α_2MACROGLOBULIN BINDING TO THE PLASMA MEMBRANE OF CULTURED FIBROBLASTS

Diffuse Binding Followed by Clustering in Coated Regions

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ABSTRACT

Using transmission electron microscopy, we have studied the interaction of $\alpha_2$macroglobulin (α_2M) with the surface of cultured fibroblasts. When cells were incubated for 2 h at 4°C with ferritin-conjugated α_2M, ~90% of the α_2M was diffusely distributed on the cell surface, and the other 10% was concentrated in "coated" pits. A pattern of diffuse labeling with some clustering in "coated" pits was also obtained when cells were incubated for 5 min at 4°C with α_2M, fixed with glutaraldehyde, and the α_2M was localized with affinity-purified, peroxidase-labeled antibody to α_2M. Experiments in which cells were fixed with 0.2% paraformaldehyde before incubation with α_2M showed that the native distribution of α_2M receptors was entirely diffuse without significant clustering in "coated" pits. This indicates that some redistribution of the α_2M-receptor complexes into clusters occurred even at 4°C.

In experiments with concanavalin A(Con A), we found that some of the Con A clustered in coated regions of the membrane and was internalized in coated vesicles, but much of the Con A was directly internalized in uncoated vesicles or pinosomes.

We conclude that unoccupied α_2M receptors are diffusely distributed on the cell surface. When α_2M-receptor complexes are formed, they rapidly cluster in coated regions or pits in the plasma membrane and subsequently are internalized in coated vesicles. Because insulin and epidermal growth factor are internalized in the same structures as α_2M (Maxfield, F. R., J. Schlessinger, Y. Shechter, I. Pastan, and M. C. Willingham. 1978. *Cell.* **14:** 805–810.), we suggest that all peptide hormones, as well as other proteins that enter the cell by receptor-mediated endocytosis, follow this same pathway.

KEY WORDS $\alpha_2$macroglobulin · coated regions · endocytosis · plasma membrane · receptors

The internalization of molecules that have receptors on the plasma membrane occurs through adsorptive or receptor-mediated endocytosis (23). Substances that exert their action subsequent to specific binding to cell surface receptors include low-density lipoprotein (LDL) (2), lysosomal enzymes (11, 15), and hormones such as insulin (9) or epidermal growth factor (EGF) (6, 10). While

LDL and lysosomal enzymes require internalization to carry out their functions, it is unclear whether any of the functions of peptide hormones (insulin, EGF) are related to their internalization.

We have been studying the binding and internalization of $\alpha_2$macroglobulin (α_2M), a large serum protein that undergoes receptor-mediated endocytosis (13, 16, 24). We have found that insulin and EGF are internalized into the same vesicular structures as α_2M (13, 21). Although the fluorescence microscope techniques used in our previous studies have provided useful information about the time and temperature-dependent nature of the internalization, several important questions could not be answered by these methods. The initial distribution of the receptors, as determined by fluorescence localization at 4°C, appeared to be diffuse, but it was impossible to determine whether there was a clustered component of the receptors in addition to the diffuse distribution. Electron microscope localization of LDL (2) indicated that a significant percentage of LDL-receptor complexes were clustered at 4°C. Also, the special characteristics of the plasma membrane at the locations where α_2M, insulin, and EGF collect could not be determined by light microscopy.

We have used two electron microscope localization methods to resolve these questions. One was similar to that previously used to localize LDL (2) in that we labeled α_2M directly by conjugation with ferritin. We also employed affinity-purified antibodies to α_2M and detected their presence on the cell surface with peroxidase-labeled antiglobulins. The results of these and other experiments presented in this paper show that α_2M initially binds to receptors that are diffusely distributed on the cell surface without any detectable clustering of the unoccupied receptors. The receptor-α_2M complexes accumulate in clusters in coated regions of the cell membrane. These coated regions or pits rapidly invaginate and pinch off to form coated vesicles. Our results and those of Anderson et al. (2) suggest a common mechanism for receptor-mediated endocytosis involving clathrin-coated (17, 4) regions of the plasma membrane.

MATERIALS AND METHODS

Cell Culture

Swiss 3T3-4 cells were obtained and propagated as previously described (26). Cells were grown in Dulbecco-Vogt's modified Eagle's medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.) at 37°C. 10-cm^2 dishes of cells were planted at subconfluent densities and incubated or fixed as indicated in the experimental protocols in Table I.

Sources of Commercial Reagents

Concanavalin A (Con A) and rabbit antibody to α_2M were obtained from Miles Laboratories Inc. (Miles Research Products, Elkhart, Ind.). Goat anti-rabbit globulin conjugated to horseradish peroxidase (GAR-HRP) was obtained from N. L. Cappel Laboratories Inc., Cochranville, Pa. Ferritin and HRP were obtained from Sigma Chemical Co. (St. Louis, Mo.). Glutaraldehyde was obtained from Tousimis Research Corp. (Rockville, Md.) and paraformaldehyde from Fisher Scientific Co. (Pittsburgh, Pa.).

Preparation of α_2M

Partially purified α_2M was prepared by the procedure of Wickerhauser and Hao (25), except that a small amount of EDTA was used to solubilize the zinc-sulfate precipitate. The α_2M was further purified on a Sepharose 6B column (0.9 × 50 cm) in 10 mM TrisCl (pH 7.8).

Preparation of Ferritin-α_2M

Rhodamine-labeled ferritin was prepared by a modification of the procedure of Clark and Shepard (8), which we have used previously for preparing fluorescein- or rhodamine-labeled α_2M (13). The rhodamine-ferritin was cross-linked to α_2M with toluene-2,4-diisocyanate (TC) using the procedure of Schick and Singer (20). Free α_2M was separated from the complex by centrifugation at 100,000 *g*. For control purposes, rhodamine-ferritin was treated with the cross-linking procedure except that buffer was used in place of the α_2M solution.

