a residue outside of RBF. Thus, platinum incorporation studies would reveal

poor *cis*-DDP incorporation within isolated RBF. Prior studies demonstrating a lack of incorporation of platinum into RBF utilized *cis*-DDP treatment of α_2M* followed by papain digest and gel filtration to resolve the RBF.[65] In this present study, we compared the yield of RBF from papain digests of α_2M* and *cis*-DDP–α_2M*. If *cis*-DDP modification involved a cross-link with a residue within the RBF and another residue outside the RBF, the amount of recovered RBF form papain digested *cis*-DDP–α_2M* would be decreased. This change in the recovery of RBF between α_2M* and *cis*-DDP–α_2M* was not demonstrated, thus providing conclusive evidence for *cis*-DDP modification of α_2M* at a site outside the RBF. (figure 7)

Our data indicates that RBF does contain sites governing internalization via LRP/α_2MR, and we hypothesize that *cis*-DDP modification of residues outside of RBF involves a site which is spatially near to the interaction site between RBF and LRP/α_2MR, thus shielding RBF binding and decreasing internalization via LRP/α_2MR. However, it is possible that the residues affected by *cis*-DDP may themselves be important in the binding of α_2M* to LRP/α_2MR. As, mentioned previously, the 40kDa carboxy-terminal receptor binding fragment possess a charge cluster and is sensitive to *cis*-DDP modification.[43] *Cis*-DDP has highest affinity for reacting with sulfur containing amino acids, but it does react well with arginine, lysine, and histidine.[66] These are the same amino acids which are typically found in positive charge clusters.[70,71]. Therefore, it is reasonable to postulate that reaction with *cis*-DDP may involve modifying a charge cluster in the 40kDa region which is important for interaction with LRP/ α_2MR .

Cis-DDP modification provides a unique mechanism for defining effects of α_2M* binding to each of its cellular receptors. (Figure 8) Additionally, this modification provides further delineation of sites of α_2M* which are inherent for LRP/ α_2MR binding, yet pose no effect upon α_2MSR interaction. Apparently, the *cis*-DDP-sensitive site outside of RBF is important to maintaining binding of α_2M* to facilitate clearance via LRP/ α_2MR. It will be interesting to determine whether this *cis*-DDP-sensitive site directly interacts with

LRP/ α_2MR or whether it is simply located near to the sites of RBF interacting with LRP/ α_2MR. Notably, this *cis*-DDP-sensitive site does no appear to be important in the binding of α_2M* to α_2MSR. Certainly, this modification tool could be an important step in defining the roles of each binding pathway for α_2M*.

