Selective Mutations in Cloned and Expressed \( \alpha_2 \)-Macroglobulin Receptor Binding Fragment Alter Binding to Either the \( \alpha_2 \)-Macroglobulin Signaling Receptor or the Low Density Lipoprotein Receptor-related Protein/\( \alpha_2 \)-Macroglobulin Receptor*

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\( \alpha_2 \)-Macroglobulin (\( \alpha_2 \)M) \(^{1} \) is a member of a superfamily containing both proteinase inhibitors and complement components (for review, see Refs. 1 and 2). Reaction of \( \alpha_2 \)M with proteinases causes a major conformational change in the inhibitor which physically entraps the proteinase and also exposes receptor recognition sites on each of the four identical subunits of the inhibitor. Each \( \alpha_2 \)M subunit contains an internal \( \beta \)-cysteinyl-\( \gamma \)-glutamyl thiol ester which can undergo direct nucleophilic attack by methionine or ammonia. This reaction also causes a conformational change in the inhibitor exposing the receptor recognition sites. The low density lipoprotein receptor-related protein\( /\alpha_2 \)M receptor (LRP/\( \alpha_2 \)MR) has been identified as an \( \alpha_2 \)M receptor (for review, see Ref. 3). This multivalent receptor binds many ligands besides \( \alpha_2 \)M-proteinase or -methylamine-containing receptor-associated protein (RAP), apolipoprotein E, lactoferrin, lipoprotein lipase, Pseudomonas exotoxin A, plasminogen activators alone or in complex with inhibitors, and potentially other serpin-proteinase complexes. Most of these ligands do not compete for binding with each other; however, RAP is able to block binding of all known ligands to LRP/\( \alpha_2 \)MR.

We have recently identified a second \( \alpha_2 \)M receptor which activates a typical signaling cascade after ligation by \( \alpha_2 \)-methylamine or a cloned and expressed receptor-binding fragment (RBF) from rat \( \alpha_2 \)M (4–7). This receptor is coupled to a pertussis toxin-insensitive G protein and activation of this cascade causes a rapid increase in the synthesis of IP3 with a related increase in intracellular calcium ([Ca\(^{2+} \)].) Ligation of \( \alpha_2 \)MSR also promotes activation of protein kinase C (8) and several phospholipases, including activation and phosphorylation of phospholipase C\( \gamma \) (7). \( \alpha_2 \)MSR ligation also promotes alkalinization of the cell cytoplasm (7), and these latter two effects are typical of those seen when certain growth factors, such as epidermal growth factor, bind to their respective cellular receptors. More recently, studies have shown that RBF regulates the growth of smooth muscle cells (9). We suggest that the role of \( \alpha_2 \)MSR is to sense proteolysis in the environment and initiate macrophage responses, some of which may be growth factor-like (7).

Recent studies examining binding characteristics of RBF with murine macrophages have demonstrated two classes of cell surface binding sites (10). The higher affinity class of sites comprises approximately 4–8% of the total \( \alpha_2 \)M binding sites. In the present study, we identify two similar classes of binding sites using wild-type RBF and mutant RBF ligands and show a correlation between the binding to the high and low affinity sites and signal transduction and ligand internalization and degradation, respectively. The lower affinity site demonstrates characteristics of LRP/\( \alpha_2 \)MR since RAP competes for binding to this class of receptors while it fails to compete for binding to the higher affinity site, a characteristic of \( \alpha_2 \)MSR. Site-directed mutagenesis together with secondary structure prediction was employed to probe regions of RBF which might be important for binding to LRP/\( \alpha_2 \)MR and \( \alpha_2 \)MSR.

