The Binding of Receptor-recognized $\alpha_2$-Macroglobulin to the Low Density Lipoprotein Receptor-related Protein and the $\alpha_2M$ Signaling Receptor Is Decoupled by Oxidation*

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Receptor-recognized forms of $\alpha_2$-macroglobulin ($\alpha_2M^*$) bind to two classes of cellular receptors, a high affinity site comprising approximately 1500 sites/cell and a lower affinity site comprising about 60,000 sites/cell. The latter class has been identified as the so-called low density lipoprotein receptor-related protein (LRP). Ligation of receptors distinct from LRP activates cell signaling pathways. Strong circumstantial evidence suggests that the high affinity binding sites are responsible for cell signaling induced by $\alpha_2M^*$. Using sodium hypochlorite, a powerful oxidant produced by the H$_2$O$_2$-myeloperoxidase-Cl$^-$ system, we now demonstrate that binding to the high affinity sites correlates directly with activation of the signaling cascade. Oxidation of $\alpha_2M^*$ using 200 $\mu$M hypochlorite completely abolishes its binding to LRP without affecting its ability to activate the macrophage signaling cascade. Scatchard analysis shows binding to a single class of high affinity sites ($K_d = 71 \pm 12$ pm). Surprisingly, oxidation of native $\alpha_2$-macroglobulin ($\alpha_2M$) with 125 $\mu$M hypochlorite results in the exposure of its receptor-binding site to LRP, but the ligand is unable to induce cell signaling. Scatchard analysis shows binding to a single class of lower affinity sites ($K_d = 0.7 \pm 0.15$ nm). Oxidation of a cloned and expressed carboxyl-terminal 20-kDa fragment of $\alpha_2M$ (RBF), which is capable of binding to both LRP and the signaling receptor, results in no significant change in its binding $K_d$, supporting our earlier finding that the oxidation-sensitive site is predominantly outside of RBF. Attempts to understand the mechanism responsible for the selective exposure of LRP-binding sites in oxidized native $\alpha_2M$ suggest that partial protein unfolding may be the most likely mechanism. These studies provide strong evidence that the high affinity sites ($K_d = 71$ pm) are the $\alpha_2M^*$ signaling receptor.

$\alpha_2$-Macroglobulin ($\alpha_2M$) is a highly conserved, homotetrameric, 720-kDa glycoprotein found in high concentration in the plasma (2–4 mg/ml). It has the unique ability to inhibit all mechanistic classes of proteinases by “entrapping” the proteinase and thereby sterically blocking the access of high molecular weight substrates (reviewed in Refs. 1 and 2). Proteinases first cleave the “bait” region of native $\alpha_2M$ exposing the internal $\gamma$-glutamyl-lys-cysteinyl-thioester bond. Reaction of the thioester bond with a free amino lysyl residue on the surface of the proteinase results in bond rupture and a major conformational change in native $\alpha_2M$. The resulting molecule is much more compact as evidenced by faster migration on a native acrylamide gel (3), electron microscopy (4, 5), sedimentation behavior (6), and circular dichroism (7). Consequently the receptor-recognition site is exposed. Small amine nucleophiles, such as methylamine, can initiate this reaction by directly attacking the thioester bond generating the conformational change and the exposure of the receptor-recognition site without bait region cleavage. Receptor-recognized $\alpha_2M$ ($\alpha_2M^*$) can rapidly eliminate the “entraped” proteinase from the circulation by binding to a cell surface clearance receptor, the low density lipoprotein receptor-related protein (LRP) (8, 9).

LRP is a multiligand receptor that binds to a wide variety of unrelated ligands (reviewed in Ref. 10). Binding of all ligands to LRP can be effectively competed by receptor-associated protein (RAP), which co-purifies with LRP. Prior investigation of $\alpha_2M^*$ binding to LRP has shown that the binding mechanism involves a cluster of positively charged residues on $\alpha_2M^*$ interacting with the second complement-like repeat on LRP, which contains clusters of negatively charged residues (11). Analysis of the receptor-binding site on $\alpha_2M^*$ using monoclonal antibody (12, 13) and recombinantly expressed protein (14, 15) demonstrates that the carboxyl terminus of $\alpha_2M^*$ is involved in receptor binding.