VI. Acknowledgments

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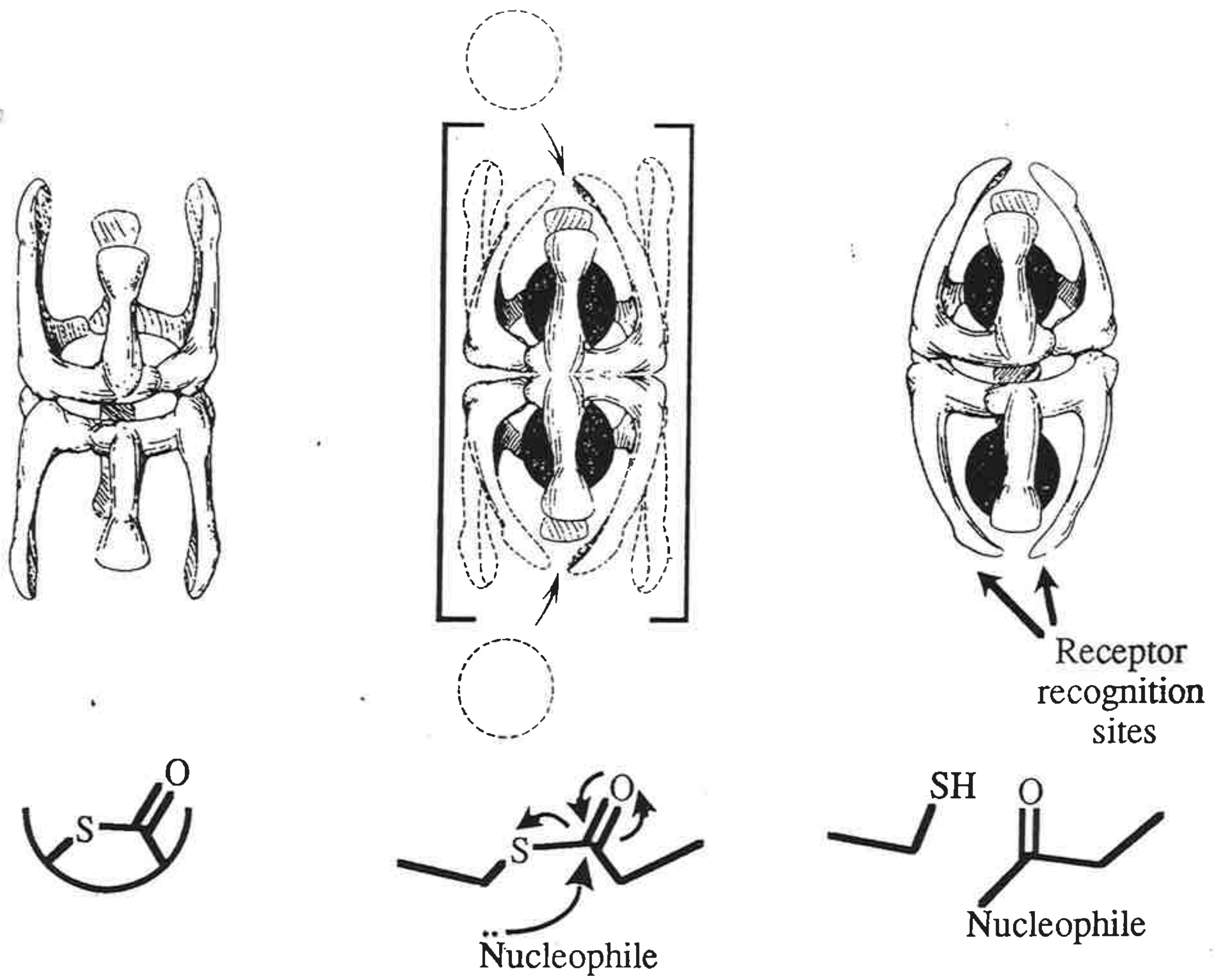


Figure 1. α_2M Trap Mechanism.
This figure provides a schematic representation of the α_2M proteinase inhibitory ability. Each of the four subunits of 180kDa have a characteristic short and long arm. Movement of these long arms following the proteinase interaction with the bait region sterically entraps the proteinase. Previously buried receptor recognition sites are exposed and the reactive internal thiol ester bond is broken.