The (rhodamine-ferritin)-α_2M complex formed fluorescent patches on Swiss 3T3 cells which were visualized by video-intensification microscopy (26, 13). In double-labeling experiments, fluorescein-α_2M and (rhodamine-ferritin)-α_2M were observed in the same patches (results not shown). The TC-treated rhodamine-ferritin did not form fluorescent patches on cells. This indicates that the α_2M in the complexes is recognized by its receptor on the cell surface.

Affinity-Purified Rabbit Anti-Human α_2M

Commercial rabbit antibodies (Miles) to α_2M showed binding to the surface of cells in the absence of added α_2M which was not removed by preabsorption with purified human α_2M. For this reason, we prepared affinity-purified antibody to α_2M by the method of Yamada (29). 5.4 mg of purified α_2M was coupled to 2 g of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). A globulin fraction, prepared from 10 ml of antiserum by precipitation with 50% ammonium sulfate, was

applied to the column, and 4 mg of affinity-purified antibody was eluted.

Experimental Controls

For experiments with ferritin-α_2M (Table I, exp 1), sections from 25 cells incubated with and without α_2M at 3 mg/ml were examined. This concentration of α_2M reduced the amount of ferritin-α_2M associated with the cell surface in both a diffuse and clustered form by >90%. Nonspecific binding to the extracellular matrix was still present under these conditions. Incubations with ferritin alone which had been treated by the same coupling procedure showed only nonspecific binding to extracellular proteins, but no specific binding to the cell surface.

As a control for experiments with antibodies to α_2M (Table I, exps 2 and 3), cells were incubated without added α_2M before fixation, and neither the affinity-purified antibody to α_2M nor a normal rabbit globulin control showed binding of any kind to the plasma membrane. In experiments in which 0.2% paraformaldehyde prefixation was employed, normal globulin and affinity-purified anti-α_2M failed to show any surface binding in the absence of added α_2M. When cells were incubated for 5 min with α_2M and then fixed with 0.2% paraformaldehyde, the clustered pattern of α_2M was not altered by this fixation step. By absorbing the affinity-purified antibody with calf serum, we obtained an antibody that was specific for human α_2M. Using this reagent, we tested the specificity of the diffuse pattern seen with prefixation by competing the added human α_2M with a fourfold excess of calf serum α_2M. The loss of label by this competition showed that the diffuse pattern was specific for α_2M (results not shown) and not caused by nonspecific binding. (A 20-fold excess of bovine albumin failed to compete for α_2M labeling.)

For experiments with Con A (Table I, exp 4), cells incubated either in the absence of Con A, or with Con A together with α-methylmannoside, were unlabeled.

Electron Microscopy

After fixation in 2% glutaraldehyde, cells were washed in PBS, incubated in 300 mM glycine buffer, pH 10, for 5 min to neutralize excess aldehydes, reacted with the diaminobenzidine substrate when peroxidase was employed (as in reference 5), postfixed in 1.5% OsO_4 in PBS, dehydrated in ethanol, and embedded in situ in Epon 812. Thin sections were cut with a diamond knife and mounted on 200-mesh nickel grids. Sections were taken both parallel to and perpendicular to the plastic substratum. In Figs. 1–4 are micrographs of cells from nearly parallel sections which were otherwise unstained. Fig. 5 is from perpendicular sections which were poststained with the osmium-thiocarbohydrazide-osmium (OTO) procedure (22) followed by saturated uranyl acetate in methanol and lead citrate (19).

RESULTS

The surface of cells growing in medium with 10% calf serum has α_2M bound to it. To detect the location of newly added α_2M by immunological methods, the surface of the cells must be cleared of previously bound α_2M. This was accomplished by incubating the cells in serum-free medium (SFM). Using peroxidase-labeled antibody to α_2M, we determined that, following incubation in SFM for 4 h, no α_2M remained on the cell surface (see below, Fig. 3*c*). Therefore, a 4-h incubation in SFM was used before each experiment. In the experiments reported here, 200 μg/ml (0.25 μM) of α_2M or its labeled derivatives were used. However, similar results have been obtained with a wide range of concentrations (10–500 μg/ml).

Van Leuven et al. (24) have reported that protease-α_2M complexes are taken up by human fibroblasts more rapidly than native α_2M. Using rhodamine-labeled α_2M (R-α_2M) (13, 26), we examined the possibility that protease-α_2M complexes might also be taken up preferentially by Swiss 3T3 cells. We have previously reported that excess unlabeled α_2M blocks the binding of R-α_2M (13); trypsin-α_2M complexes block R-α_2M binding at approximately the same concentration as untreated α_2M (not shown). Also, trypsin-treated R-α_2M binds to Swiss 3T3 cells and collects in the same cell surface clusters as untreated R-α_2M. The binding of trypsin-treated R-α_2M is inhibited by the same concentrations of α_2M used to inhibit the binding of R-α_2M. The differences between our results and those of Van Leuven et al. (24) probably are a result of differences in cell type. Because trypsin treatment does not significantly alter the way Swiss 3T3 cells handle α_2M, we have used untreated α_2M in all the experiments described in this paper.