Although LRP is the only $\alpha_2M^*$ receptor identified to date, some important cellular regulatory functions ascribed to $\alpha_2M^*$ suggest that an alternate receptor must exist. $\alpha_2M^*$, but not native $\alpha_2M$, suppresses the production of superoxide anion (16), enhances the release of prostaglandin E$_2$ (17, 18) and platelet activating factor (19), and stimulates the proliferation of vascular smooth muscle cells (20). Moreover, our laboratory has characterized a novel signaling cascade and found that it does not appear to be LRP-mediated (21–23). Furthermore, we have identified two classes of $\alpha_2M^*$ binding sites on peritoneal macrophages and human trabecular meshwork cells, both of which demonstrate activation of signaling cascades after exposure to $\alpha_2M^*$ (24). The lower affinity binding site is 10 times more abundant than the high affinity binding site and has clearly been identified as LRP (25, 26). The identity of the signaling receptor remains elusive; however, using site-directed mutagenesis, we have found that a lysine residue (1374, human numbering) within a 20-kDa fragment constituting the carboxyl terminus of $\alpha_2M$ (RBF) is important for signaling (26).
Very recently, however, some investigators reported that residue 1374 is involved in binding to LRP as well, raising the possibility that the αM* signaling receptor may not be a separate receptor (27). Our previous attempts to study αM* binding to LRP and the signaling receptor using cis-dichlorodiamine-platinum(II) (cis-DDP) modification have shown that cis-DDP modifies a region upstream of the 20-kDa carboxyl-terminal of αM* and that this modification results in decreased binding to LRP while having no effect on cell signaling (25, 28, 29). This observation together with other immunological studies (12, 13, 30) suggest that a region outside of RBF may be involved in αM* binding to LRP. Further characterization of the receptor-binding sites using RBF demonstrated that this fragment both binds to LRP and retains the ability to induce αM* signaling cascade (23, 26). Mutational studies have suggested that LRP and the αM* signaling receptor are distinct entities. To date, however, no data have demonstrated a complete dissociation between αM* binding to LRP and to the signaling receptor.

Previous studies have shown that 25 μM sodium hypochlorite completely abolishes the anti-proteinase activity of native αM* (31, 32). Its effects on αM* receptor-recognition have not been examined. In this study we demonstrate that hypochlorite oxidation of αM* completely destroys its ability to bind to LRP without affecting its ability to bind to the signaling receptor. This modification occurs predominantly outside of the carboxy-terminal 20 kDa, consistent with our previous finding that the cis-DDP-sensitive site is upstream of RBF. Surprisingly, we also found that although hypochlorite oxidation of native αM* results in the selective exposure of the receptor-recognition site to LRP, the ligand cannot signal, thereby providing direct evidence for the dissociation of αM* binding to LRP from binding to the signaling receptor.

EXPERIMENTAL PROCEDURES

Materials—Recombinant RAP construct was a kind gift of Dr. Joachim Herz (University of Texas, Southwestern, Dallas, TX). Recombinant RBF construct was prepared as in (14): The production of both recombinant RAP and RBF is described in detail in Ref. 26. RPMI 1640, fetal bovine serum, Hanks’ balanced salt solution (HBSS), and HBSS without CaCl2, MgCl2, MgSO4 were purchased from Life Technologies, Inc. Bovine serum albumin (BSA), HEPES, sodium hypochlorite, L-methionine, and EDTA were purchased from Sigma. Tris base was purchased from Boehringer Mannheim. PD-10 column was purchased from Pharmacia Biotech Inc. (Upplands, Sweden). Carrier-free Na125I and 125I-Bolton-Hunter reagent for protein iodination was obtained from NEN Life Science Products. 1-2-[5-Carboxyoxazol-1-yl]-6-aminobenzofuran-5-oxyl-2-(2’-aminosulfonylphenyl)ethane-N,N,N,N’-tetraacetic acid acetylomethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR). BCA protein assay kit, IODO-Beads®, and 10,000 molecular weight cut-off Slide-A-Lyzer® dialysis cassettes were purchased from Pierce. All other reagents were of the highest quality commercially available.

Preparation of Activated αM (αM*)—Human native αM was purified according to a previously published protocol (33). Native αM was activated with 200 mM manganese in a buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, for 16–18 h at room temperature in the dark. Unreacted manganese was removed by dialysis for 48 h with four changes of buffer containing 150 mM NaCl, 25 mM sodium phosphate, pH 7.4. Diaoyzed αM* was sterile-filtered using 0.22-μm syringe microfilters from Millipore (Bedford, MA), stored at 4 °C, and used within 2 weeks. Native αM and αM* were iodinated using either IODO-Beads® or 125I-Bolton-Hunter reagent according to the manufacturer-specified protocol. Specific activity of 125I-αM* varied from 1000 to 1500 cpm/μg for IODO-Beads® labeling and 500–700 cpm/μg for 125I-Bolton-Hunter method. The molecular mass of αM* used in these experiments is 720 kDa (34).