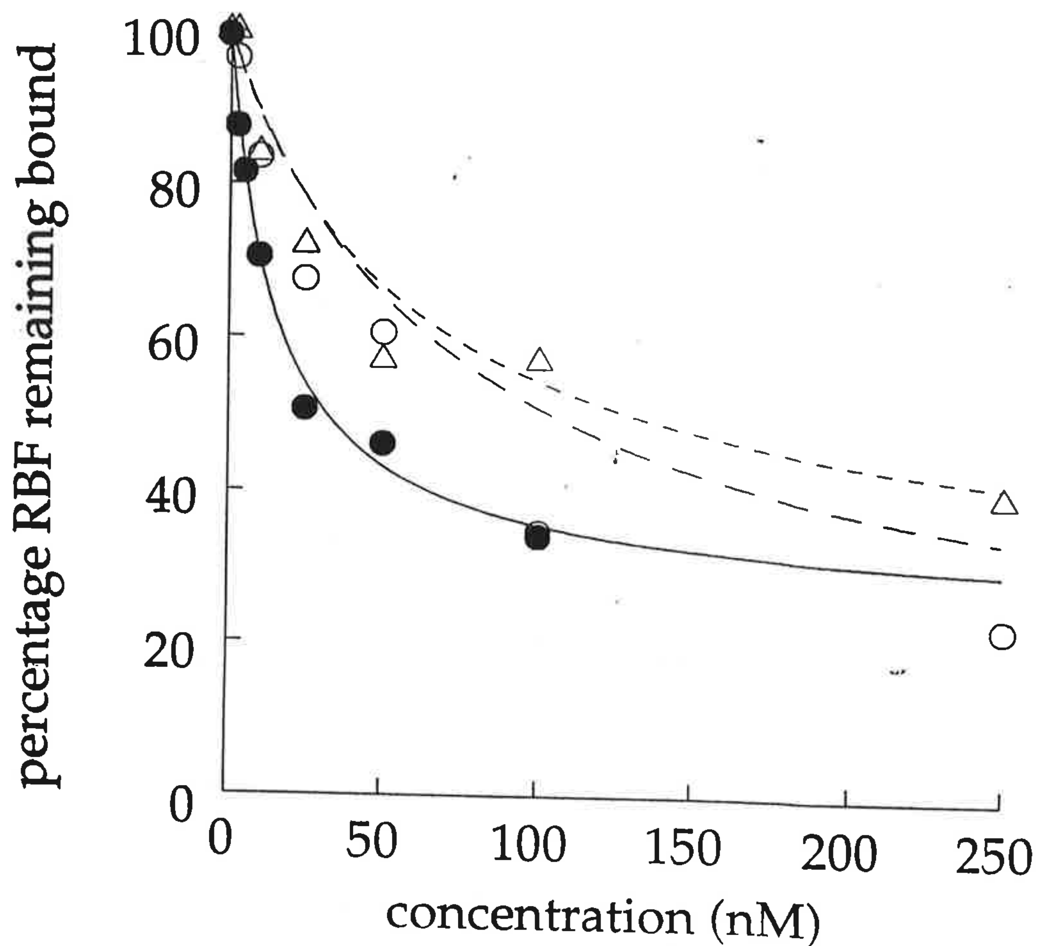


Figure 2. Binding displacement of ^{125}I- α_2M* from macrophages. Displacement of bound ^{125}I- α_2M* (1.0nM) from macrophages by increasing concentrations of unlabeled α_2M* (+), unlabeled *cis*-DDP-α_2M*(o), and unlabeled RAP (Δ). Values are mean ± Standard error of the mean (SEM) from three or more independent experiments performed in duplicate. Specific binding was calculated as total binding minus nonspecific binding as determined in the presence of 5mM EDTA. The lines of this graph represent the dissociation binding model which best fits the data as determined by the Sysstat program for each ligand [(-------) α_2M*, (———) cis-DDP-α_2M*, and (— — —) RAP.]

