# $\alpha_2$-Macroglobulin Adsorbed to Colloidal Gold: A New Probe in the Study of Receptor-mediated Endocytosis

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ABSTRACT $\alpha_2$-Macroglobulin ($\alpha_2$M) was adsorbed to colloidal gold and used as a new tool in the study of receptor-mediated endocytosis. $\alpha_2$M-gold is easy to prepare and is clearly visualized at the electron microscope level. When cells were incubated with $\alpha_2$M-gold at 0°C, gold was visualized both diffusely over the cell surface and concentrated in coated pits. After cells to which $\alpha_2$M-gold had been bound at 0°C were warmed, the gold was rapidly internalized into uncoated vesicles, previously termed receptosomes. After 30 min of incubation or longer, gold was found in small lysosomes and, later, in large lysosomes and very small vesicles in the region of the Golgi complex. This pattern of localization is similar to that previously described, using peroxidase-labeled anti-$\alpha_2$M antibodies. By incubating cells with both $\alpha_2$M-gold and vesicular stomatitis virus (VSV), we studied the internalization of these two markers simultaneously. VSV and $\alpha_2$M-gold rapidly clustered in the same coated pits and were internalized in the same receptosomes. Proteins and hormones adsorbed to gold may be useful in the study of receptor-mediated endocytosis.

A variety of hormones, proteins, and viruses enter cells by the process of concentrative adsorptive endocytosis (2, 14, 17, 19). Receptor-bound ligands rapidly cluster in clathrin-coated pits on fibroblast cell membranes and are then internalized. A newly defined organelle, termed the receptosome, has been identified as the initial intracellular location of hormone-receptor complexes (23). Receptosomes are uncoated, phase-neutral vesicles 1,500–3,000 Å in diameter that move in the cytoplasm by saltatory motion. After a delay of 15–30 min, they deliver their contents to the Golgi region and finally to lysosomes (19–23). Information in many of these previous studies was based on visualization of ligands by peroxidase- or ferritin-labeled antibodies directed against the ligand or antibodies to the ligand. Although providing useful information concerning the intracellular localization of ligands, these procedures are complex and, in the case of peroxidase, require a histochemical reaction.

$\alpha_2$-Macroglobulin ($\alpha_2$M) is a large, tetrameric protein found in plasma and other body fluids of vertebrates. Its precise biological role is unknown but it is able to form complexes with a wide variety of endopeptidases and zinc (8, 16). Previous studies using fluorescent (9, 11, 12) or ferritin- or peroxidase-labeled (20–23) $\alpha_2$M have demonstrated that $\alpha_2$M binds to membrane receptors on fibroblasts and is internalized by the coated pit–receptosome pathway outlined above. We now report that colloidal gold can be used to visualize this process. Colloidal gold is an easily visualized, discrete label for electron microscopy that has previously been adsorbed to various proteins including immunoglobulins and lectins to localize cell surface antigens (3, 7, 9). We have used $\alpha_2$M-gold in this study to examine the pathway of receptor-mediated endocytosis. We have then simultaneously compared the pathways of entry of $\alpha_2$M-gold and vesicular stomatitis virus (VSV).

## MATERIALS AND METHODS

$\alpha_2$M was prepared from whole human plasma as previously described (18, 20). $\alpha_2$M was iodinated with $^{125}$I using the chloramine-T procedure (13) and used as tracer radioactivity to quantify adsorption to colloidal gold.

Normal rat kidney (NRK-2T) and Swiss mouse 3T3 cells were grown in Dulbecco-Vogt's modified Eagle's medium containing 10% (vol/vol) calf serum. For experiments, cells were plated in 35-mm Falcon tissue culture dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, Calif.) at $1.5 \times 10^5$ cells/dish in 2 ml of medium supplemented with calf serum that had been heated for 1 h at 60°C. Cells were usually used 4 d after plating. At this time, confluent monolayers had formed. Cells were incubated in serum-free medium for 1 h at 37°C before any experiment, to remove most of the cellular-bound $\alpha_2$M derived from calf serum, and then washed extensively with serum-free medium.