Production of Polyclonal Antiserum against RBF—BALB/c mice were immunized three times at 4-week intervals with 50 μg of antigen (RBF) and Titermax® adjuvant (Vaxcel, Norcross, GA) (35). Two weeks after the third immunization, retro-orbital blood was collected, and polyclonal sera from the individual mice were screened for antigen activity by ELISA with RBF as the coating antigen. The mouse with the best titer was boosted with 50 μg of antigen. Four days following the final boost, the mouse was euthanized and bled by cardiac puncture.

Oxidation of Native αM, αM*, and RBF—Oxidation of native αM, αM*, and RBF was performed essentially as described previously (32). In brief, native native αM, αM*, and RBF were incubated with sodium hypochlorite (1.25 mM to 2 mM) for 15 min at 37 °C in phosphate-buffered saline. At the end of the incubation, 20 mM of L-methionine was added to the mixture to quench residual oxidants. The sodium hypochlorite concentration was determined by measuring the absorption at a wavelength of 292.5 nm using 206 μM cm−1 as the extinction coefficient for H2O2 at 7.5 (36). To ensure that oxidation did not result in a loss of iodine label from proteins, trichloroacetic acid precipitation of 250 μM hypochlorite-oxidized 125I-αM* was performed. No significant loss of labeling (i.e., less than 5%, n = 4) was found.

Spectrophotometric Analysis of Oxidized Native αM, αM*, and RBF—The spectral differences between hypochlorite oxidized native αM, αM*, and RBF, and nonoxidized native αM, αM*, and RBF, were analyzed on a DU 640 spectrophotometer (Beckmann Instruments) as described previously (37) with the following modifications. Native αM, αM*, or RBF (0.25 mg/ml) was first oxidized according to the published protocol. 1 ml of each sample was then added to the sample cuvette and measured against nonoxidized native αM, αM*, or RBF (0.25 mg/ml) in the reference cuvette. The absorption difference at 220 nm to 260 nm was calculated. As controls, the absorptions of L-methionine and methionine sulfoxide at these wavelengths were found to be negligible.

Polyacrylamide Gel Electrophoresis (PAGE)—Nondenaturing, nonreducing gradient (5–15%) PAGE, or reducing SDS-PAGE (7.5%) were performed to determine the effects of oxidation on the structure of αM. To visualize the protein bands, gels were fixed in acetic acid with Coomassie Brilliant Blue. Oxidized protein with a concentration up to 125 μg had no effect on the ability of αM to be stained by the dye. Oxidation at greater 250 μg, however, appears to decrease the ability of αM to be Coomassie-stained.

ELISA—Proteins to be tested were incubated in 96-well Immulon plates (Dynatech, Chantilly, VA) for 1 h in 0.1 M NaHCO3, pH 9.6, at room temperature. Following incubation, each well was washed twice in PBS-TBS (phosphate-buffered saline, 0.05% Tween-20) to remove unbound proteins. 50 μl of PBS-TBS with 4% BSA were then added to each well for 0.5 h at room temperature to block nonspecific binding sites. Following incubation, each well was washed twice with PBS-TBS. To each well was then added 50 μl of 1:100 dilution of primary antibody against the protein to be tested and then incubation was continued for 1 h at 25 °C. Following incubation, each well was washed twice with PBS-TBS and then 50 μl of 1:400 dilution of anti-mouse IgG-horseradish peroxidase-conjugated antibody was added. After 1-h incubation at 25 °C, the unbound anti-mouse IgG antibody was removed by washing with PBS-TBS and 50 μl of o-phenylenediamine dihydrochloride substrate solution was added to each well. The enzyme-substrate reaction was allowed to proceed for 15 min at room temperature. A wavelength of 450 nm was determined with a microplate reader (Molecular Devices, Menlo Park, CA). Since oxidation may alter the binding of oxidized αM to the plate, we compared the quantity of the oxidized proteins bound to the plate with that of the nonoxidized protein. We found no statistically significant difference (n = 3) in the amount of proteins bound between oxidized and nonoxidized αM.

Macrophage Harvesting—Pathogen-free female C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC). Peritoneal macrophages were obtained as described previously (38). Thioglycolate-elicited peritoneal macrophages were harvested by peritoneal lavage using 5 ml of 150 mM NaCl, 20 mM HEPES, pH 7.4. The macrophages were pelleted by centrifugation at ~800 x g for 5 min and suspended in RPMI 1640 medium containing 12.5 units/ml penicillin, 6.254 mg/ml streptomycin, and 10% fetal bovine serum. Cell viability was assayed by the trypan blue method (Hausser Scientific, Horsham, PA).