Ferritin-Labeled α_2M

Incubation of cells with ferritin-labeled α_2M at 4°C for 2 h, followed by fixation with glutaraldehyde, showed that α_2M was present on the cell surface (Table I, exp 1). Part of the α_2M was diffusely distributed and part was clustered over coated regions of the membrane and in coated pits (Fig. 1). Coated pits were often seen at the base of microvilli, and in these coated pits the ferritin-α_2M conjugate was concentrated and easily seen. The morphologic appearance of coated pits has been extensively described (1–4). Although the clathrin coat is not completely visible in unstained sections,

TABLE I

Experimental Protocols for Electron Microscopy

Exp	Prefixation-0.2% paraformaldehyde (5 min)	1st incubation (4°C)	Fixation-2% glutaraldehyde (10 min)	2nd incubation (20 min)	3rd incubation (20 min)	Results
1	–	α_2M-ferritin (2 h)	+			D,C
2	–	α_2M (5 min)	+	AP anti-α_2M	GAR-HRP	D, C
3	+	α_2M (30 min)	+	AP anti-α_2M	GAR-HRP	D
4	–	Con A (20 min)	–	HRP	2% glutaraldehyde	D,C*, I

The following concentration of reagents was used: α_2M-ferritin (200 μg/ml), α_2M (200 μg/ml), Con A (20 μg/ml), affinity-purified (AP) anti-α_2M (100 μg/ml), GAR-HRP, 100 μg/ml, HRP, 100 μg/ml. Con A and HRP incubations were at 4°C followed by warming to 37°C for 1 min before fixation. Incubations with α_2M in exp 3 included 5 mg/ml BSA to compete for any nonspecific binding induced by fixation. D, diffuse; C, clustered in coated regions; C*, clustered in coated and uncoated regions; I, internalized in coated and uncoated vesicles.

the coated pits could be easily identified by their characteristic shape. The characteristic appearance of both uncoated and coated pits is shown in Fig. 2 in a variety of planes of section. This figure shows the marked difference in size, shape, and distribution of uncoated pinosomal invaginations and coated pits in these Swiss 3T3-4 cells.

When we compared the number of ferritin particles associated with the coated regions, which represent a small percentage of the total cell surface (2), to the number of particles associated with the uncoated regions which make up most of the cell surface, it was evident that a majority of the α_2M was in a diffuse pattern. On a typical cell, 11% of the ferritin-α_2M molecules were inside and 89% were outside of coated pits. This result is different from observations with LDL (2), where 70% of the LDL-receptor complexes are located inside coated pits after incubating cells with ferritin-LDL at 4°C. Competition of this ferritin-α_2M with a 15-fold excess (3 mg/ml) of unlabeled α_2M abolished ~95% of both the diffuse and clustered labeling, indicating that the ferritin visualized in these locations represented ferritin coupled to α_2M and bound to the α_2M receptor (Fig. 1 *c*). When cells with ferritin α_2M bound to their surface were incubated at 37°C for 1 min before fixation and the cells were sectioned perpendicular to the substratum, ferritin α_2M was observed in coated vesicles in the cytoplasm (results not shown).

The ferritin-conjugated α_2M also bound nonspecifically to large fibrillar aggregates of extracellular protein matrix which were clearly separated from the plasma membrane. This nonspecifically bound ferritin α_2M on the extracellular matrix could easily be distinguished from the specifically bound ferritin α_2M on the cell surface in experiments where the cells were not warmed above 4°C. However, when cells were warmed to 37°C, some of the nonspecifically bound ferritin-α_2M in these heavily labeled aggregates was eventually detected still bound to aggregated protein in large, uncoated pinosomes within the cell. This type of nonspecific binding was also detected with ferritin treated by the cross-linking procedure but without α_2M. This result makes it difficult to determine the eventual intracellular fate of the specifically bound ferritin α_2M. It is known that uncoated pinosomes rapidly fuse with lysosomes on entry into the cell (27). It is also known that the phase-neutral endocytic vesicles seen with fluorescently labeled α_2M (13, 26) do not rapidly fuse with phase-dense lysosomes. Therefore, the detection of ferritin in "secondary" lysosomes at early times of incubation could be caused by nonspecific uptake of the heavily labeled extracellular matrix material rather than by transfer from vesicles that contain specifically bound α_2M. For this reason, we believe that the ferritin conjugate is most useful for obtaining quantitive data about the distribution of α_2M-receptor complexes on the cell surface at 4°C.