Colloidal gold was prepared as previously described (9) and its pH adjusted to 6.0 (the pI of $\alpha_2$M is 5.4). $\alpha_2$M was dialyzed extensively against distilled, deionized water at pH 6.0. Then, 50 ml of gold solution was reacted with 12 mg (10 ml) $\alpha_2$M (containing tracer $^{125}$I-$\alpha_2$M). 1.5 ml of 1% polyethylene glycol was then added to stabilize the mixture, and the gold-bound and free $\alpha_2$M were

separated by centrifugation (20,000 *g*, 1 h). The gold was resuspended and washed several times in phosphate-buffered saline (pH 7.4) containing 1.5 mM $CaCl_2$ and 0.01% polyethylene glycol (PBS-Ca-PEG). The gold adsorbed to $\alpha_2$M did not flocculate upon addition of 1% NaCl (unlike nonadsorbed gold), indicating that adsorption occurred (3, 7, 9). The gold particles appeared to be relatively homogeneous in size (100–200 Å in diameter) by electron microscopy. On the basis of radioactivity associated with the gold, we estimated 400 $\alpha_2$M molecules were bound to each gold particle (1.2 mg $\alpha_2$M/6 mg colloidal gold). For cellular studies, $\alpha_2$M-gold (resuspended at 50 $\mu$g $\alpha_2$M/ml) in PBS-Ca-PEG was incubated with cell monolayers at 0°C for 4 h with or without 2 mg/ml $\alpha_2$M to compete for receptor binding. Then the cell monolayers were washed five times with PBS containing 1.5 mM $CaCl_2$. The cells were then fixed at 0°C or warmed to 37°C for various times and fixed at 37°C. Fixation was carried out with 2% glutaraldehyde (Tousimis Research Co., Rockville, Md.) in PBS. Cells were then postfixed in $OsO_4$, ethanol-dehydrated, and embedded *in situ* in Epon 812 (20–23). Thin sections were counterstained with lead citrate and viewed at 50 kV with a Hitachi HU-12A electron microscope.

When binding of $^{125}$I-$\alpha_2$M-gold to cell monolayers was studied, cells were first incubated with the $\alpha_2$M-gold at 0°C for 4 h. Then the cells were washed five times with PBS-Ca at 0°C. Radioactivity was solubilized in 0.1 N NaOH and counted in an LKB Mini-gama spectrometer (LKB Instruments, Inc., Rockville, Md.).

Monovalent, hybrid anti-$\alpha_2$M/antiferritin antibodies were prepared to directly visualize $\alpha_2$M bound to gold. Anti-$\alpha_2$M antiserum (Miles Laboratories Inc., Elkhart, Ind.) and antiferritin were purified by standard methods (ammonium sulfate fractionation and DEAE Affi-Blue (Bio-Rad Laboratories, Richmond, Calif.) chromatography) (4). Then the purified IgG fractions were pepsin digested and F(ab)$_2$′ fragments were cleaved with $\beta$-mercaptoethanolamine (Sigma Chemical Co., St. Louis, Mo.), mixed, dialyzed, and oxidized under $O_2$ to yield hybrid F(ab)$_2$′ species (1). The hybrid antibody was purified by affinity chromatography (5). Hybrid antibody (10 $\mu$g) was incubated with 0.25 ml of $\alpha_2$M-gold for 15 min at room temperature in the presence or absence of 1 mg $\alpha_2$M. Then the gold was pelleted by centrifugation for 15 min in an Eppendorf centrifuge 5412 (Brinkmann Instruments Inc., Westbury, N. Y.). After two washes in PBS-Ca-PEG, the $\alpha_2$M-gold was incubated for 15 min at room temperature in 100 mg/ml ferritin (Sigma Chemical Co.) and rewashed twice. The resuspended gold was spread on a Formvar-coated electron microscope grid and viewed without further treatment.

Swiss 3T3 cells were incubated for 4 h at 4°C in serum-free medium with $10^3$ plaque-forming units/cell vesicular stomatitis virus (VSV) mixed with either 50 $\mu$g/ml $\alpha_2$M-gold or $\alpha_2$M-gold plus excess $\alpha_2$M (3 mg/ml). The dishes were then washed in serum-free medium at 4°C and warmed to 37°C for 2 min. They were then fixed in 3% glutaraldehyde in PBS for 10 min at 23°C, followed by $OsO_4$ postfixation, ethanol-dehydration, and Epon embedding. From other studies, VSV, like $\alpha_2$M, is known to be found in coated pits when observed within 30 s of warming to 37°C, and internalized into the cell 1.5–4.0 min after warming (2, 17). In our experiments, 2 min was chosen as a time that would yield images of both clustering in coated pits on the surface and some internalized material in receptosomes (23).

## RESULTS AND DISCUSSION

### *Characterization of $\alpha_2$M-Gold*

On the basis of the association of $^{125}$I-$\alpha_2$M with colloidal gold, we calculated that ~400 molecules of $\alpha_2$M were bound to each gold particle. The $\alpha_2$M-gold was reacted with anti-$\alpha_2$M/antiferritin antibodies and then ferritin to determine whether the immunoreactivity of $\alpha_2$M was retained during the adsorption and washing procedures. Fig. 1 *B* shows that ferritin was associated with the colloidal gold, indicating that the $\alpha_2$M was still immunoreactive. When excess $\alpha_2$M was included during the incubations (Fig. 1 *A*), it effectively competed for binding by the antibodies, resulting in very little ferritin-gold association.