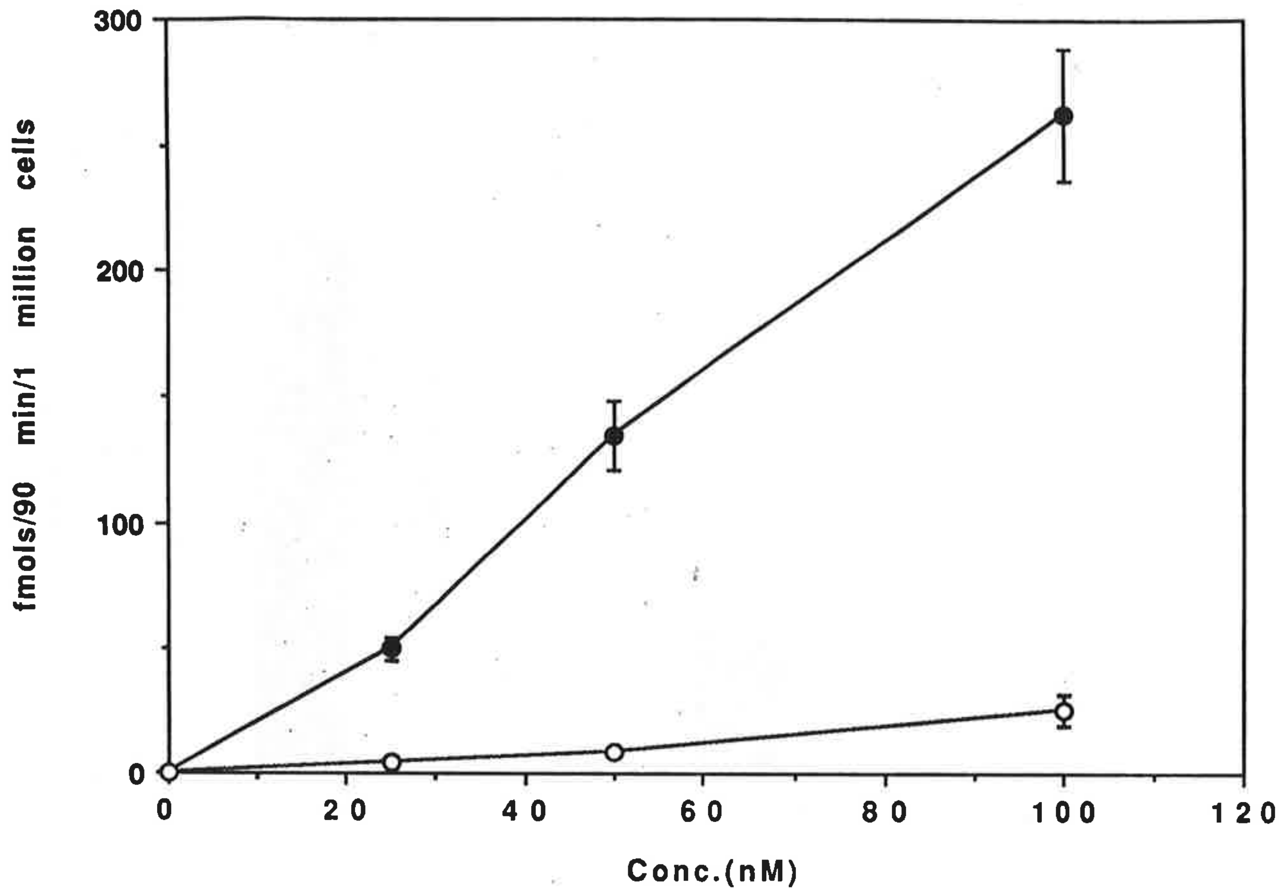
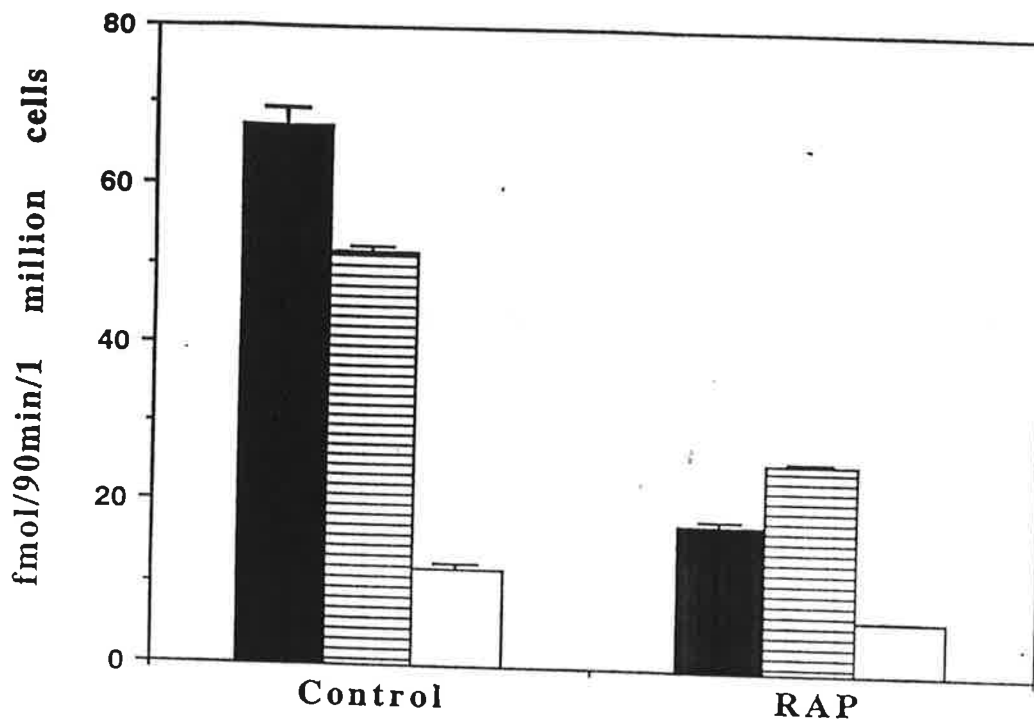


Figure 3. Internalization of ^{125}I-Ligands by macrophages.

