The binding and internalization of $^{125}$I-labeled $\alpha_2$-macroglobulin ($^{125}$I-$\alpha_2$M) was studied in cultured fibroblasts. Two classes of binding sites were detected on cell surfaces. One class corresponds to the previously described, high affinity and low capacity sites. The other class of binding sites may mediate uptake of high physiological blood levels of $^{125}$I-$\alpha_2$M and has not been described previously. At 0 °C, this lower affinity class saturates at approximately 1,000 µg/ml and has a capacity of approximately 600,000 sites/cell. The lower affinity class accounts for the vast majority of cellular receptors for $\alpha_2$M. An assay employing pepstatin at pH 4 was developed to distinguish between surface-bound and internalized $^{125}$I-$\alpha_2$M. Cellular uptake of $^{125}$I-$\alpha_2$M at 37 °C has a component which saturates between 200 and 1,000 µg/ml and the rate of internalization of this component was approximately 1,700,000 molecules/cell/h. One mM Ca$^{2+}$ was required for cell uptake of $^{125}$I-$\alpha_2$M at 37 °C. Ca$^{2+}$ was also required for binding at 0 °C to both low and high affinity classes of binding sites for $^{125}$I-$\alpha_2$M. The transglutaminase inhibitors bacitracin, monodansylcadaverine, and N-benzoxycarbonyl-5-diazo-4-oxonorvaline paraminophenyl ester all inhibited cellular internalization of $^{125}$I-$\alpha_2$M at 37 °C. Each of these three compounds selectively reduced $^{125}$I-$\alpha_2$M binding to the high affinity, low capacity component at 0 °C. Based on the current binding studies and previous studies using electron microscopy which showed that bacitracin and other transglutaminase inhibitors block clustering of $\alpha_2$M-receptor complexes in coated pits, we suggest that the inhibitors block the accumulation of occupied lower affinity $\alpha_2$M receptors in coated pits where they acquire a higher apparent affinity.

Many proteins as well as small molecules interact with binding sites on cell surfaces. Such binding sites are usually termed "receptors" when binding of the ligand occurs within the physiological range of ligand concentrations, when the binding is saturable, and when some degree of ligand specificity can be demonstrated. $\alpha_2$-Macroglobulin is a large, tetrameric protein found in plasma and other body fluids of many vertebrates. Among its properties are its abilities to form complexes with a wide variety of endopeptidases (modifying their activity) and to bind zinc (1, 2). The exact biological role of $\alpha_2$M is unknown. Using rh-$\alpha_2$M it has been recently demonstrated that $\alpha_2$M at near physiological concentrations is able to bind to a receptor on the surface of a variety of fibroblastic cell lines (3-5). Binding is followed by the rapid appearance of fluorescent spots within the cell that correspond to phase-neutral vesicles. After $\frac{1}{2}$ h or more, fluorescence appears in phase-dense, lysosomal structures (3-5). Using peroxidase-labeled, anti-$\alpha_2$M antibodies, the fate of receptor-bound $\alpha_2$M has been followed using electron microscopy. By this technique, $\alpha_2$M-receptor complexes appear to rapidly associate with clathrin-coated pit structures on the plasma membranes. After a rapid, as yet unclear transition, the $\alpha_2$M-receptor complexes appear in uncoated vesicles and these vesicles eventually transfer their contents to lysosomes (6-8). Using rh-$\alpha_2$M, several properties of the $\alpha_2$M-receptor internalization process have been inferred. The appearance of fluorescent spots does not occur in the absence of Ca$^{2+}$ or in the presence of a wide variety of primary amines. Since Ca$^{2+}$ dependence and amine sensitivity are also characteristic of transglutaminase, it has been hypothesized that a transglutaminase-like activity is involved in the process of $\alpha_2$M-receptor uptake by cells (9, 10). Study of this uptake process would be facilitated by more quantitative methods.