To determine whether $\alpha_2$M-gold retained receptor-recognition properties, $^{125}$I-$\alpha_2$M-gold was bound to 0°C to NRK-2T cell monolayers in the presence or absence of an excess of $\alpha_2$M. Fig. 2 shows that cell binding measured by $^{125}$I was clearly demonstrable. It was reduced to 10% of control in the presence of an excess of $\alpha_2$M. The data indicated that ~$2.5 \times 10^3$ particles of gold ($10^6$ molecules of $\alpha_2$M) were bound per cell. Similar results (data not shown) were obtained with Swiss 3T3 fibroblasts. Because the $\alpha_2$M-gold is multivalent, it is not clear how many receptors were occupied by each particle of gold. It has been previously demonstrated[1] that at 0°C a fibroblast can bind up to $6 \times 10^5$ molecules of $^{125}$I-$\alpha_2$M to its surface receptors. A small portion of these receptors ($10^4$) are of high affinity and may be associated with coated pits.

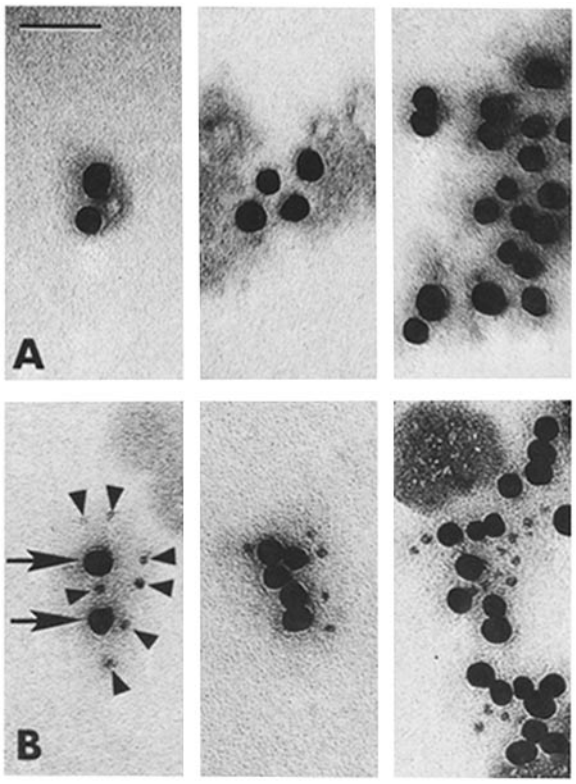


FIGURE 1 Visualization of immunoreactivity of $\alpha_2$M adsorbed to gold. Colloidal gold was bound to $\alpha_2$M and then washed. A hybrid anti-$\alpha_2$M/antiferritin antibody was then added to the $\alpha_2$M gold in the absence (*B*) or presence (*A*) of an excess of $\alpha_2$M. After addition of ferritin and then washing, ferritin was localized in association with gold to verify the presence of immunoreactivity, adsorbed $\alpha_2$M. Arrows, colloidal gold particles; arrowheads, ferritin cores. Bar, 500 Å. × 210,000.

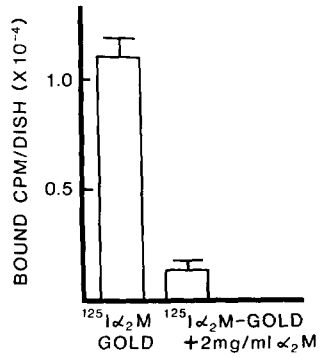


FIGURE 2 Binding of $^{125}$I-$\alpha_2$M-gold to fibroblast surfaces. NRK-2T cells were incubated with $^{125}$I-$\alpha_2$M-gold at 0°C in the absence or presence of an excess of nonradioactive $\alpha_2$M. After washing cell monolayers at 0°C, cell-associated radioactivity was determined, verifying receptor-specific binding of $\alpha_2$M-gold. Bars indicate average ± SEM of triplicate determination.

[1] Dickson, R. B., M. C. Willingham, and R. H. Pastan. Binding and internalization of $^{125}$I-$\alpha_2$-macroglobulin by cultured fibroblasts. *J. Biol. Chem.* In press.