α_2M Localized by Peroxidase-Labeled Antibody

Using affinity-purified antibody to α_2M, fol-

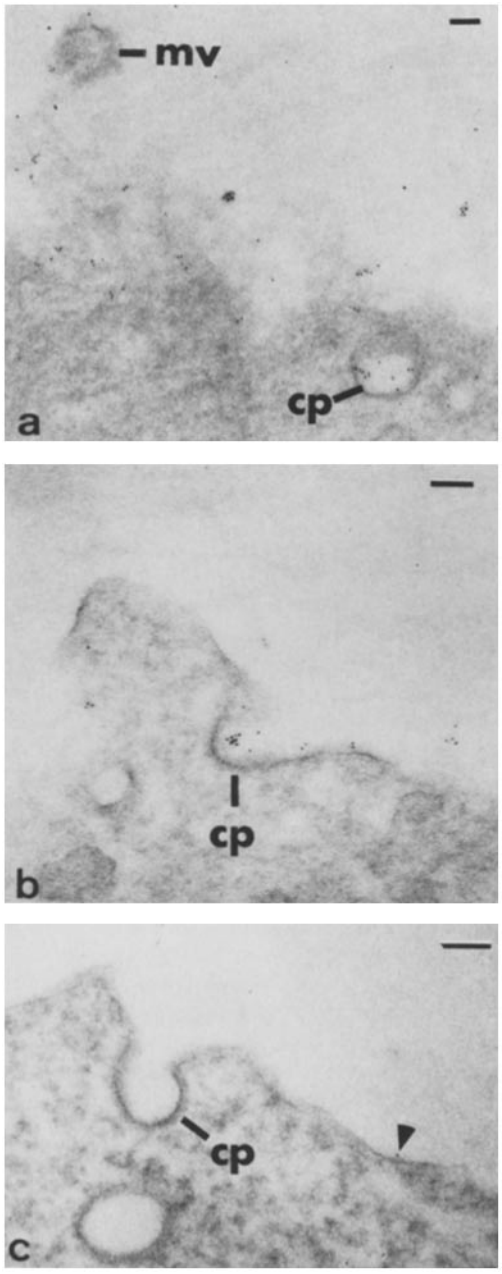


FIGURE 1 Exp 1: Binding of ferritin-α_2M at 4°C. After incubating in SFM for 4 h at 37°C, cells were transferred to 4°C and incubated in 200 μg/ml ferritin-α_2M conjugate; after 2 h, the cells were washed and fixed at 4°C in 2% glutaraldehyde. Note the ferritin particles in the tangential sections of plasma membrane in Fig. 1 *a* and *b* which show diffuse labeling of the entire membrane and some concentration of label in coated pits (*cp*). The location of coated pits at the base of microvilli (*mv*) is commonly observed. Competition of this label with a 15-fold excess of unlabeled α_2M virtually abolished the surface labeling (*c*) Arrowhead, single ferritin core. (*a*) × 39,000; (*b*) × 54,000; (*c*) × 63,500. Bars, 0.1 μm. Unstained.

lowed by peroxidase-labeled antiglobulin antibody, we were able to indirectly label the α_2M bound to the cell surface without encountering nonspecific binding of one of the reagents. Furthermore, the cytochemical detection of peroxidase allowed us to show the distribution of receptor-bound α_2M more clearly than was possible with ferritin. In one such experiment, cells were incubated with purified human α_2M at 4°C for 5 min, fixed at 4°C with glutaraldehyde, and then incubated sequentially with affinity-purified anti-α_2M and peroxidase-labeled antiglobulin (see Table I, exp 2). This method also demonstrated that α_2M was bound to the surface in both a diffuse and clustered pattern, and that the clusters were exclusively located over coated regions (Fig. 3 *a* and *b*; also Fig. 2 *G* and *H*). The small amount of clustering observed at 4°C could not be detected by fluorescence microscopy (14, 21). When α_2M was omitted from the incubation medium, no surface reaction product was detected (Fig. 3 *c*). This result showed that nonspecific binding of the globulin reagents did not occur and that there was no residual α_2M left on the surface from the original calf-serum-containing medium. Because the cells were kept at 4°C during the incubation with α_2M and subsequent fixation, none of the surface-bound α_2M should have been internalized by endocytosis. Unfortunately, this labeling technique was not useful for following α_2M after endocytosis, because once α_2M was sequestered intracellularly it was not available to antibody. Further, allowing α_2M to react with this divalent antibody on the surface of living cells before internalization might not show the clustering and internalization characteristic of α_2M itself, but rather an effect induced by antibody.

Prefixation of α_2M Receptors

To determine whether the clusters observed at 4°C occurred after binding of α_2M or represented the native distribution of α_2M receptors, we used prefixation to immobilize the α_2M receptors in the membrane without destroying their ability to bind α_2M. To perform this experiment, we fixed cells with a variety of agents and found that cells fixed

with 0.2% paraformaldehyde still specifically bound α_2M. Concentrations of paraformaldehyde >1% abolished any binding of α_2M. Although the morphological preservation of cells fixed with only 0.2% formaldehyde is poor, there was sufficient preservation to allow us to determine the distribution of α_2M receptors on the surface. Further, tangential sections are shown so that a large amount of membrane surface can be observed (Fig. 4). By prefixing cells at 23°C with 0.2% paraformaldehyde and subsequently labeling with α_2M, affinity-purified anti-α_2M (Table I, exp 3), and peroxidase-labeled antiglobulin, the native distribution of the α_2M receptor could be seen. Fig. 4 shows that under these conditions, α_2M receptors are diffusely distributed over the entire membrane. Only in rare instances was a very small amount of clustering observed over coated regions. In no instance was a coated pit seen which contained the amount of label shown in pits without prefixation (Fig. 3 *a* and *b*). Thus, clustering was vastly decreased relative to the clustering observed in cells incubated with α_2M at 4°C wihtout prefixation (Fig. 3 *a* and *b*) or with ferritin α_2M at 4°C (Fig. 1). We conclude that most of the α_2M receptors are diffusely distributed.

Because our results differ from those obtained with LDL (1–4), we considered the possibility that prefixation might selectively inactivate diffuse or clustered receptors, or decrease total binding to all receptors. We believe that this is unlikely because we were unable to detect any difference in the amount of α_2M bound between prefixed cells subsequently exposed to α_2M or live cells to which α_2M had been bound at 4°C for 5 min before fixation (from the appearance of cells treated with α_2M and peroxidase labeling). In addition, the competition control (in experimental controls) indicates that the binding observed in prefixed cells is still specific.