The purpose of the present study is to develop a quantitative, biochemical method to study cellular binding and uptake of $\alpha_2$M. $^{125}$I-$\alpha_2$M is used to study the kinetics and other properties of binding and internalization of this ligand. Some studies have already been carried out which demonstrate that cells appear to take up and degrade $^{125}$I-$\alpha_2$M (11, 12) and rh-$\alpha_2$M (3-5) at near physiological concentrations. Only a few studies have attempted to characterize cellular binding components for $\alpha_2$M. A high affinity, low capacity binding component for $^{125}$I-$\alpha_2$M complexed with trypsin ($\alpha_2$M-T) has been described in cultured human fibroblasts (11, 12) and macrophages (13-15). High affinity binding of $^{125}$I-$\alpha_2$M could not be detected and it was proposed that it did not exist (11, 12). A slightly lower affinity binding component for $^{125}$I-$\alpha_2$M in cultured fibroblasts has been described in a recent report (16). This component appears to be saturated at less than 1% of physiological plasma $\alpha_2$M concentration. The relationship between binding sites for $^{125}$I-$\alpha_2$M and $^{125}$I-$\alpha_2$M-T has been unclear. The present report for the first time characterizes and compares $^{125}$I-$\alpha_2$M and $^{125}$I-$\alpha_2$M-T binding and internalization over a wide range of ligand concentrations. Previous studies have shown that cellular uptake at 37 °C of rh-$\alpha_2$M and $^{125}$I-$\alpha_2$M (16) reaches saturation at much higher concentrations. 

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PBS, phosphate-buffered saline; DCD, monodansylcadaverine; BDONV, N-benzoxycarbonyl-5-diazo-4-oxonorvaline paraminophenyl ester; dansyl, 5-dimethylaminonaphthalene-1-sulfonfyl. 

The abbreviations used are: $\alpha_2$M, $\alpha_2$-macroglobulin; rh-$\alpha_2$M, rhodamine $\alpha_2$-macroglobulin; $\alpha_2$M-T, $^{125}$I-$\alpha_2$M complexed with trypsin; $\alpha_2$M-M. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
levels of α2M than does binding at 0 °C to high affinity receptors. Thus, the relationship between high affinity binding sites and cellular uptake of α2M is unclear.

A biochemical technique is described which makes it possible for the first time in fibroblasts to quantify and distinguish surface-bound and internalized 125I-α2M and 125I-α2M-T. Using this method, the effects of Ca2+ and of inhibitors of transglutaminase are then studied.

**MATERIALS AND METHODS**

α2M was prepared from whole human plasma as previously described (8, 18). α2M was iodinated using the chloramine-T procedure (19). The specific activity of 125I-α2M was 4 × 10⁶ cpm/μg. 125I-α2M-T and α2M-T were prepared (12, 19) by reaction of a 6 μM excess of trypsin (Sigma) over α2M for 30 min at 37 °C. Then a 10 μM excess of soybean trypsin inhibitor (Sigma) over trypsin was added to stop the reaction. The α2M-T complex was then chromatographed on Sephadex G-100 in PBS, pH 7.4 (Gibco), to remove unreacted trypsin and trypsin inhibitor. Recovery of α2M-T ranged from 75 to 95% as determined by radioactivity or A280. 125I-α2M and 125I-α2M-T were greater than 95% precipitable with 10% trichloroacetic acid. Using soybean trypsin, gel electrophoresis (5% polyacrylamide) and autoradiography, 75% of 125I-α2M-T co-migrated in the ranges of the α2M monomer (M₀ = 180,000) and the monomeric α2M-protease complex (M₄ = 80,000). 125I-α2M contained less than 8% of the 125I-α2M protease complex, while the 125I-α2M-T contained 20% of unreacted 125I-α2M.