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EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis of RBF—The FLAG-1/rat \( \alpha_2 \)M construct, containing coding sequences for residues 1313–1451 (human numbering; residues 1336–1476 rat numbering) of the rat \( \alpha_2 \)M RBF, served as a template for polymerase chain reaction (11). Plasmid DNA was amplified using the following oligonucleotides: 5’-GGCGGATCCGGCTGGG-GAGGAGAAGCACCC-3’ and 5’-GGAGATCTGTCGACATG-3’. To facilitate cloning, these primers contained restriction sites for BamHI and NotI. Following amplification, the DNA was cloned into pBluescript SK+ (Stratagene, La Jolla, CA). Mutagenesis was performed on the RBF using the Muta-Gene Phagemid In Vitro mutagenesis kit, version 2 (Bio-Rad). Manipulations were carried out according to the manufacturer’s instructions and the uracil-laden single-stranded DNA was purified using the Prep-A-Gene DNA purification kit (Bio-Rad). 24-Base oligonucleotides were used to create silent mutations and silent restriction sites. Mutant plasmids were transformed into Escherichia coli strain DH5α and screened by colony polymerase chain reaction, restriction digest, and DNA sequencing using Sequenase version 2 (U.S. Biochemical Corp., Cleveland, OH). Using the BamHI and SalI restriction sites, wild-type or mutant RBF was cloned into the pGEX-4T-3 expression vector (Pharmacia Biotech, Piscataway, NJ). Mutant RBFs are represented as follows: original amino acid/human RBF numbering/new amino acid, such as K1370A in which a lysine at residue 1370 (human RBF numbering) has been mutated to an alanine.

Expression and Purification of RBF—RBF was expressed and purified by the method of Herz et al. (12) with the following modifications. DH5α bacteria harboring the pGEX-4T-3 pM1 construct, containing an \( \alpha_2 \)M sequence of 0.6–0.8 kDa, were used for induction (9). Following induction, the cells were harvested by centrifugation at 14,000 × g for 40 min. Affinity chromatography was performed using glutathione-Sepharose 4B (Pharmacia Biotech). The RBF was cleaved from the GST-RBF fusion protein by incubating 1 mg of fusion protein with 10 units of bovine thrombin (U. S. Biochemical) in a 1-mL volume for 2 h at room temperature. Free RBF was obtained by applying the incubation mixture to a glutathione-Sepharose 4B column and collecting the flow-through. Protein samples were dialyzed against 20 mM HEPES, pH 7.3, containing 150 mM NaCl, sterile-filtered, and stored in aliquots at −80 °C. Protein concentration was determined by the BCA Protein Assay (Pierce) using bovine serum albumin as a standard. Yields of GST-RBF fusion protein varied from 1 to 8 mg/L of culture, with approximately 30% recovery after thrombin cleavage and repurification.

Preparation of RAP—The pGEX-39 kDa expression construct was the kind gift of Dr. Jachim Herz (University of Texas-Southwestern, Dallas, TX). The protein was expressed and purified as described in Herz et al. (12) with the following modifications. Before passing the bacterial lysate through needles, DNase and MgCl2 were added to a final concentration of 20 mg/mL and 10 mM, respectively. After a 30-min incubation on ice, the cell debris was removed by centrifugation at 14,000 × g for 40 min. Affinity chromatography step, glutathione-Sepharose 4B was substituted for glutathione-agarose. Protein-containing fractions were pooled, and dialyzed extensively against 20 mM HEPES, pH 7.3, containing 150 mM NaCl, sterile-filtered, and stored in aliquots at −80 °C. The GST has been cleaved from the recombinant RAP-GST product and removed from the RAP preparation.

Secondary Structure Prediction—A secondary structure profile for RBF was computed from the amino acid sequence of RBF with the Profile-fed neural network system (3).2 The primary sequences of seven proteins in the \( \alpha_2 \)-macroglobulin/complement 3-5 family were used in constructing the profile. The homologous protein domains of these molecules ranged from 31 to 62% in sequence identity to rat \( \alpha_2 \)M RBF. Primary sequences used in the analysis were accessed directly from release 2.80 of the EMBL/Swiss-Prot data base by the PHD program and included (code and accession number in parentheses): muri-noglugulin-1 precursor (mug1, a2mg-mouse, P28665); muringluogulin-2 precursor (mug2) (a2mg-mouse, P28666); a-inhibitor III precursor (a1i3-rat, P14460); pregnancy zone protein precursor (pro-human, P02742); rat \( \alpha_2 \)-macroglobulin precursor (\( \alpha_2 \)M-G-rat, P06238); human

2 Input to the program (the primary sequence of RBF, with program instructions) was submitted by e-mail to PredictProtein@EMBL-Heidelberg.DE. Program help information can be obtained by e-mail to PredictProtein@EMBL-Heidelberg.DE.