Con A

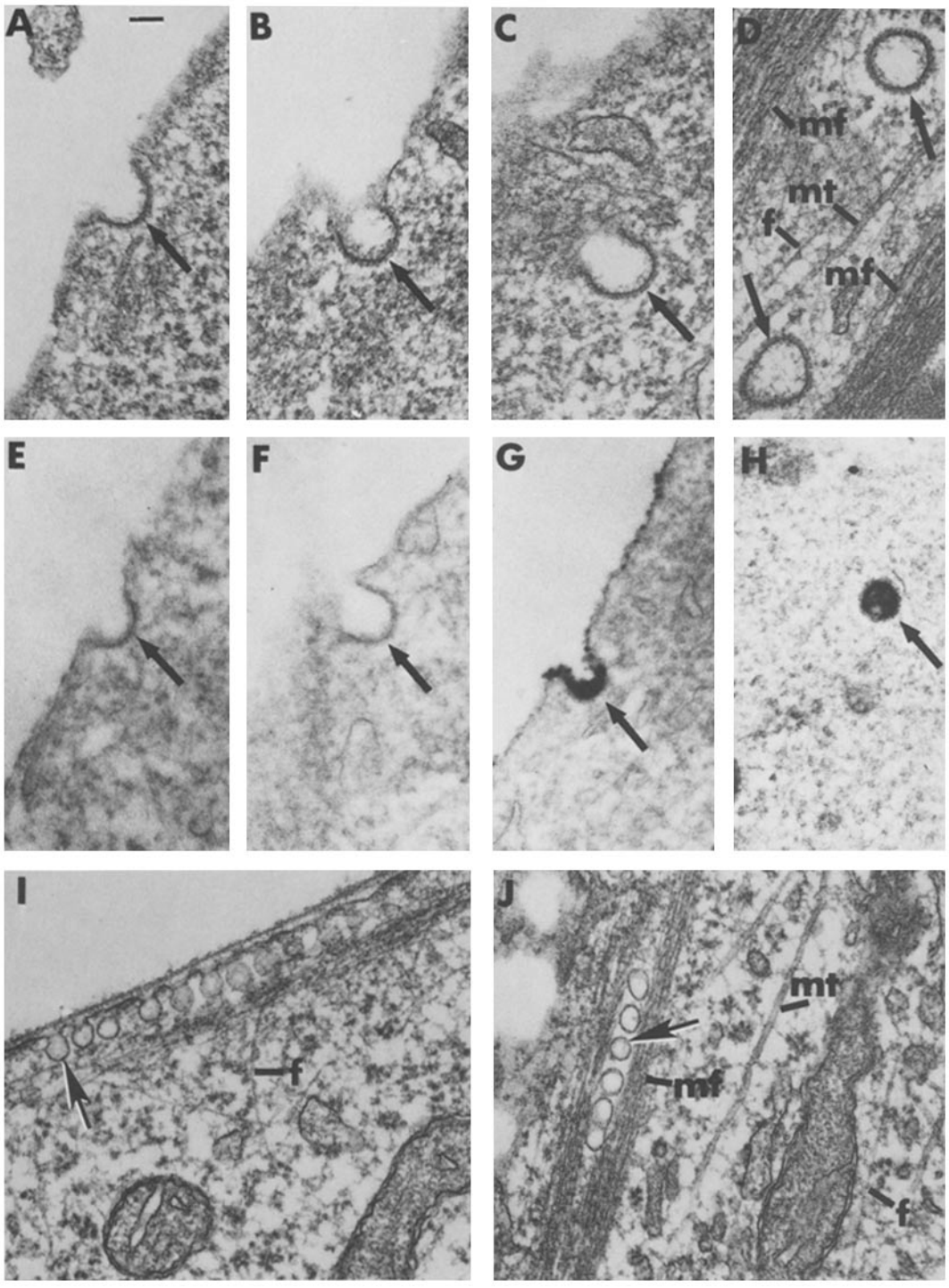
Con A binds to a large number of glycoproteins on the cell surface. We thought that it would be of interest to compare Con A with α_2M. When cells were incubated with Con A at 4°C, washed, and then exposed to HRP (5), we could determine the location of the Con A. When cells were incubated with these molecules at 4°C and then warmed to 37°C for only 1 min (Table I, exp 4; Fig. 5), Con A was seen on the surface and within the cell. Part of the surface-bound Con A was diffusely distributed or collected in patches away from coated regions, and part was clustered in coated regions. The internalized Con A was also in two locations. Some was in coated vesicles (Fig. 5 *b*) and some in small uncoated vesicles (Fig. 4 *c*). The uncoated vesicles had a thin layer of reaction product presumably representing the concentration of Con A that was diffusely bound over the entire surface. We have never observed α_2M in small uncoated vesicles although it is conceivable that small amounts at low concentrations could enter the cell in this way. In contrast to its distribution in uncoated vesicles, Con A is concentrated in coated vesicles, producing vesicles almost completely filled with reaction product (Fig. 5 *b*).

DISCUSSION

The data presented here show that α_2M is internalized by clustering in coated regions or pits on the plasma membrane. These structures rapidly pinch off to form coated vesicles. Because insulin and EGF are known to follow the same pathway as α_2M (13), the coated vesicle is the presumptive endocytic organelle for these molecules as well. Previously, LDL has been shown to bind to specific receptors and enter the cell in coated vesicles (2). Recently, Anderson et al. have shown that LDL binds to the clathrin-coated regions using antibodies to clathrin (4). Therefore it seems likely that this pathway represents the major route of physiologic receptor-mediated endocytosis. Other candidates for this pathway would be other peptide hormones and the receptor-mediated uptake of lysosomal enzymes (11, 15). The reason for the involvement of coated vesicles is not clear. One possibility is that the clathrin coat prevents early fusion of physiologically valuable endocytosed molecules with the destructive mechanisms in lysosomes. Because α_2M is a broad-spectrum protease inhibitor, is present in serum in large amounts, and also enters clathrin-coated vesicles, its simultaneous endocytosis with biologically potent molecules may serve to protect them or their receptors from the action of proteases. This mechanism also provides the cell with a means of efficiently concentrating specific molecules on the cell surface.