NRK-2T cells (a cell line with fibroblastic morphology) were grown in Dulbecco-Vogt's modified Eagles medium containing 10% (v/v) calf serum. For experiments, cells were plated in 35-mm Falcon tissue culture dishes at 1 × 10⁵ cells/dish in 2 ml of medium supplemented with calf serum which had been heated for 1 h at 80 °C. Cells were usually used 4 days after plating. At this time, confluent monolayers had formed and each dish contained approximately 5 × 10⁵ cells.

Binding experiments were usually performed in duplicate 35-mm dishes in 1 ml of Dulbecco-Vogt's medium containing 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.2% bovine serum albumin (DV-IA medium). Cells were washed three times and reincubated in the medium at 37 °C for 1 h preceding experiments. Then fresh medium containing 125I-α2M was added. If binding at 0 °C was to be studied, cells and medium containing 125I-α2M were placed on ice prior to incubation. After binding incubations at 0 °C or at 37 °C, cells were cooled on ice and washed five times with phosphate-buffered saline containing 1.5 mM CaCl₂ (PBS-Ca buffer). Total cellular radioactivity was solubilized with 1 ml of RIPA buffer (50 mM Tris, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5% aprotinin from Sigma) or 0.1 N NaOH. If surface and internalized labeled a2M were to be determined, the incubated cell cultures were then washed 1 ml of 5 mM of pepsin (Sigma) in 70 mM acetic acid (pH 4.0) and 50 mM NaCl was added to the cells on ice. The cells were then incubated at room temperature for 15 min, and the supernatant (containing radioactivity previously bound to cell surfaces) was removed. Remaining cell-associated radioactivity was then solubilized. Radioactivity was determined in an LKB mini-γ-spectrometer. Usually binding assays were carried out using 125I-α2M with or without an excess (3-5 μg/ml) of α2M to compete for "specific binding." At low concentrations of 125I-α2M, non-specific binding at 0 °C was 5-10% of the total, while at 100 μg/ml of 125I-α2M (made by dilution of specific activity of 125I-α2M or nonradioactive α2M-T by 4.0) 75% of 125I-α2M-T bound to cells with greater avidity are completely saturated at only 1% physiological α2M levels.

**RESULTS**

**Cellular Binding of 125I-α2M and 125I-α2M-T at 0 °C**—The characteristics of cellular binding of 125I-α2M and 125I-α2M-T were examined. Fig. 1 shows that binding at 1 and 100 μg/ml of 125I-α2M-T was apparently equilibrated by 8 h. There were no striking differences between α2M and α2M-T. Cell monolayers were then incubated for 8 h with a wide range of concentrations of each ligand and the data analyzed by the method of Scatchard (21). For either ligand, two classes of binding sites were detected (see Fig. 2). For 125I-α2M, the classes of binding sites were: I) 10,500/cell, Kᵦ = 0.20 nm (0.17 μg/ml); II) 600,000/cell, Kᵦ = 100 nm (85 μg/ml). For 125I-α2M-T (data not shown), the classes of binding sites were: I) 11,500, Kᵦ = 0.11 nm (0.09 μg/ml); II) 800,000/cell, Kᵦ = 42 nm (37 μg/ml) (data not shown). When 1 μg/ml of 125I-α2M or 125I-α2M-T was incubated at 0 °C for 8 h with a 100 × excess of nonradioactive α2M or α2M-T, the displacement of radioactivity was similar by either nonradioactive ligand (data not shown). These data indicate that α2M and α2M-T bind to the same sites. Plasma levels of α2M in man are approximately 3 mg/ml (18). The lower affinity sites are saturated by a near physiological level of α2M, whereas the higher affinity sites which bind 125I-α2M-T with greater avidity are completely saturated at only 1% physiological α2M levels (17).