A. Fmols of internalization of α_2M^* (solid circles) and *cis*-DDP–α_2M^* (open circles) at 25, 50 ,and 100nM by 1 x 10^6 macrophages in 90 min. Values are mean ± SEM from ≥ 5 independent experiments performed in duplicate. Fmols of internalization was calculated as total internalization minus nonspecific internalization as determined in the presence of 5mM EDTA.



B. Fmols of internalization of 25nM α_2M^* (solid black bar), RBF (lined bar) and *cis*-DDP-α_2M^*(solid white bar) per 1 x 10^6 macrophages in 90 min. Control internalization was calculated as total internalization minus nonspecific internalization as determined in the presence of 5mM EDTA. Values are given for internalization of ligands in the presence of 80-fold excess RAP.

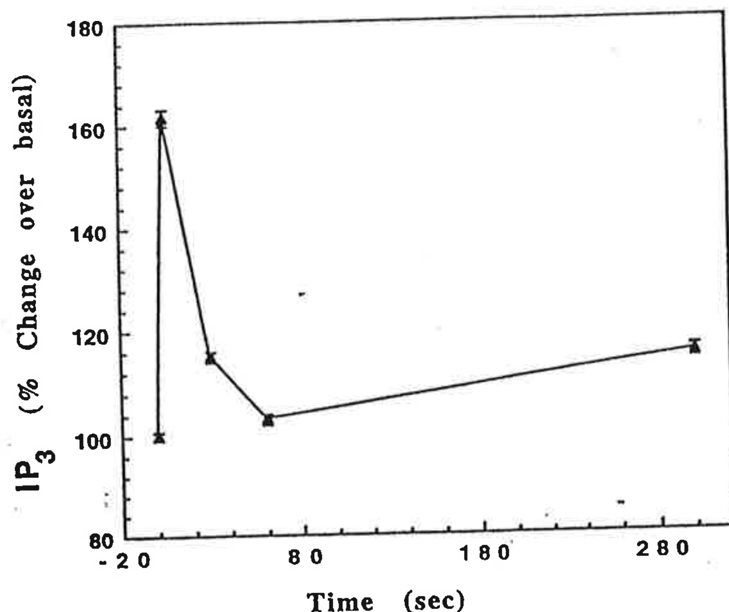


Figure 4. Inositol (1,4,5)-tirphosphate formation in ligand-stimulated macrophages. 2-[^{3}H]myo-inositol labeled macrophages were treated with α_2M^* and *cis*-DDP-α_2M^* (40nM) for various time periods and processed for quantitation of IP^3 radioactivity. Values are expressed as percentage change in radioactivity over basal value form 0 to 300 sec ± SEM.