Diffuse α_2M Receptors Cluster in Coated Regions

The results in the prefixation experiment (Fig. 4) demonstrate the diffuse distribution of unoc-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cupied receptors. Apparently, ligand-induced clustering in coated pits occurs to a small degree at 4°C (Fig. 3). However, at 37°C the ligand-receptor complexes are rapidly and preferentially redistributed into coated regions. Thus, the coated region, rather than being the site of unoccupied α_2M receptors, is the nidus for the accumulation of receptor-ligand complexes, ensuring that endocytosis of most of the ligand occurs through the coated vesicle pathway. Our finding differs from that with LDL, which is found to be partly concentrated in coated regions even in prefixed cells (1, 2). We also find less clustering of α_2M-receptor complexes at 4°C (11% on coated pits) than has been reported for cells incubated with LDL at 4°C. The differences between LDL and α_2M may reflect differences between specific receptors, cell types, or experimental conditions.

Significance for Insulin and EGF

The diffuse appearance of most of the bound α_2M at 4°C is in agreement with its diffuse appearance when observed in fluorescence experiments with video intensification microscopy (VIM) (13, 26). Previous experiments with VIM (13) had shown that insulin, EGF and α_2M cluster in the same region of the plasma membrane. Therefore, insulin and EGF bound to their receptors must also form clusters in coated regions of the membrane (also see reference 10). It is also clear that a great deal of the surface-bound α_2M is still diffuse at these early times, but this remaining diffuse component most likely follows the same pathway as the early clusters by collecting in newly exposed coated regions until all the surface α_2M has been cleared (Fig. 3 *c*).

Coated Pits Form Coated Vesicles

We have seen coated vesicles containing ferritin-α_2M in perpendicular sections as early as 1 min after raising the incubation temperature from 4° to 37°C (results not shown). A similar result was seen with Con A incubations when warmed to 37°C (Fig. 5 *a* and *b*). This is in agreement with experiments with ferritin-LDL (1) in which it was shown that coated pits pinch off to form coated vesicles.

Coated Vesicles Fail to Fuse with Lysosomes

In experiments using VIM (26), we have failed to observe fusion of α_2M-containing phase-neutral endocytic vesicles with phase-dense lysosomes for up to 4 h after the uptake of rhodamine-α_2M by fibroblasts (M. C. Willingham and I. Pastan, unpublished results). Similarly, we did not find clear examples of rapid fusion of coated vesicles with lysosomes in the electron microscope experiments described in this paper. Uncoated vesicles (micropinosomes) freely fuse with lysosomes (27; also Fig. 5 *c*). These uncoated pinosomes are not, however, the vesicles in which highly concentrated α_2M enters the cell. It is known that after 12–24 h

FIGURE 2 Morphologic character of coated and uncoated pits in Swiss 3T3-4 cells. The appearance of coated pits (arrows) on the surface of Swiss 3T3-4 cells in sections counterstained with uranyl acetate and lead citrate are shown in Fig. 2 *A–D* in increasingly tangential orientations to the plasma membrane. While Fig. 2 *A* is almost perpendicular to the membrane, Fig. 2 *D* is so tangential that the communication with the outside is masked (also see Fig. 2 *H*). Fig. 2 *E* and *F* represent unstained sections of areas quite similar to Fig. 2 *A* and *B* showing that the clathrin coat is not so visible under these conditions. In Fig. 2 *G*, a section from an experiment with peroxidase labeling of α_2M (similar to Table I, exp 2) shows concentration of label in association with the coated pit. Fig. 2 *H* shows a similar pit, but from an area sectioned in a plane more similar to that shown in Fig. 2 *D*. The presence of label (in this experiment performed at 4°C to prevent endocytosis and fixed before addition of antibody) shows that this pit is still in communication with the outside. This picture (*H*), therefore, demonstrates a section through the bottom of a coated pit. Fig. 2 *I* and *J* show the appearance of uncoated pinosomes (large arrow) still connected to the plasma membrane, in a near perpendicular section in Fig. 2 *I* and more tangential in Fig. 2 *J*. The alignment in rows of these vesicles is often seen because, in very flat cells with many microfilament bundles, the only area of membrane not attached to the substratum lies in between the bundles. This "herding" phenomenon is often seen with other vesicles which require an opening to the outside such as the coated vesicles shown in Fig. 2 *D*. *mf*, microfilament bundle; *mt*, microtubule; *f*, 10-nm filament. All Figs. × 50,000. Bar in (*A*), 0.1 μm. (*A–D, I,* and *J*), uranyl acetate-lead citrate (UALC) counterstained; (*E–H*), unstained.