**Development of an Assay to Distinguish Noninternalized and Internalized 125I-α2M**—On the basis of studies using ferritin-α2M, rh-α2M, and 125I-α2M, it appears that cellular uptake of membrane receptor-bound α2M is a temperature-dependent process. Virtually no α2M-receptor complexes are internalized by cells at 0 °C (3-6, 20). After cells were incubated at 0 °C with 125I-α2M, cells were washed and incubated either with or without inhibitors of transglutaminase (9, 10, 20) DCD (Pluka), bactracin (Sigma), and BDONV (Melloy). BDONV is an irreversible inactivator of cellular transglutaminase (20). Bactracin was dissolved at 30 mg/ml in PBS and 30 μl was added/dish of cells for 1 mg/ml of final concentration. DCD was dissolved in an equimolar amount of HCl and then brought to 1 mg/ml of concentration with PBS. Thirty μl was used/dish of cells for 100 μM final concentration. BDONV was dissolved in dimethyl sulfoxide at 3 mg/ml. Ten μl was added/dish of cells for 100 μM/ml of final concentration. In some experiments, trypsin (Sigma) was briefly incubated with cell monolayers to deactivate binding sites for 125I-α2M. Treatment of cells with Ca²⁺-free medium, trypsin, or transglutaminase inhibitors did not result in detachment of cells from the dishes.

For analysis of the Scatchard (21) plots of data on the concentration dependence of binding by 125I-α2M (Fig. 2) and 125I-α2M-T to cells, a computer program called SCATFIT (22) was implemented. This program employs a least squares analysis of data and allows automatic determination of the number of classes of binding sites and their parameters. Using this method of analysis, it was determined that two classes of binding sites (four parameters) were the best fit to the data. The computer-generated theoretical classes of binding sites are indicated on Fig. 2. Statistical analysis was performed on the data presented in Figs. 7 and 9. Three or more independent observations were averaged for each data point. Data are averages ± S.E. Student's t test was performed and p values determined using the criterion p < 0.05 to indicate data significantly different from controls.

**FIG. 1. Time dependence of binding by 125I-α2M and 125I-α2M-T at 0 °C**—Cell monolayers were incubated for various times at 0 °C with 1 or 100 μg/ml of 125I-α2M-T. Then cells were washed and solubilized and the radioactivity determined. Parallel incubations contained 3 μg/ml of the appropriate nonradioactive ligand to give a measure of nonspecific binding. This was 50-100 × the Kᵦ and enough to displace receptor-bound ligand. Nonspecific binding (not shown) was 5-10% of the total with the 1 μg/ml concentrations and 30% of the total with the 100 μg/ml concentrations. Total binding - nonspecific binding = specific binding. Each point is the average of duplicate determinations and this experiment was representative of three independent experiments.
bated at 0 °C with 125I-α2M and washed, the bound ligand could be released from the cells by incubation at room temperature with 8 mg/ml of pepsin at pH 4.0 (Fig. 3). If instead, the washed cell monolayers to which 125I-ligand was bound were warmed to 37 °C for 15 min, the 125I-α2M or 125I-α2M-T became resistant to pepsin treatment. It is likely that pepsin resistance is a consequence of 125I-α2M internalization. The pepsin removal of surface-bound radioactivity occurred rapidly, while release of internalized 125I-α2M was minimal even after 1 h pepsin treatment. Treatment of cell monolayers with pH 4 enzyme buffer without pepsin did not release significant levels of radioactivity from cells treated with 125I-α2M at either 0 °C or 37 °C.

When cells were incubated continuously at 37 °C with 125I-α2M (Fig. 5) or 0.5 μg/ml of 125I-α2M-T (data not shown), uptake was linear for at least 1 h. Fig. 4A shows the concentration dependence of cellular uptake of 125I-α2M at 37 °C. Half-maximal "specific" or saturable uptake averaged 150 μg/ml (n = 3) and was near saturation at 1 mg/ml of 125I-α2M. The pepsin assay was then used to examine the internalization of 125I-α2M during cellular uptake at 37 °C. In Fig. 4B, the 125I-α2M concentration dependence of specific cellular uptake at 37 °C was studied using the pepsin assay. Half-maximal surface binding and internalization averaged 150 μg/ml of 125I-α2M (n = 3). The maximal rate of specific internalization of 125I-α2M averaged 1,700,000 molecules of 125I-α2M/cell/h (n = 3).