α2M Lysine Mutations and Receptor Binding

Circular dichroism spectroscopy was performed on wild-type RBF in order to empirically corroborate the secondary structure prediction. The studies were performed using an AVIV 62 DS instrument at 25 °C. RBF (0.16 mg/mL) in 2 mM HEPES, pH 7.2, containing 15 mM NaCl, was placed into the cell over the wavelength range of 200 to 260 nm, averaging times. For each sample nine scans were taken. Secondary structure content was estimated with the program CONTIN (14).

Screening Binding Competition Assays—Macrophages were obtained from C57B1/6 mice and were plated in 48-well plates (1 × 105 cells/well) and incubated for 3 h at 37 °C in a humidified CO2 incubator. After 30 min of incubation at 4 °C, monocytes were rinsed three times with ice-cold buffer A (Hanks’ balanced salt solution containing 25 mM HEPES (HHBSS) containing 12.5 units/mL penicillin, 6.5 μg/mL streptomycin, 5% bovine serum albumin). To assess nonspecific binding, some wells were rinsed three times with ice-cold buffer B (HHBSS without Ca2+ and Mg2+ containing 12.5 units/mL penicillin, 6.5 μg/mL streptomycin, 5% bovine serum albumin, 5 mM EDTA). Increasing concentrations of unlabeled competitor ligands were co-incubated with 15.0 nM 125I-labeled wild-type RBF in 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 in ice-cold buffer A or B. Solubilization solution was added to the wells (1.0 mL NaOH, 0.1% SDS) and allowed to incubate at room temperature for ~5 h before transferring the solution to tubes to be assayed in a γ-counter.

Screening Signaling Studies—

Intracellular calcium concentration \([\text{[Ca}^{2+}])\) was determined by digital imaging microscopy in cells pre-loaded with 1-[2-(5-carboxyoxazol-1-yl)-6-aminobenzofuran-5-oxyl-2-(2’-aminop-5’-methyleneoxy)ethane-N,N,N’-tetraacetic acid acetoxymethyl ester (Fura-2/AM) as described in detail previously (4–6). Briefly, macrophages were plated on glass coverslips sitting in 35-mm Petri dishes at a density of 1.5 × 105 cells/mL and incubated for 16–18 h. The cells were pre-loaded with 4 μM Fura-2/AM for 30 min in the dark. [Ca2+]i, measurements were obtained using a digital imaging microscope. After taking baseline measurements for 5 min, ligands were added and multiple [Ca2+]i measurements were taken.

Competition Signaling Studies—

Intracellular calcium concentration \([\text{[Ca}^{2+}])\) was determined by digital imaging microscopy in cells pre-loaded with Fura-2/AM as described under “Screening Sig-naling Studies (Ca2+).” After baseline measurements, ligands were added and multiple measurements were taken. A second ligand was added later in the assay in order to investigate competition between ligands for the signaling receptor.

Direct Binding Assays and Scatchard Analysis—

Macrophages were obtained and prepared as described under “Screening Binding Competition Assays.” Monolayers were equilibrated to 4 °C and rinsed with buffers A and B as described under “Screening Binding Competition Assays.” Increasing concentrations of 125I-ligands were added to each well and allowed to incubate at 4 °C for 16–18 h. Radioligand solutions were removed from the wells which were rinsed two times and counted as described under “Screening Binding Competition Assays.” Specific binding achieved was determined for each concentration of unlabeled competitor and evaluated using the Sysstat® program for estimation of a Ks, value. This Ks, value equals Ki/(Ki + [Ca2+]i) × 100% inhibition of ligand concentration or IC50 as previously (15). The term, Ks, is used because this assay examines binding to a mixed complement of receptors on the cell surface. Two or more experiments in duplicate were performed for each ligand tested. Standards errors of the mean (S.E.) for these experiments were ±10%, and r values for each curve were ≥0.98.