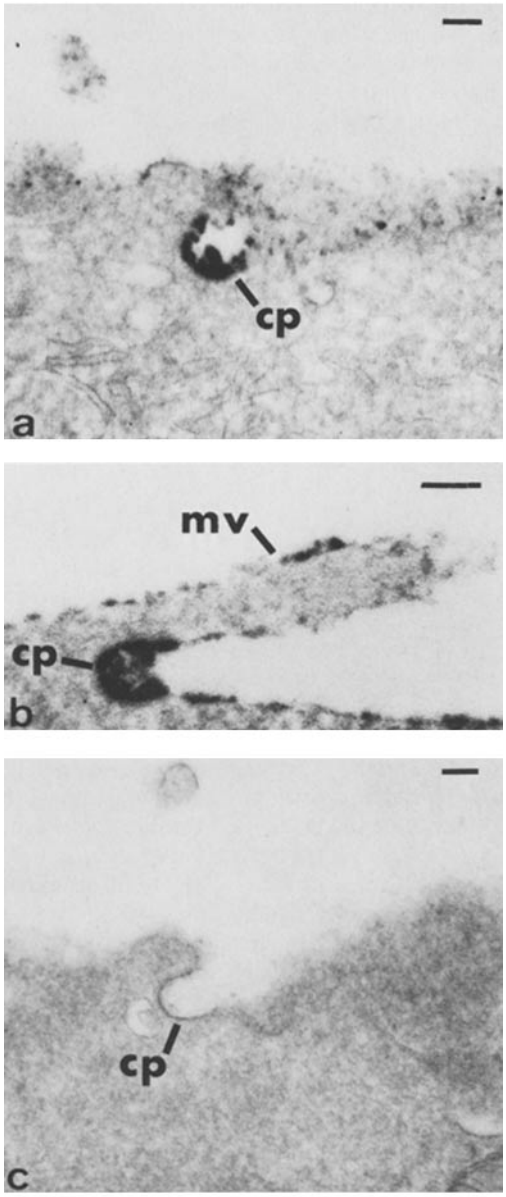


FIGURE 3 Exp 2: Affinity-purified antibody labeling of bound α_2M at 4°C. After incubating in SFM for 4 h at 37°C, cells were incubated at 4°C either with 200 μg/ml α_2M for 5 min (*a* and *b*) or without α_2M (*c*). After washing at 4°C, the cells were fixed in 2% glutaraldehyde at 4°C, then incubated in affinity-purified rabbit antibody to α_2M for 20 min, followed by washing and incubation in GAR-HRP for 20 min at 23°C. Note the diffuse labeling of the tangentially sectioned membrane in Fig. 3*a* with concentration in a coated pit (*cp*) located under the tangentially sectioned base of a microvillus, seen in Fig. 3*a* as well as Fig. 3*c*. A semiperpendicular section of the microvillus (*mv*)–coated pit (*cp*) complex is shown in Fig. 3*b* with diffuse labeling present on the plasma membrane and concentration of label in the coated pit (*cp*). The absence of label in Fig. 3*c* demonstrates the specificity of the antibody in the absence of added α_2M and the lack of residual α_2M present on the surface after the SFM preincubation. (*a*) × 49,500; (*b*) × 79,000; (*c*) × 43,700. Bars, 0.1 μm. Unstained.

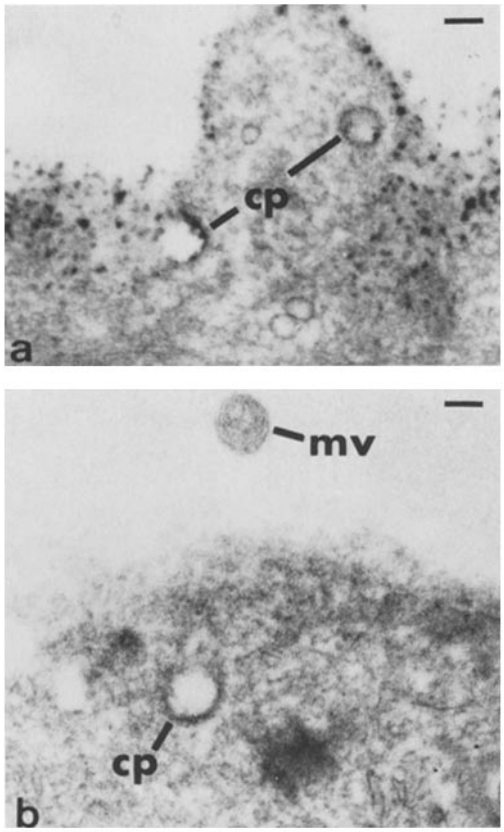
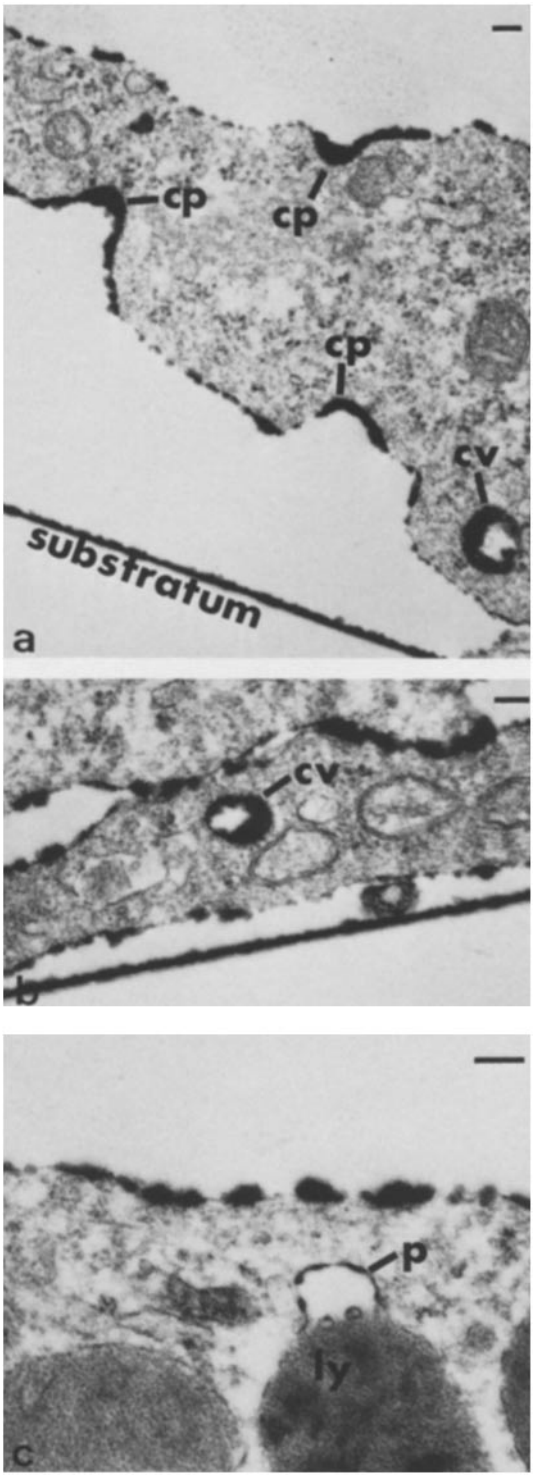