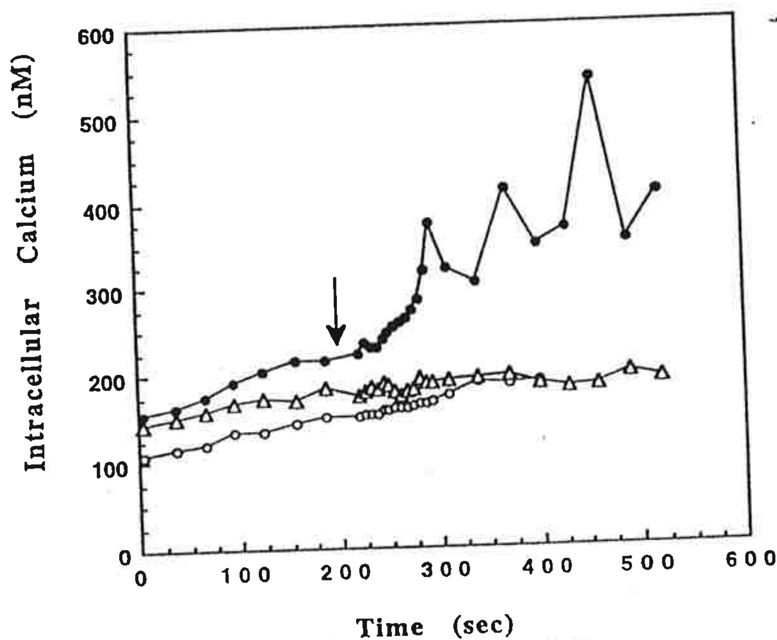


Figure 6. Representative $[Ca^{2+}]_i$ signal upon *cis*-DDP-α_2M^* stimulation. Macrophages were preloaded with 4μM FUra-2/AM for 30 minutes at 37°C and changes in $[Ca^{2+}]_i$ after stimulation with α_2M^* or *cis*-DDP-α_2M^* (40nM) were measured. Typical cell responses to stimulation with *cis*-DDP-α_2M^*(●), *cis*-DDP alone(o), and boiled *cis*-DDP-α_2M^* (Δ) are shown. The arrow indicated the time and addition of ligand. The average increase in $[Ca^{2+}]_i$ for stimulation with α_2M^* and *cis*-DDP-α_2M^* is shown in figure 5.

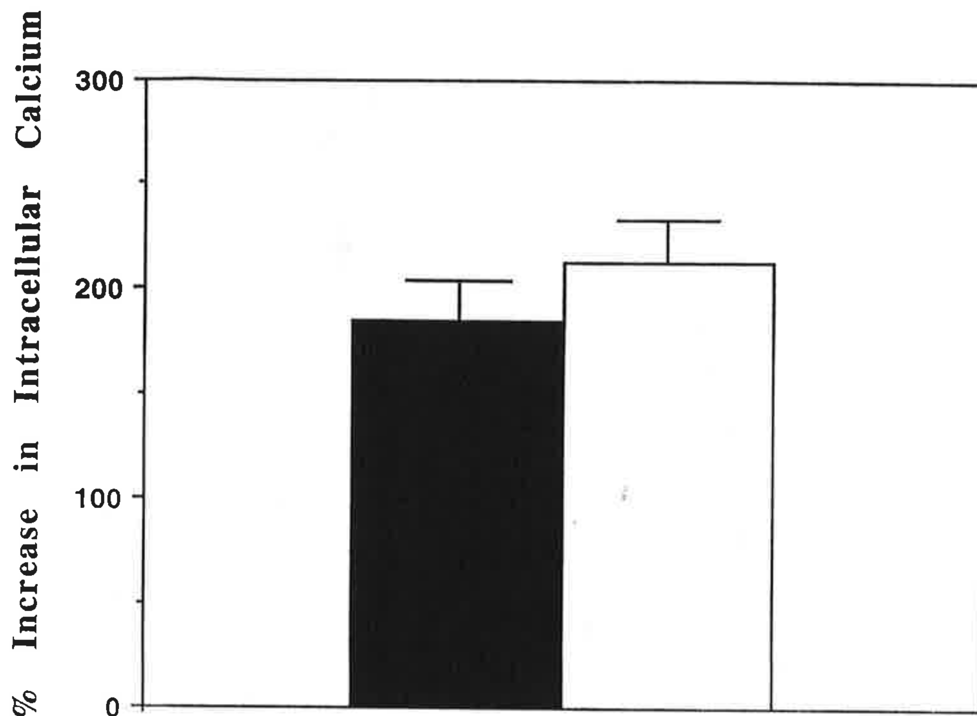


Figure 5. Calcium response in ligand-stimulated macrophages. $[Ca^{2+}]_i$ in ligand stimulated macrophages. Macrophages were preloaded with 4μM Fura-2/AM for 30 min and changes in $[Ca^{2+}]_i$ after stimulation with various ligands (40nM) were measured. Values are given for α_2M^* (solid black bar), and *cis*-DDP-α_2M^*(solid white bar).