Intracellular Calcium Measurements—

Intracellular calcium concentration \([\text{[Ca}^{2+}])\) was determined by digital imaging microscopy in cells pre-loaded with Fura-2/AM as described under “Screening Signaling Studies (Ca2+).” After baseline measurements, ligands were added and multiple measurements were taken. A second ligand was added later in the assay in order to investigate competition between ligands for the signaling receptor.

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well contents were solubilized as described under “Screening Binding Competition Assays.” Nonspecific uptake averaged ≤15% of total uptake over several assays. Specific uptake is reported as total uptake minus nonspecific uptake. A 100-fold molar excess of RAP was included with the radioligand in some wells to investigate competition for internalization of the radioligand. Trichloroacetic acid (15%) and 5% bovine serum albumin were added to the well solution samples and the mixtures were centrifuged and incubated at 4°C for several hours. Aliquots were then removed for counting on a γ-counter. Nonspecific degradation averaged ≤10% of total degradation in two assays performed in triplicate.

The stimulated value represents the peak response which occurred between 1 and 2 min after exposure of macrophages to ligand in experiments which were performed at 37°C. The values for [Ca2+]i are the mean ± S.E. Ratio = stimulated value/basal value.

The Ki values represent 1/2 (50% inhibitory concentration) determined from binding competition studies with macrophages at 4°C (15). The S.E. for Ki values for the ligands was ≤10% for a minimum of two separate experiments performed in triplicate.

### RESULTS AND DISCUSSION

Previous studies have demonstrated two distinct macrophage binding sites for receptor-recognized forms of α2M-macroglubulins. Ligation of only one of these receptors, namely α2M-MSR, by receptor-recognized forms of α2M activates a signaling cascade, while LRP/α2MR appears to be important in uptake and degradation of α2M-proteinase complexes (9). By virtue of this uptake function, it can also facilitate antigen presentation mediated by macrophages (17, 18). The α2M receptor binding sites for both LRP/α2MR and α2M-MSR are located in a carboxyl-terminal region of Mγ-20,000 (5, 6, 11, 19, 20). The three-dimensional structure of the RBF is unknown, and high-resolution chemical mapping studies of RBF-receptor interactions have not been reported. We have used secondary structure prediction methods to determine which residues reside in loops or on solvent-exposed surfaces of amphipathic helices and have targeted these residues for site-directed mutagenesis. The algorithm implemented in the PHD program uses a trained neural network which acts upon a primary sequence profile derived from aligned similar sequences to develop a secondary sequence prediction. In this report, secondary sequence predictions for rat RBF were made using amino acid sequences of corresponding domains of seven proteins with sequence similarity to RBF. Rost and Sander (13) report an overall three-state prediction accuracy of 72% for globular proteins.

In agreement with previous analysis of native human α2M (21), RBF is predicted to consist mostly of β-structure (40.8%). In addition, PHD predicts that a short segment of residues in the middle of the domain (residues 1372 to 1376, human numbering) is helical. Cir-

### Table 1

The effect of wild-type RBF and mutant RBFs on [Ca2+]i in murine peritoneal macrophages and screening binding competition Ki values

| Ligand       | Number of studies | Number of cells quantified per study | Basal [Ca2+]i | Stimulated [Ca2+]i | Ratio | Screening Ki |
|--------------|-------------------|--------------------------------------|--------------|--------------------|-------|--------------|
| RBF wild-type | 2                 | 35–40                                | 134 ± 9      | 294 ± 18           | 2.2   | 25           |
| K1374R       | 2                 | 45–50                                | 123 ± 3      | 123 ± 9            | 1.0   | 30           |
| K1374I       | 2                 | 45–50                                | 172 ± 15     | 187 ± 15           | 1.1   | 100          |
| E1431V       | 4                 | 30–40                                | 261 ± 24     | 357 ± 25           | 1.4   | 25           |
| E1431A       | 2                 | 35–40                                | 311 ± 42     | 471 ± 52           | 1.5   | 30           |
| K1370A       | 3                 | 25–30                                | 216 ± 21     | 347 ± 27           | 1.6   | 1500         |

* The stimulated value represents the peak response which occurred between 1 and 2 min after exposure of macrophages to ligand in experiments which were performed at 37°C. The values for [Ca2+]i are the mean ± S.E. Ratio = stimulated value/basal value.