FIGURE 4 Exp 3: Prefixation with 0.2% paraformaldehyde followed by labeling with α_2M. After incubation in SFM at 37°C, cells were fixed for 5 min in 0.2% paraformaldehyde in PBS at 23°C, then incubated with (*a*) or without (*b*) 200 μg/ml α_2M for 20 min followed by affinity-purified rabbit antibody to α_2M, GAR-HRP, and fixation in 2% glutaraldehyde. Note the diffuse nature of label on the tangentially sectioned plasma membrane in Fig. 4*a* without significant concentration in coated pits (*cp*). The lack of labeling on the plasma membrane, coated pit, or microvillus (*mv*) in Fig. 4*B* confirms the specificity of the label for α_2M. (*A*) × 51,600; (*B*) × 50,600. Bars, 0.1 μm. Unstained.



α_2M is found in lysosomal structures in the cell (16, 28). The pathway by which this process occurs is not clear. We speculate that coated vesicles, or vesicles containing material derived from coated vesicles, eventually fuse with components of the Golgi system (28).

Con A

The uptake of Con A is complex because at the concentrations used here (20 μg/ml) two separate pathways seem to be involved. There is selective concentration of a small fraction of the surface-bound Con A into coated pits and eventually coated vesicles (Fig. 5 *a* and *b*). There is also, however, some internalization of Con A in uncoated pinosomes which rapidly fuse with lysosomes (Fig. 5 *c*). Thus, both uncoated and coated vesicles are involved in this process. Receptor molecules for protein hormones exist on the cell surface, some of which are probably glycoproteins or associated with glycoproteins (7, 12, 18). We speculate that Con A may cross-link these receptors and induce them to cluster in coated pits. It is also possible that Con A binds to glycoproteins already clustered in coated pits and allows them to be visualized. The observation that some of the Con A bound to the cell surface clustered in coated pits allowed us to use peroxidase bound to Con A as a probe to easily follow the formation of coated vesicles from coated pits after brief incubations at 37°C. The constant internalization of the entire plasma membrane in uncoated micropinosomes could account for the small amount of diffusely distributed Con A found in uncoated vesicles.

FIGURE 5 Exp 4: Labeling with concanavalin A. After washing with SFM at 4°C, cells were incubated in 20 μg/ml Con A at 4°C, followed by washing and incubation in 100 μg/ml HRP for 20 min. They were then warmed to 37°C for 1 min and immediately fixed in 2% glutaraldehyde. Sections perpendicular to the substratum which have been counterstained with the OTO procedure and uranyl acetate and lead citrate are shown here. Note the tendency for concentration of the diffuse surface label in coated pits (*cp*) in Fig. 5 *a* and the presence of coated vesicles (*cv*) which have already pinched off from the plasma membrane in Fig. 5 *a* and *b* which contain an extremely high concentration of label. In Fig. 5 *c*, an uncoated pinosome (*p*) has already pinched off from the surface and fused with a lysosome (*ly*) near the surface, showing its characteristic low concentration of label. (*a*) × 33,750; (*b*) × 45,000; (*c*) × 63,000. Bars, 0.1 μm. OTO and UALC stained.

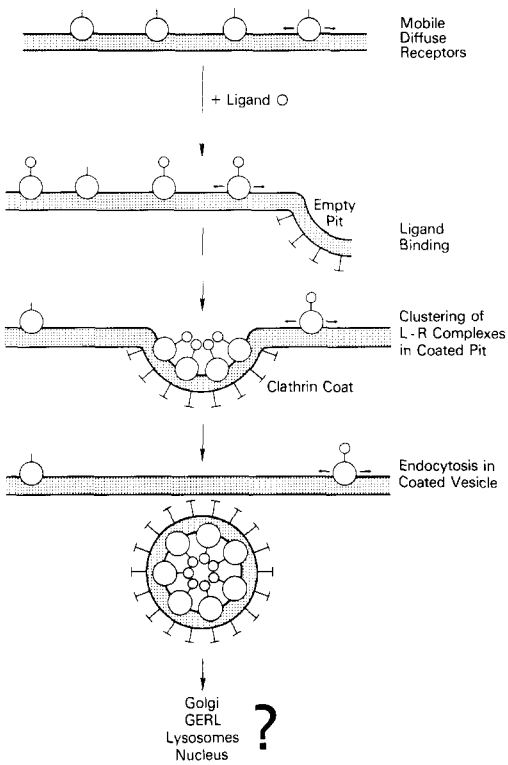


FIGURE 6 Model of receptor-mediated endocytosis.

A Model of Receptor-Mediated Endocytosis

A scheme depicting the steps involved in α_2M uptake is shown in Fig. 6. First, α_2M binds to diffusely distributed unoccupied receptors. Next, the ligand-receptor complexes cluster in coated pits. Then, the α_2M-receptor complexes are internalized in coated vesicles. Insulin and EGF follow this same pathway. We postulate that this mechanism could be the general pathway of receptor-mediated endocytosis. This could function as a mechanism to regulate the number of receptors for various ligands exposed on the cell surface, or as a mode of intracellular selective delivery of ligands to potential sites of action. α_2M could accompany other molecules in a protective role as a protease inhibitor or have other functions of its own not yet elucidated. The further fate of α_2M and other molecules internalized by this mechanism is still under investigation.

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