The rapid process of 125I-α2M internalization was also studied (Fig. 5). 125I-α2M was bound to cells at 0 °C, the cells washed, and then warmed to 37 °C for various times. With either α2M concentration, internalization was mostly complete by 2 min. At the end of the 37 °C incubations, dishes of cells

**Fig. 2.** Concentration dependence of binding by 125I-α2M. Cell monolayers were incubated at 0 °C for 8 h with a range of 125I-α2M concentrations (0.02-1000 μg/ml) with or without a 5 mg/ml excess of α2M. The non-specific component has been subtracted for each point. Data have been graphed by the method of Scatchard. Data have been analyzed by computer (see "Materials and Methods") and the computer-derived theoretical classes of binding sites indicated by solid lines. The data have been pooled from five independent experiments. Some experiments utilized a single concentration of 125I-α2M displaced by various amounts of nonradioactive α2M, while others use increasing amounts of 125I-α2M.

**Fig. 3.** Acid-pepsin assay for surface-bound 125I-α2M. A, time dependence of pepsin release of 125I-α2M. Cell monolayers were incubated at 0 °C for 4 h with 0.5 μg/ml of 125I-α2M with or without a 3 mg/ml excess of nonradioactive ligand. Then the cells were washed and retained on ice or warmed to 37 °C (in DV-HA medium) for 15 min and cooled on ice. Then 8 mg/ml of pepsin in pH 4.0 buffer was added on ice and the cells warmed to room temperature for various times. Radioactivity was then determined in the supernatant (pepsin releasable) and in the solubilized cells (pepsin resistant). The pepsin-releasable or surface-bound radioactivity is then expressed as a percent of total cell-associated radioactivity (0.02 μg/dish for 0 °C incubations and 0.015 μg/dish for 0 °C/37 °C incubations). B, pepsin assay with 125I-α2M and 125I-α2M-T. Cells were incubated as in A with 0.5 μg/ml of 125I-ligand and the surface-bound radioactivity was determined after a 10-min treatment with pepsin. During warming of the cells at 37 °C, 10-20% of the surface-bound radioactivity was released into the medium. Data in A and B are averages of duplicate determinations and expressed as specific binding. This experiment was representative of two independent experiments.

**Fig. 4 (left).** Concentration dependence of cellular uptake of 125I-α2M. A, cell monolayers were incubated in duplicate with various concentrations of 125I-α2M with or without a 5 mg/ml excess of nonradioactive α2M at 37 °C for 30 min. Total, nonspecific, and specific binding are shown. The data are representative of three independent experiments. B, cells were incubated as in A but the acid-pepsin assay was used to distinguish surface-bound and internalized 125I-α2M. Each point is the average of duplicate determinations and the data are representative of three independent experiments. Nonspecific surface binding of 125I-α2M comprised 40% of the total nonspecific radioactivity, and its increase was linear over the concentration range tested. Nonspecific internalized 125I-α2M also increased in a linear fashion with respect to 125I-α2M concentration and was 60% of the nonspecific total.

**Fig. 5 (right).** Rapid internalization of 125I-α2M receptor complexes. Cells were incubated with 125I-α2M (with or without a 2 mg/ml excess) for 4 h at 0 °C. Then the monolayers were washed and warmed at 37 °C (with DV-HA medium) for various times. Then the cells were cooled on ice and surface-bound and internalized 125I-α2M determined with the acid-pepsin assay. Data are corrected for nonspecific binding and each point is the average of duplicate determinations. In both A and B, the missing radioactivity was released by the cells into the media during warming at 37 °C. The data are representative of two independent studies.
were placed on ice, washed, and pepsin treated. Since the cells do not cool down instantaneously, internalization probably proceeds more slowly than suggested by the data.