* The Ki values represent 1/2 (50% inhibitory concentration) determined from binding competition studies with macrophages at 4°C (15). The S.E. for Ki values for the ligands was ≤10% for a minimum of two separate experiments performed in triplicate.

#### Figures

**Figure 1.** Single cell response of macrophages exposed to wildtype and mutant RBFs. Monolayers of Fura-2 loaded cells were stimulated with wild-type or mutant RBFs at a concentration of 40 nM. The arrow indicates the time of addition of the ligands. A, the symbols used are: ●, wild-type RBF; ○, K1374R; △, mutant RBF and K1374I mutant RBF. B, the symbol used is: ○, K1370A mutant RBF.
cular dichroic spectra analysis also indicates that RBF is comprised of mostly β-sheet structure (37%), with a small amount of α-helix content (8%). The predicted distribution of hydrophobic and hydrophilic residues suggests that the helical segment in the middle of the domain is likely to be amphipathic. RBF could be expected to fold into a 6–8-stranded antiparallel β-sheet sandwich in which the helical segment is exposed as a surface loop connecting opposing sheets. Such a structure is likely to be a candidate receptor binding site. Residues between 1359 and 1376 (human numbering) show greater than 95% identity for sequences occurring in receptor-recognized forms of α2-macroglobulins. Moreover, very few non-conservative substitutions occur in this region. Accordingly, we have designed degenerate oligonucleotide primers to introduce mutations at residues within the highly conserved, putative α-helical region and in other highly conserved regions of RBF.

Binding competition assays were performed for wild-type RBF and RBF mutants and the $K_v$ values were obtained (Table I). The $K_v$ value obtained using this method does not differentiate between classes of binding sites; however, it does provide a gross estimate of alterations in cell surface binding of the ligand. RBF mutants found to have a significant change in this $K_v$ value or loss of signal transduction were then further analyzed using direct binding methods which provide detailed characterization for one or more classes of binding sites. RBF mutant K1370A was found to have a $K_d$ value of 1500 nM as compared to 25 nM for the wild-type RBF and was selected for more detailed characterization of binding and IP₃ studies.

As a means to screen for alterations in signal transduction, [Ca²⁺]ᵢ studies were performed for wild-type RBF and RBF mutants (Table I). Ligands identified to have lost the ability to stimulate an increase in [Ca²⁺]ᵢ were then selected for studies of IP₃ synthesis. Mutant RBFs K1374R and K1374I did not demonstrate a significant [Ca²⁺]ᵢ response. Five to 10% of the macrophages exposed to these RBF mutants show a very minimal response while 80–85% of cells show a significant response to wild-type RBF. The single cell response of macrophages exposed to wild-type RBF and mutant RBFs K1374R, K1374I, and K1370A are shown in Fig. 1. RBF mutant K1370A which shows a significantly increased $K_v$ value in the screening binding competition assay elicits a 61% increase in [Ca²⁺]ᵢ (Table I), a response comparable to that obtained in other
studies with wild-type RBF (5, 6) which suggests that the interaction of RBF mutant K1370A with α2MSR is similar to that of wild-type RBF. Signaling studies were also performed to investigate cross-competition between some of the ligands. We have previously shown that exposure of macrophages to a ligand for α2MSR results in a refractory period during which time addition of a second dose of ligand for α2MSR does not result in signaling. Wild-type RBF and mutant RBF K1370A demonstrate such cross-competition for the signaling binding site (Fig. 2). Lactoferrin, a LRP/α2MR ligand which does not signal through α2MSR but through LRP/α2MR (6), fails to show cross-competition with mutant RBF K1370A for binding to a signaling receptor site.