Cell Surface Binding Assay—Macrophages were plated in 24-well cell culture plates (Becton Dickinson, Lincoln, Park, NJ) at 1 x 106 cells/well with a maximum of 10% variation. In some wells, n = 4 and increases for 3 h at 37 °C in a humidified 5% CO2 incubator. These plates were then cooled to 4 °C, and unbound cells were removed by rinsing three times with ice-cold HBSS containing 20 mM HEPES, 5% BSA, pH 7.4 (buffer A). As a control for nonspecific αM* binding, some of the wells were rinsed three times with ice-cold HBSS without CaCl2, MgCl2, MgSO4 containing 20 mM HEPES, 5% BSA, and 5 mM EDTA, pH 7.4 (buffer B) to assess calcium-independent binding. For RAP compe-
Receptor Binding and Cell Signaling of Oxidized $\alpha_2M$

**RESULTS**

**Spectrophotometric Absorption Analysis of Oxidized Native $\alpha_2M$, $\alpha_2M^*$, and RBF**—To quantitate the concentration dependence of native $\alpha_2M$, $\alpha_2M^*$, and RBF modifications, the spectrophotometric absorption differences between oxidized and nonoxidized native $\alpha_2M$, $\alpha_2M^*$, and RBF from $A_{220 \text{ nm}}$ to $A_{400 \text{ nm}}$ were determined. As shown in Fig. 1, the peak difference between oxidized and nonoxidized native $\alpha_2M$ and $\alpha_2M^*$ occurred at approximately $A_{242 \text{ nm}}$ and, to a lesser extent, at $A_{300 \text{ nm}}$. The absorption at $A_{242 \text{ nm}}$ corresponds to a mixture of species with chloramine modification of amino terminus and lysine side chain being the most abundant as determined by trinitrobenzene sulfonate titration (39, 40). The absorption at $A_{300 \text{ nm}}$ corresponds to the formation of dichloramine (41). The hypochlorite oxidation of RBF resulted in minimal modification (Fig. 1C). This is not unexpected since our previous attempts to modify RBF using cis-DDP and hydrogen peroxide, which react...

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**Fig. 1.** Difference spectra analyses of oxidized native $\alpha_2M$, $\alpha_2M^*$, and RBF. $\alpha_2M^*$ (A) and native $\alpha_2M$ (B) were oxidized using 12.5 (single line) or 37.5 (open square or circle) $\mu$m hypochlorite for 15 min at 37°C. Following incubation, 20 nm of l-methionine was added to quench residual oxidants. The spectra of oxidized proteins were measured from wavelengths of 220–400 nm using nonoxidized $\alpha_2M^*$ or native $\alpha_2M$ spectra as base lines. The absorption spectrum of RBF (C) oxidized with 50 $\mu$m of hypochlorite was also measured. The concentration dependence of $\alpha_2M^*$ (open square), native $\alpha_2M$ (open circle), and RBF (filled circle) modification by hypochlorite is shown in D. These data represent the average of two independent experiments performed in duplicate.

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**Data Analysis**—In direct binding studies, the $B_{max}$ and $K_i$ were derived from the x intercept and the slope of the Scatchard plot, respectively. These numbers were also verified using least square analysis based on single-site binding using the Systat® 5.0 computer program. The apparent dissociation constant for the unlabeled ligand, $K_i$, was determined using the equation $K_i = IC_{50}/(1 + L/K_{d})$, where $L$ and $K_{d}$ are the concentration and the dissociation constant for the radiolabeled protein, respectively (37). The concentrations of radiolabeled proteins used in oxidized native $\alpha_2M$ and oxidized $\alpha_2M^*$ cold competition experiments equal the dissociation constant. This reduces the above equation to $K_i = IC_{50}/2$.
at the same residues, have both failed to affect RBF (42–44).

**Fig. 1**

D. Shows the concentration dependence of native $\alpha_2M$, $\alpha_2M^*$, and RBF oxidation at $\lambda_{242}$ nm. Both native $\alpha_2M$ and $\alpha_2M^*$ were equally susceptible to hypochlorite oxidation at all concentrations. Moreover, this modification plateaued at an oxidant concentration of approximately 2 mM. On the other hand, RBF oxidation showed minimal protein modification even at high oxidant concentrations.