Sensitivity of \( ^{125}\text{I-}{\alpha_2}\text{-Macroglobulin} \) Binding and Uptake to \( \text{Ca}^{2+} \), Protease, and Transglutaminase Inhibitors—Using fluorescent \( \alpha_2\text{-M} \), previous studies have demonstrated that \( \alpha_2\text{-M} \) uptake required \( \text{Ca}^{2+} \) and could be blocked using a variety of inhibitors of transglutaminase (9, 10, 20). The sensitivity of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) uptake was examined using the pepsin assay to distinguish internalized and noninternalized ligand. Fig. 6 shows that bacitracin inhibits both internalization and accumulation of surface-bound \( ^{125}\text{I-}{\alpha_2}\text{-M} \) for at least 2 h. Fig. 7 demonstrates that \( \text{Ca}^{2+} \)-free medium or any of the three transglutaminase inhibitors (following 37 °C preincubation with cells) could block uptake of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) or \( ^{125}\text{I-}{\alpha_2}\text{-M-T} \) (measured after 30 min). The 37 °C preincubation step was required for DCD but not for bacitracin blockage of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) uptake (data not shown).

To see if these effects could be due to effects on the binding sites themselves, cells were incubated at 0 °C with various inhibitors of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) uptake. By using \( \text{Ca}^{2+} \)-free medium (Fig. 8), it was shown that \( \text{Ca}^{2+} \) is required for binding of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) (0.5 µg/ml or 100 µg/ml) or \( ^{125}\text{I-}{\alpha_2}\text{-M-T} \). The low binding in the absence of \( \text{Ca}^{2+} \) probably explains the \( \text{Ca}^{2+} \) requirement for cellular uptake of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) at 37 °C. One mM \( \text{Ca}^{2+} \) was required for binding of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) to either class of binding sites. Cells were incubated at 0 °C with \( ^{125}\text{I-}{\alpha_2}\text{-M} \) and the transglutaminase inhibitors bacitracin, DCD, or BDONV (Fig. 9A). Bacitracin almost completely abolished binding of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) to the higher affinity class of binding sites but had little effect on the lower affinity class. BDONV also selectively reduced binding to the higher affinity class of binding sites, and DCD had no effect on either class of sites. The inhibition of \( ^{125}\text{I-}{\alpha_2}\text{-M} \) uptake by DCD and of fluorescent \( \alpha_2\text{-M} \) uptake by DCD and some other amines (9) requires preincubation of cells with inhibitor at 37 °C before exposure to \( \alpha_2\text{-M} \) (in contrast to bacitracin). When cells were preincubated with bacitracin, DCD, or BDONV at 37 °C and then cooled to 0 °C to assay binding (Fig. 9B), the higher affinity binding was substantially reduced by all of the inhibitors while the lower affinity binding was not reduced. Thus, all three transglutaminase inhibitors could selectively...
of binding sites on the surfaces of cultured fibroblasts. In agreement with previous studies in macrophages (13-15) and normal human fibroblasts (11, 12), the \[^{125}\text{I}\alpha_2\text{M-T}\] complex binds with a higher affinity than \[^{125}\text{I}\alpha_2\text{M}\] to a limited capacity, high affinity class of binding sites. The apparent \(K_d\) values of the high affinity class of binding sites are in the range of 0.1-1 \(\mu\)g/ml. This value is much below the level of plasma \(\alpha_2\text{M}\) (17). The previously unreported, major class of binding sites reached saturation with \[^{125}\text{I}\alpha_2\text{M}\] and \[^{125}\text{I}\alpha_2\text{M-T}\] in the near physiological range of \(\alpha_2\text{M}\) concentrations. Other studies may have not identified this class of sites because of shorter equilibration time at 0 °C or because the binding was not characterized at high enough levels of \[^{125}\text{I}\alpha_2\text{M}\]. It is likely that this class of lower affinity binding sites was involved in the clustering and internalization of near physiological blood levels of peroxidase-labeled \(\alpha_2\text{M}\), rh-\(\alpha_2\text{M}\), and high levels of rh-\(\alpha_2\text{M-T}\) previously described (3-10).