Direct binding studies of wild-type RBF and mutants K1374R and K1370A are shown in Figs. 3, 4, and 5 and the Scatchard analyses are summarized in Table II. Wild-type RBF displays binding to two distinct sites, one class having 1,500 sites/cell with an apparent Kd of 90 pM and the other site having 60,400 sites/cell with an apparent Kd of 40 nM. Only binding to the lower affinity site is compatible by RAP. The Kd of 40 nM for the lower affinity site is consistent with published values for cloned and expressed RBF binding to cells (20 nM) (11, 19, 22, 23). It is possible that the data determined by Scatchard analysis to fit a two-site model may actually represent a complicated one-site model, such as dimerization of receptors by a multivalent ligand as in the case of human α2M which may create a high affinity component, or negative cooperativity of binding to a receptor which may create a low affinity component. Neither of these explanations seems likely given the use of a monomeric ligand and the selective inhibition of only one binding site by the competitor RAP. K1374R mutant RBF demonstrates comparable binding to lower affinity sites; however, binding to high affinity sites is reduced by 83%. Mutation of Lys-1370 to Ala results in an increase in the apparent Kd to the lower affinity site from 40 to 150–200 nM, while binding to the high affinity site is unaltered. This decreased affinity for the low affinity site makes the K1370A mutant RBF a poor competitor against wild-type RBF for binding to cells (Table I), with only 2–3% of cell surface α2M receptors being the high affinity class, to which the K1370A mutant
RBF still binds comparably to wild-type RBF. Studies completed with E1431A mutant RBF show that no apparent alterations result in either binding assay or the signal transduction assay from this mutation. Two classes of binding sites are identified with similar characteristics as the wild-type RBF binding sites: apparent Kd values = 40 nM (1100 receptors/cell) and 10 nM (40,500 receptors/cell).

Internalization and degradation studies were performed with mutant RBFs (Fig. 6). Internalization is comparable for wild-type RBF, K1374R, and E1431A mutant RBFs and is RAP-inhibitable. Internalization and degradation of K1370A mutant RBF, however, is negligible as compared to wild-type RBF, and K1374R and E1431A mutant RBFs.

Effects of wild-type RBF and mutant RBFs on the generation of IP3 by macrophages was studied (Fig. 7). Binding of wild-type RBF induces synthesis of IP3 as previously reported (4–6). However, neither K1374R nor K1374I mutant RBF induce any increase in IP3 synthesis. Consistent with these results, while wild-type RBF induces an increase in [Ca2+]i, neither of the two mutant RBF’s elicit a significant increase in [Ca2+]i (Fig. 1). Table II summarizes the IP3 response data.

The only information known concerning the interaction of RBF with α2MS is that RAP does not affect its binding (5, 6). By contrast, RAP does block the binding of RBF to LRPA/MR (5, 20). The data presented in this study indicate that mutation of K1374 affects binding of RBF to α2MSR while not altering binding to LRPA/MR significantly. This suggests that K1374 is part of the ligand binding site for α2MSR. It is possible that mutation in this region disrupts tertiary structure, however, gross alteration of tertiary structure is unlikely since mutant RBFs can still bind LRPA/MR, with a decreased affinity only in the case of RBF mutant K1370A. It is known that interaction of RBF with LRPA/MR is very dependent on conformation (23). In addition, mutant RBFs were expressed and purified as effectively as the wild-type RBF. Mutations were also introduced into a second highly conserved sequence in RBF (residues 1424 and 1435; human numbering) (19). These mutants signaled comparably to wild-type RBF with none showing decreased binding to LRPA/MR (Table II), and further studies of E1431A mutant RBF show no alteration in binding to either site as compared to wild-type RBF.

Lys-1370, which is predicted to be in a loop immediately upstream of the helix containing Lys-1374, is conserved in all receptor-binding α-macroglobulins (19). In frog α-macroglobulin, which does not bind to macrophages (24), this residue is an alanine. Interestingly, mutation of Lys-1370 to alanine in the rat protein results in a significantly increased Kd for binding to α2M Lysine Mutations and Receptor Binding