**Binding Competition of Oxidized $\alpha_2M^*$ to Macrophages**—The effect of hypochlorite modification on the receptor binding properties of $\alpha_2M^*$ was determined. Two different sets of experiments were performed. The first set showed that oxidation significantly decreased the ability of $\alpha_2M^*$ to compete for the binding of nonoxidized $\alpha_2M$ (Fig. 2A). This effect appeared to take place only when $\alpha_2M^*$ was oxidized at a hypochlorite concentration of at least 37.5 $\mu$M. At 75 $\mu$M hypochlorite, oxidized $\alpha_2M^*$ was unable to compete for more than 90% of the binding of nonoxidized $\alpha_2M^*$. The second set of experiments showed that oxidation can abolish the RAP-sensitive binding of $\alpha_2M^*$ (Fig. 2B). Consistent with the results in the first set of experiments, no significant effect on $\alpha_2M^*$ binding was observed when the protein was oxidized by less than 37.5 $\mu$M hypochlorite. At concentrations equal to or greater than 37.5 $\mu$M, pronounced inhibition of $\alpha_2M^*$ binding was observed. The slight increase in the amount of RAP-insensitive binding when the protein is modified by high concentration of hypochlorite pos-
sibly reflects an increase in electrostatic interaction between oxidized α2M* and the cell surface.

Binding Competition of Oxidized Native α2M to Macrophages—As a control for the binding of oxidized proteins to the cell surface, we also determined the ability of oxidized native α2M to compete for the binding of α2M*. Since native α2M does not bind to either LRP or the signaling receptor, we expect that oxidized native α2M also would not bind to either receptor. Fig. 3A shows that oxidation of native α2M with either 12.5 or 25 μM hypochlorite resulted in an increased ability of oxidized native α2M to compete for the binding of α2M*, suggesting that its receptor-recognition site had been exposed. Oxidation with concentrations of hypochlorite greater than 25 μM resulted in a concentration-dependent decrease in its ability to compete for α2M* binding (data not shown). These findings are confirmed in Fig. 3B showing that native α2M that has been oxidized with hypochlorite can bind specifically to LRP, since its binding can be competed by RAP.

Comparison of the Effects of Different Labeling Methods on Oxidized α2M Binding to Macrophages—In Figs. 2 and 3, the iodinated proteins used in the binding experiments were labeled with IODO-BEADS®. Since this method can potentially oxidize α2M, we performed additional experiments using 125I-Bolton-Hunter-labeled native α2M and α2M* (125I-BH-native α2M, 125I-BH-α2M*, respectively) to verify the results. Fig. 4A shows that oxidation of 125I-BH-α2M* at a hypochlorite concentration greater than 200 μM resulted in the complete absence of binding to LRP. Fig. 4B shows that oxidation by as little as 25 μM of hypochlorite can enhance the binding of 125I-BH-α2M. Maximal enhancement, however, occurs at a hypochlorite concentration of 125 μM. Oxidation appears to effect 125I-BH-α2M similarly compared with IODO-BEADS®-labeled α2M; however, higher concentrations of oxidants were necessary to achieve the same results. Given the greater resistance to oxidation by 125I-BH-α2M as demonstrated by receptor binding, we compared the susceptibility of Bolton-Hunter or IODO-BEADS®-labeled α2M to structural damage by oxidation as a mean of determining which labeling method gave a product that is more represent-
ative of the unlabeled protein. A previous study has shown that \( \alpha_{2M} \) oxidation results in fracturing of the protein along its dimeric axis (32). Fig. 4C shows the tetramer to dimer transition of \( \alpha_{2M} \) upon oxidation. IODO-BEADS\(^{\text{a}} \) labeled \( \alpha_{2M} \) appear to be more sensitive to oxidation compared with unlabeled or Bolton-Hunter labeled \( \alpha_{2M} \) confirming the results from receptor binding.

**Competition Binding of Oxidized RBF to Macrophages**—Since RBF binds to both LRP and the signaling receptor, we investigated whether oxidation altered its receptor-binding properties. Consistent with the spectral analyses data, oxidation at a hypochlorite concentration up to 125 \( \mu \text{M} \) had no significant effect on the ability of RBF to compete for the binding of nonoxidized RBF to cell surface receptors (Fig. 5). Additional calcium signaling experiments revealed that oxidation had no effect on the ability of RBF to signal (data not shown).