Surface-bound and Internalized \[^{125}\text{I}\alpha_2\text{M}\]—Using electron microscopy (8, 9) and fluorescence microscopy (3), it has been demonstrated that \(\alpha_2\text{M}\) receptors are diffusely distributed over cell surfaces. These receptors are mobile and when high levels of \(\alpha_2\text{M}\) are incubated with cells at 0 °C, the ligand is visualized both in coated pits and diffusely over cell surfaces (8). When cells are warmed at 37 °C, peroxidase-labeled \(\alpha_2\text{M}\) and rh-\(\alpha_2\text{M}\) rapidly enter cells and appear in vesicular structures termed "receptosomes" (6-8). In the present study, we have developed a biochemical technique to quantitatively study this internalization process. It is probable that surface-bound \[^{125}\text{I}\alpha_2\text{M}\] is accessible to pepsin release and that internalized ligands are inaccessible to the brief enzyme treatment. When cells with surface-bound \[^{125}\text{I}\alpha_2\text{M}\] were warmed at 37 °C, the internalization of ligand occurred rapidly. In 2 min (at low and high levels of receptor occupancy), the process was essentially complete. A rapid internalization rate for \[^{125}\text{I}\alpha_2\text{M}\] has been previously inferred using macrophages and an assay based on the releasability of surface-bound \[^{125}\text{I}\alpha_2\text{M}\] by EDTA treatment (13-15). The present report extends these studies to fibroblasts and to the class of binding sites which is presumably involved in uptake of physiological levels of \(\alpha_2\text{M}\).

**Inhibitors of \(\alpha_2\text{M}\)-Receptor Internalization—**Bacitracin, dansylcadaverine, and BDQNV inhibit internalization of \[^{125}\text{I}\alpha_2\text{M}\]. These inhibitors blocked binding of \[^{125}\text{I}\alpha_2\text{M}\] to the high affinity class of cellular binding sites (Fig. 9). These two observations have also been independently demonstrated for dansylcadaverine and methyamine in another recent report (23). It is unlikely that these compounds inhibited uptake of \(\alpha_2\text{M}\) simply by binding to or preventing the recycling of a small fraction of the binding sites. A possibility that we favor is that after binding \(\alpha_2\text{M}\), the low affinity sites move to coated pits, and the \(\alpha_2\text{M}\) acquires a higher apparent affinity for receptors when sequestered in the pit environment. We suggest that by inhibiting a transglutaminase-like activity, bacitracin reduces the accumulation of receptor-ligand complexes in the coated pit regions, thereby preventing their appearance as a class of high affinity binding sites.

**DISCUSSION**

**Low Affinity, High Capacity Binding Sites for \[^{125}\text{I}\alpha_2\text{M}\]**—\[^{125}\text{I}\alpha_2\text{M}\] and \[^{125}\text{I}\alpha_2\text{M-T}\] appear to bind to the same two classes of binding sites on the surfaces of cultured fibroblasts. The effect of trypsin pretreatment of cellular binding sites was also examined (Fig. 10). Cells were preincubated with various concentrations of trypsin for 5 min at room temperature. Then they were washed and binding assays were performed at 0 °C for 8 h. Binding data are corrected for nonspecific binding, and control levels of binding were the same in A and B. Data are the averages of three or more independent experiments. (Error bars indicate the S.E.; * indicates \(p < 0.05\) compared with control.)