| Ligand    | Number of studies | Site 1 Kd (nM) | Site 1 (receptors/cell) | Site 2 Kd (nM) | Site 2 (receptors/cell) |
|-----------|-------------------|----------------|-------------------------|----------------|-------------------------|
| RBF wild-type | 4                 | 0.09 ± 0.02   | 1,500 ± 300             | 40 ± 14       | 64,000 ± 5,200          |
| RAP       | 2                 | 0.19 ± 0.06   | 1,500 ± 500             | 36 ± 11       | 25,500 ± 3,600          |
| K1374R    | 3                 | 0.02 ± 0.01   | 200 ± 50                | 17 ± 6        | 42,000 ± 5,100          |
| RAP       | 2                 | 0.01 ± 0.01   | 300 ± 80                | 5 ± 3         | 14,200 ± 2,200          |
| K1370A    | 3                 | 0.08 ± 0.02   | 900 ± 200               | 200 ± 100     | 62,500 ± 4,800          |
| RAP       | 2                 | 0.03 ± 0.01   | 800 ± 200               | 150 ± 80      | 23,500 ± 2,100          |
| E1431A    | 2                 | 0.04 ± 0.02   | 1,100 ± 400             | 10 ± 4        | 40,500 ± 6,500          |

A: Internalization and degradation of wild-type 125I-RBF, 125I-K1374R, 125I-K1370A, and 125I-E1431A mutant RBFs by murine macrophages at 37°C. A, picomoles of 125I-wild-type RBF and 125I-E1431A mutant RBF internalized in the absence (●) and (▲), respectively) and presence (○) of 100-fold molar excess of RAP. B, picomoles of 125I-K1374R mutant RBF and 125I-K1370A mutant RBF internalized in the absence (●) and (▲), respectively) and presence (○) of 100-fold molar excess of RAP. C, picomoles of 125I-wild-type RBF (●) and 125I-E1431A RBF (▲), 125I-K1374R RBF (○), and 125I-K1370A RBF (△) degraded by macrophages at 37°C for 2 h. Degraded ligand was defined as soluble in 15% trichloroacetic acid, 5% bovine serum albumin samples of the well solutions. Nonspecific degradation was determined in wells containing 5 mM EDTA and subtracted from the total degradation at each concentration tested.

3 In collaboration with the laboratory of Dr. Jan Enghild, we have cloned this frog α-macroglobulin. The sequence is as yet unpublished.
macrophages (Kd = 1500 nM) (Table I). Macrophages exposed to this mutant show augmented IP3 synthesis (Fig. 7B) and [Ca2+] increases like those of wild-type RBF (Fig. 1, Table II).

Initial estimates suggested that as many as 20% of α2M receptors on macrophages are α2M-MSR (5). The more detailed binding studies presented in this report suggest that less than 5% of the total receptors on macrophages are α2M-MSR (10), agreeing with studies of rat vascular smooth muscle cells (less than 3–5%) (9). An analogy to this situation may be that of thrombin-binding sites on platelets, where high affinity binding sites for thrombin are estimated to comprise 20–50 sites/platelet out of a total of 85,000–591,000 thrombin binding sites/platelet (25, 26). This extremely low percentage of sites is within the error of typical binding competition or displacement assays, and it appears that the percentage of α2M cellular receptors which are α2M-MSR is similarly well within the 5–10% error of these assays. Based on these observations and the fact that the K1370A mutation resulted in an increase in Kd of about 5–10-fold without significant loss of signal transduction properties, we hypothesize that this observed decrease in receptor affinity is to LRP/α2MR.

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FIG. 7. IP3 synthesis in macrophages exposed to wild-type RBF and mutant RBFs. These studies were performed with macrophages in multiwell plates at 37°C. The cells were prelabeled with myo-[2-3H]inositol to assess IP3 synthesis as described by Berridge (16) and detailed for macrophages by Misra et al. (4). A, the symbols used are: ●, wild-type RBF; ▲, K1374R mutant RBF; and △, K1374I mutant RBF. B, the symbols used are: ●, wild-type RBF and ○ K1370A mutant RBF. The cells were exposed to wild-type and mutant RBFs at a concentration of 40 nM.
Selective Mutations in Cloned and Expressed α-Macroglobulin Receptor Binding Fragment Alter Binding to Either the α₂-Macroglobulin Signaling Receptor or the Low Density Lipoprotein Receptor-related Protein/α₂-Macroglobulin Receptor

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