**Intracellular Calcium Signaling Studies of Oxidized Native \( \alpha_{2M} \) and \( \alpha_{2M}^{\text{a}} \)—Since our cell surface binding studies showed that oxidation can completely abolish the RAP-sensitive binding of oxidized \( \alpha_{2M}^{\text{a}} \) and expose the receptor-recognition site of native \( \alpha_{2M} \) to LRP, we investigated whether oxidized native \( \alpha_{2M} \) and oxidized \( \alpha_{2M}^{\text{a}} \) also increase intracellular calcium levels by binding to the \( \alpha_{2M}^{\text{a}} \) signaling receptor. Fig. 6A shows that oxidation at a concentration of hypochlorite up to 200 \( \mu \text{M} \) had no effect on the ability of \( \alpha_{2M} \) to generate an increase in \([\text{Ca}^{2+}]_i\). The intracellular calcium rose from base-line levels between 100 and 150 \( \text{nM} \) to peak levels between 400 and 500 \( \text{nM} \) within the first 10 s of cell exposure to the ligands. Boiled \( \alpha_{2M} \) generated no increase in \([\text{Ca}^{2+}]_i\). To determine whether oxidized native \( \alpha_{2M} \) also bound to the signaling receptor, we measured the changes in intracellular calcium levels when cells were treated with native \( \alpha_{2M} \) that was oxidized with 50 and 125 \( \mu \text{M} \) hypochlorite. These concentrations correspond to the concentrations that generated the maximal exposure of LRP binding sites. Fig. 6B shows that no increase in \([\text{Ca}^{2+}]_i\) was observed with the addition of either oxidized or nonoxidized native \( \alpha_{2M} \).

**Reduced SDS-PAGE of Trypsin-treated Oxidized Native \( \alpha_{2M} \)—To determine the mechanism responsible for the oxidative exposure of the receptor-recognition site of native \( \alpha_{2M} \) for LRP, we performed a SDS-PAGE under reducing conditions of oxidized native \( \alpha_{2M} \) that was treated with 20-fold molar excess of trypsin. We hypothesized that if the exposure of the receptor-recognition site is associated with the unfolding of the protein, then oxidized native \( \alpha_{2M} \) should be more susceptible to digestion by trypsin. Fig. 7 shows that as the concentration of hypochlorite used to modify native \( \alpha_{2M} \) increased, fewer large molecular weight protein bands remained, indicating that the oxidized protein is digested more efficiently by trypsin. Oxidation at up to 5 \( \mu \text{M} \) hypochlorite appeared to have no effect on the susceptibility of native \( \alpha_{2M} \) toward trypsin. However, at oxidant concentrations greater than 5 \( \mu \text{M} \), native \( \alpha_{2M} \) became more susceptible to trypsic digestion. This is in agreement with our receptor binding data showing that exposure of the receptor-recognition site begins only after native \( \alpha_{2M} \) has been oxidized by at least an oxidant concentration of 5 \( \mu \text{M} \). To investigate whether other mechanisms may account for the oxidative exposure of the receptor-binding site of native \( \alpha_{2M} \) for LRP, we performed thioester bond titration of oxidized native \( \alpha_{2M} \). Thioester bond rupture results in exposure of the receptor-recognition site, analogous to the reaction of methylamine with native \( \alpha_{2M} \). This could complicate our interpretations of these results. In data not presented, we found no thioester bond rupture by hypochlorite oxidation. These data are consistent with previous studies of native \( \alpha_{2M} \) oxidation (32).

**ELISA of Anti-RBF Antibodies against Oxidized Native \( \alpha_{2M} \)—To further probe whether unfolding of the \( \alpha_{2M} \) secondary structure may result in exposure of RBF, we performed an ELISA using polyclonal antisera raised against RBF. The carboxyl-terminal 20 kDa of \( \alpha_{2M} \) is partially exposed in the native conformation (45), and therefore, it is not unexpected that it would be partially recognized by a polyclonal antisera to RBF. Upon exposure of native \( \alpha_{2M} \) to proteinase or methylamine, this region becomes more exposed so that it binds to cell surface receptors and should, therefore, show greater recognition by antibodies raised against RBF. Fig. 8 shows that native \( \alpha_{2M} \) was partially recognized by the anti-RBF antibodies whereas RBF and \( \alpha_{2M}^{\text{a}} \) were both fully recognized. Oxidized native \( \alpha_{2M} \) was also fully recognized by anti-RBF antibodies; however, this occurred only after it was oxidized by at least 12.5 \( \mu \text{M} \) hypochlorite.

**Direct Binding of Oxidized Native \( \alpha_{2M} \) and \( \alpha_{2M}^{\text{a}} \) to Macro-
phage Cell Surface—Since α2M* has two different Kd and Bmax values, we performed a concentration-dependent binding experiment using oxidized 125I-BH-α2M and 125I-BH-α2M* to determine whether the binding of 125I-oxidized α2M* to the signaling receptor corresponds with its binding to the high affinity site and whether the absence of binding by 125I-oxidized native α2M corresponds with its binding to the low affinity site. Fig. 9A shows the results from direct binding experiments of 125I-oxidized native α2M and oxidized native α2M. Polyvalent antisera raised against RBF (full bar) or normal mouse serum (open bar) were incubated in 96-well plates coated with either BSA (a), RBF (b), native α2M (c), α2M* (d), and native α2M oxidized at 5 (e), 12.5 (f), 25 (g), and 50 (h) μM hypochlorite. Following incubation, plates were washed twice with PBS-Tween 20 to remove unbound proteins. Anti-mouse IgG antibody conjugated with horseradish peroxidase was then added, and the plates were read at λ450 nm for 15 min. These data represent the mean and S.E. of two independent experiments performed in triplicate.