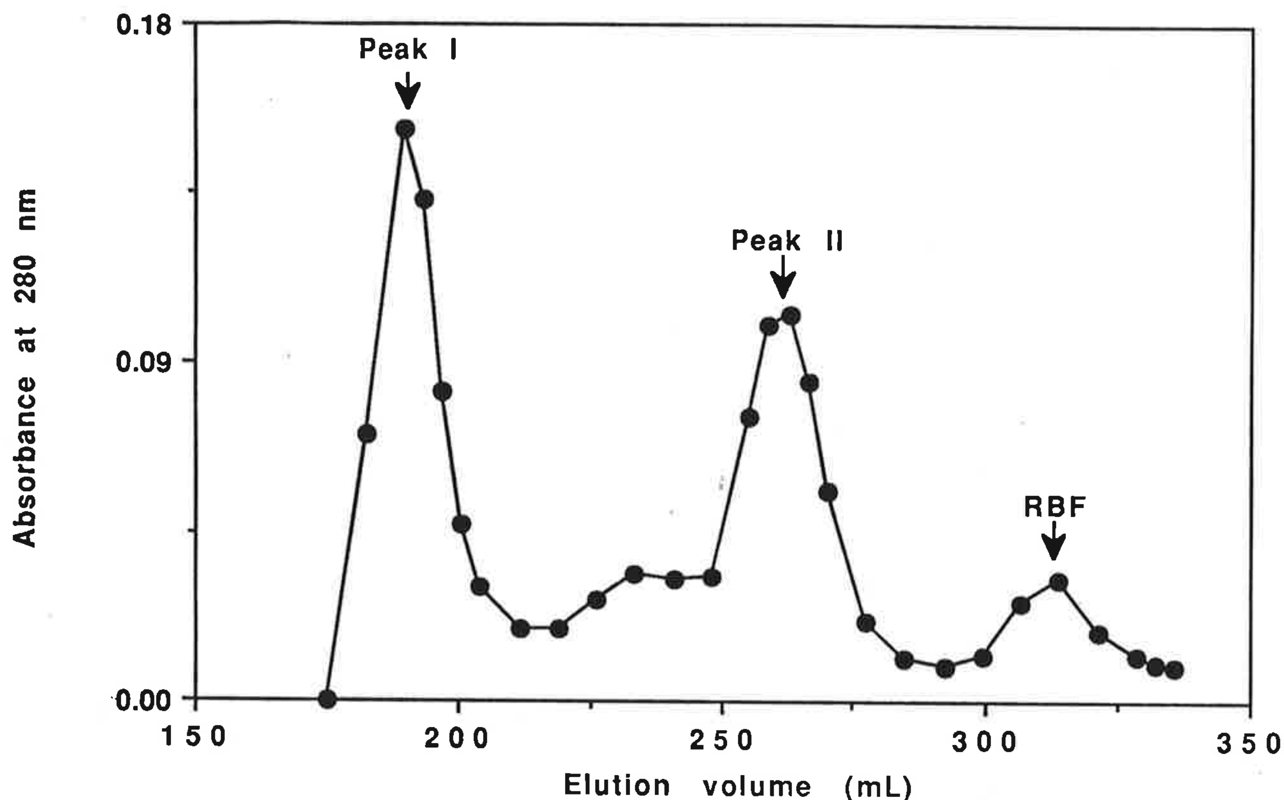


Figure 7. Elution profile of papain digested *cis*-DDP-α_2M* from Sephacryl S-200 column. Following papain digest of *cis*-DDP-α_2M*, the material was loaded onto a Sephacryl s-200 column and eluted in HEPES/ NaCl, pH 7.4 buffer. Elution volume (ml) is given along with the absorbance at 280nm for each fraction measured. Peak I refers to the high molecular weight material (600kDa) as described in Material and Methods. Peak II refers to the 55kDa material. This graph represents a typical elution profile from $\geq$ 3 independent experiments. The % yield of RBF from α_2M* and *cis*-DDP-α_2M* is 1.7±1.3% and 3.7± 2.3% , respectively.

Figure 8. Schematic of proposed orientation of α_2M* binding sites for LRP/α_2MR and α_2MSR. A schematic for the proposed interaction of α_2M* with its two cellular receptors, is shown. The moon-shaped region of α_2M* represents the domain which contains the binding information for both receptors. The hatched areas within this domain represent RBF, and the "X" indicates the *cis*-DDP sensitive site contained in the region upstream of the RBF. The sensitivity to Pertussis toxin (Ptx) of the G-protein coupled to LRP/α_2MR and α_2MSR are given. "E" represents the effectors of the signaling pathway.