**Fig. 10. Effect of trypsin pretreatment of cells on binding by \[^{125}\text{I}\alpha_2\text{M}\].** Cells were preincubated with various concentrations of trypsin for 5 min at room temperature. Then they were washed and binding assays were performed at 0 °C for 8 h. Binding data are corrected for nonspecific binding, and control levels of binding were the same in A and B. Data are the averages of three or more independent experiments. (Error bars indicate the S.E.; * indicates \(p < 0.05\) compared with control.)
plexes/coated pit and 1,000 coated pits/cell. If cells are preincubated with bacitracin or methylamine (9) and then α2M is bound to cells at 0°C, binding in the coated pits is selectively reduced, while diffuse binding to other surface areas remains unaffected. Using 125I-low density lipoprotein, other investigators have estimated that human fibroblasts can bind 7,500-15,000 molecules of low density lipoprotein/cell (24, 25). Using ferritin-low density lipoprotein, they have also reported that 70–80% of the cellular binding is to coated pit regions (26). Using these data, it may be calculated that coated pit regions may contain 5,000–12,000 receptors for low density lipoproteins. Thus, coated pits may be able to contain approximately equal numbers of receptors for low density lipoprotein and α2M.

Receptor Turnover—When cells were incubated with various concentrations of 125I-α2M, half-maximal binding and internalization averaged 150 μg/ml, in agreement with data using rh-α2M or 125I-α2M (11, 12, 16). At these high concentrations of 125I-α2M, two-thirds of the cellular binding represented internalized 125I-α2M. The maximal internalization rate averaged 1,700,000 molecules of 125I-α2M/cell/h. Since there were 600,000 receptors for α2M measured at 0°C, it appears that at 37°C each receptor is recycled in 20 min. Alternatively, there could be an additional intracellular pool of receptors which continually appears on the surface. Since the coated pits on the cell surfaces appear at 0°C to contain only 10,000–20,000 α2M-receptor complexes, internalization must occur very quickly to allow for subsequent rounds of clustering and internalization.

The Role of Calcium—Using fluorescence microscopy, we previously showed that rh-α2M required Ca2+ to enter the cell (9, 10). The fluorescent images suggested that Ca2+ was not required for binding to the cell surface, but it is difficult to evaluate by this technique the amount of rh-α2M or other fluorescent ligands diffusely bound to the cell. The present study using 125I-α2M confirms that Ca2+ is required for binding to cellular receptors. We previously suggested that extracellular Ca2+ was required for the activation of a cellular transglutaminase that promoted the clustering of α2M-receptor complexes in coated pits (9, 10). The current finding that Ca2+ is required for α2M binding to cell surface receptors suggests that intracellular Ca2+ may be required for transglutaminase activation and extracellular Ca2+ for binding.

In summary, we confirm previous reports of high affinity binding sites for 125I-α2M-T and 125I-α2M and demonstrate a new class of lower affinity sites which could mediate uptake of the high levels of α2M found in the blood. A new assay was developed to distinguish surface-bound and internalized 125I-α2M. Internalization of 125I-α2M occurred very rapidly. Binding and internalization were both much reduced in the absence of Ca2+. Both classes of binding sites were abolished by brief protease treatment of cell monolayers, suggesting that the receptors were at least partly protein. In the presence of inhibitors of transglutaminase, both internalization and the high affinity class of binding sites were reduced. Low affinity receptors may bind α2M and accumulate in coated pit regions. This process may involve a transglutaminase-like activity. In coated pit regions, the receptors may acquire a higher apparent affinity for α2M. We speculate that such a process may be a general phenomenon for receptors internalized by the coated pit pathway and may in part explain the widespread reports of biphasic Scatchard plots of binding data for many ligands, peptide hormones (27), neurotransmitters (28), and prostaglandins (29).

Acknowledgments—We wish to thank Elizabeth Lovelace for her invaluable assistance with cell culture, Maria Gallo for purification of α2M, and Dr. Harry Haigler and Dr. Sheue-yann Cheng for helpful discussion.

REFERENCES


* M. C. Willingham, unpublished observation.