DISCUSSION

In this study we demonstrate that hypochlorite oxidation of native α2M or α2M* can generate exposure of the receptor binding sites to either LRP or the α2M signaling receptor.
respectively. Oxidation of $\alpha_{2}$M* by 200 $\mu$M hypochlorite completely abolished its binding to LRP without affecting its ability to bind to the high affinity sites or the signaling receptor. Oxidation of native $\alpha_{2}$M by 125 $\mu$M hypochlorite resulted in the exposure of the previously buried receptor-binding site to LRP without exposing the binding site to the signaling receptor. Oxidation of RBF showed no decrease in its ability to bind to cell surface receptors, supporting our earlier work showing that the oxidation-sensitive site in $\alpha_{2}$M* is outside of the carboxyl-terminal 20-kDa receptor-binding domain. Studies of the mechanism of oxidative exposure of the LRP-binding site in native $\alpha_{2}$M suggested that protein unfolding may be responsible for this phenomenon. These experiments provide strong proof for the existence of two distinct $\alpha_{2}$M* receptors and the presence of two independent receptor-binding regions on $\alpha_{2}$M*.

Our earlier studies using cis-DDP modification of $\alpha_{2}$M* and RBF have shown that the cis-DDP-sensitive site in $\alpha_{2}$M* is outside of the 20-kDa carboxyl terminus and appears identical to the oxidation-sensitive site (28, 29, 37). Although cis-DDP and oxidation are capable of modifying similar residues, such modification caused only a 4–5-fold decrease in the binding affinity of $\alpha_{2}$M* for LRP. Subsequent studies demonstrate that a lysine 1370 mutant has decreased binding to LRP, and a lysine 1374 mutant is unable to activate the signaling cascade (26). This has been the best evidence for the existence of two distinct $\alpha_{2}$M* receptors; however, recent work by Nielsen et al. (27) suggested that lysine 1374 mutants also have decreased binding to LRP.

To investigate further the identity of the two classes of binding sites, we searched for ligands that could exclusively bind to either class of binding sites and tested their abilities to signal. Hypochlorite is a potent oxidant of native $\alpha_{2}$M (31, 32). Treatment of native $\alpha_{2}$M with 25 $\mu$M hypochlorite resulted in complete destruction of its anti-protease activity. We hypothesized that hypochlorite could also inhibit the ability of $\alpha_{2}$M* to bind to cell surface receptors. In this study, we show that hypochlorite treatment completely eliminated the RAP-sensitive binding of $\alpha_{2}$M* to macrophages without affecting its ability to activate the signal transduction cascade or to bind to the high affinity cell surface receptors. This confirms and extends our previous observation that RAP competes for the binding of $\alpha_{2}$M* to the low affinity sites but is unable to inhibit the ability of $\alpha_{2}$M* to signal or to bind to the high affinity sites (24–26).

Since hypochlorite oxidation is also able to cause the exposure of LRP binding sites in native $\alpha_{2}$M without inducing its ability to signal or to bind to the high affinity sites, our studies provide the best direct evidence to date that the high affinity sites represent the $\alpha_{2}$M* signaling receptors.

The oxidative exposure of the $\alpha_{2}$M-binding site to LRP but not to the signaling receptor, is unique in a number of ways. All of the known naturally occurring $\alpha$-macroglobulins or recombinantly expressed receptor binding fragments activate the signaling cascade (21, 23, 46). RBF mutant 1374 is the first ligand that does not induce a signal, yet it still binds to the high affinity site, albeit with lower affinity. Our hypochlorite oxidized native $\alpha_{2}$M is the first ligand produced that is incapable of signaling and binding to the high affinity sites. The fact that it is still capable of binding to LRP suggests that the binding site on $\alpha_{2}$M* for the signaling receptor is distinct from the LRP binding site. That hypochlorite oxidation can selectively expose only the LRP binding sites in native $\alpha_{2}$M or the signaling receptor binding sites in $\alpha_{2}$M* demonstrates that the ability of $\alpha_{2}$M* to bind to its two receptors can be uncoupled. Efforts are currently being made using oxidized $\alpha_{2}$M* to isolate and purify the signaling receptor.

Our investigation of the mechanism that may explain the oxidative exposure of LRP binding site in native $\alpha_{2}$M suggests that partial protein unfolding may be responsible. Earlier works by Davies et al. (47–50) have shown that protein oxidation results in a partial unfolding of the protein secondary structure, which results in greater susceptibility to intracellular degradation by proteosomes. Similar finding has been reported by Ossanna et al. (51) showing that extracellular proteins such as $\alpha_{1}$-antitrypsin may undergo oxidative inactivation resulting in partial protein unfolding and greater susceptibility to protease digestion.

It is interesting that the exposure of the LRP binding site is dependent on the concentration of hypochlorite used to treat $\alpha_{2}$M and on the labeling method. With IODO-BEADS® labeling, the amount of hypochlorite needed to generate the exposure of LRP-binding sites begins with as little as 5 $\mu$M and peaks at 25 $\mu$M. This is in marked contrast with $\alpha_{2}$M that has been labeled with Bolton-Hunter reagent, which generates LRP binding sites with as little as 25 $\mu$M of hypochlorite but does not peak until 125 $\mu$M. At hypochlorite concentrations greater than 125 $\mu$M, oxidized $\alpha_{2}$M binding to LRP decreased with the concentration of the oxidant. The results obtained from the two labeling methods raise important questions regarding the effects of radiolabeling on receptor binding. Radiolabeling with IODO-BEADS® involves oxidation of tyrosine residues where as Bolton-Hunter labeling modifies amino terminus and lysine side chains. It is possible that the Bolton-Hunter reagent may protect lysine residues from hypochlorite oxidation, thereby generating a ligand that is more resistant to oxidation. Our results, however, show that Bolton-Hunter-labeled $\alpha_{2}$M has similar susceptibility to oxidation as unlabeled $\alpha_{2}$M, whereas IODO-BEADS®-labeled $\alpha_{2}$M is significantly more susceptible to oxidation. This suggests that receptor binding studies with $\alpha_{2}$M should use the Bolton-Hunter labeling method to minimize protein oxidation.

The selective exposure of the LRP binding site in oxidized $\alpha_{2}$M suggests that the two receptor binding regions have distinct properties. We performed an ELISA using polyclonal antisera against RBF to determine if unfolding of the oxidized native $\alpha_{2}$M is associated with an increase in the exposure of RBF. We found that oxidation of $\alpha_{2}$M at greater than 12.5 $\mu$M hypochlorite results in full recognition of RBF by polyclonal antibodies. This exposure, however, is not associated with the ability of the ligand to signal. It is possible that recognition by the signaling receptor requires a more stringent three-dimensional conformation in the receptor binding domain of $\alpha_{2}$M* than recognition by LRP. This is supported by data showing that residues important for LRP binding appear to fall within a short consensus sequence having a predominance of positively charged residues, while the receptor binding region for the signaling receptor appears to require participation by residues from an exposed helix and from other regions of RBF (9, 26, 27). It is also possible that the binding site to the signaling receptor is exposed by oxidation but quickly destroyed; however, the fact that binding to the signal receptor is retained in oxidized $\alpha_{2}$M* even when the protein is treated with 200 $\mu$M hypochlorite suggests otherwise.

Oxidative inactivation of $\alpha_{2}$M* receptor binding to LRP suggests an interesting pathophysiological process that may occur during inflammation. $\alpha_{2}$M is ubiquitous in serum and extracellular fluids (6, 52). During inflammation, neutrophils secrete hypochlorite and proteinases as a defense mechanism against invading foreign organisms (40, 51, 53–55). In the presence of oxidants $\alpha_{2}$M that has reacted with proteinase will lose its ability to bind to its endocytic receptor (LRP) while retaining its ability to signal. This may have significant pathophysiological consequences given that $\alpha_{2}$M* signaling has been...
associated with increased production of prostaglandins and platelet-activating factor as well as increased mitogenesis in vascular smooth muscle cells (17–20). α2M that has not reacted with proteinase will lose its anti-proteinase capacity and the ability to bind to the signaling receptor. The physiological significance of these mechanisms is highlighted by the finding that activated neutrophils can create an environment that contains 124 μM hypochlorite in 2 h (40, 51) and that oxidized α2M* can be isolated from inflammatory lesions in humans (56). Further investigation of the ability of α2M to inhibit proteinases, bind to cell surface receptors, and carry cytokines in the presence of oxidants should provide novel insights into the biological role of this complex molecule during inflammation.

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The Binding of Receptor-recognized \( \alpha_2 \)-Macroglobulin to the Low Density Lipoprotein Receptor-related Protein and the \( \alpha_2 \)M Signaling Receptor Is Decoupled by Oxidation

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