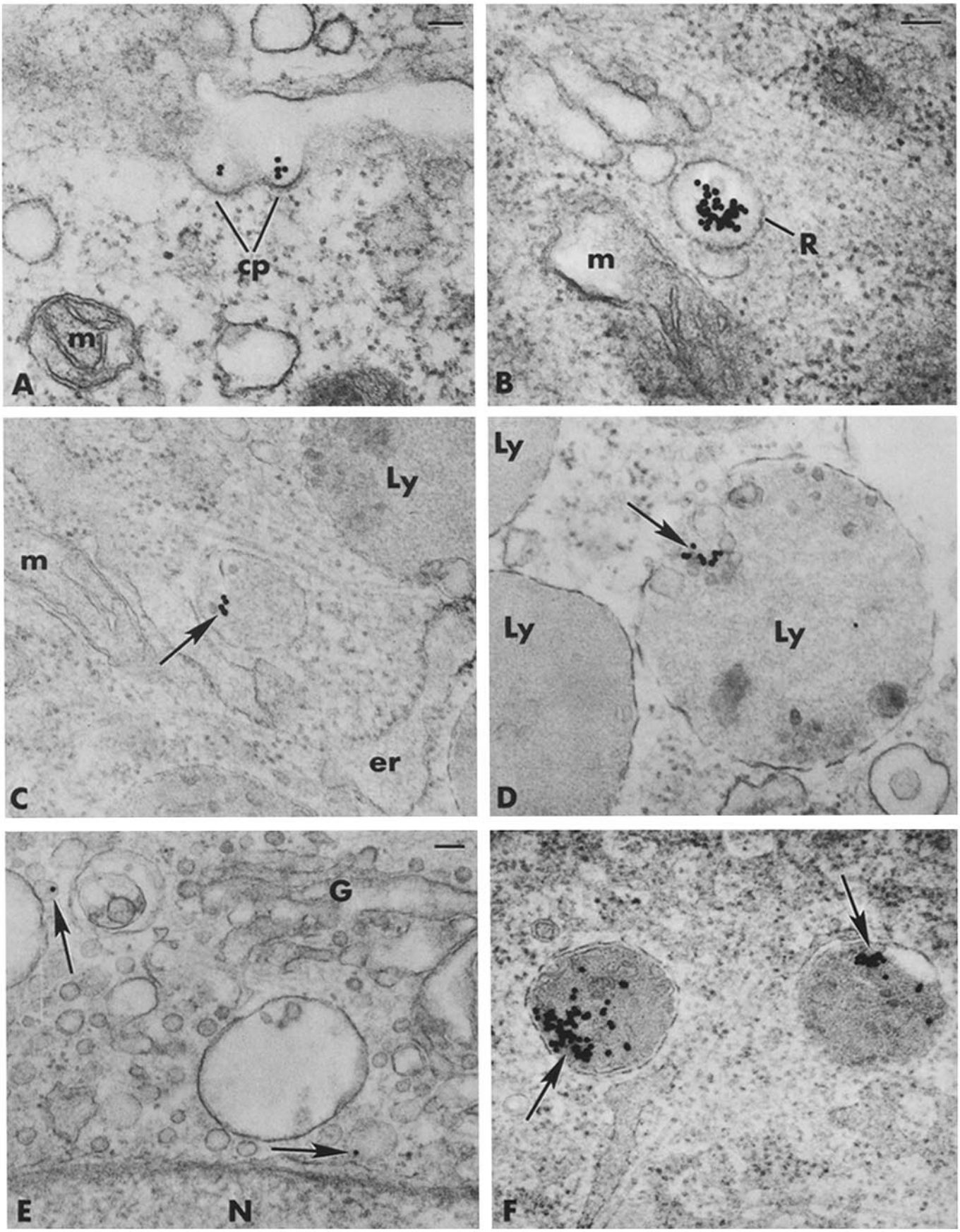


FIGURE 3 Internalization of $\alpha_2$M-gold by fibroblasts. Swiss 3T3 cells were incubated with $\alpha_2$M-gold at 4°C as described in Materials and Methods. *A* shows labeling of coated pits after incubation of cells with $\alpha_2$M-gold for 4 h at 0°C. *B* shows localization of $\alpha_2$M-gold in uncoated vesicles (receptosomes) after cell surface labeling as in *A*, followed by 10 min at 37°C. For *C*, cells were labeled as in *A* and then warmed for 30 min at 37°C showing $\alpha_2$M-gold (arrow) located in a small lysosome. For *D* and *E*, cells were labeled as in *A* and then warmed for 2 h at 37°C. Gold (arrows) was visualized in secondary lysosomes (*D*) and small vesicles in the Golgi region (*E*). In *F*, cells were incubated for 4 h at 37°C in the continuous presence of $\alpha_2$M-gold. Gold (arrows) was highly concentrated in lysosomes. Lead citrate counterstain; Coated pit (*cp*), mitochondrion (*m*), receptosome (*R*), lysosome (*ly*), endoplasmic reticulum (*er*), Golgi complex (*G*), nucleus (*N*). Bars, 0.1 μm. (*A*, *C*, and *D*) × 65,000, (*B*) × 77,000, (*E* and *F*) × 54,000.

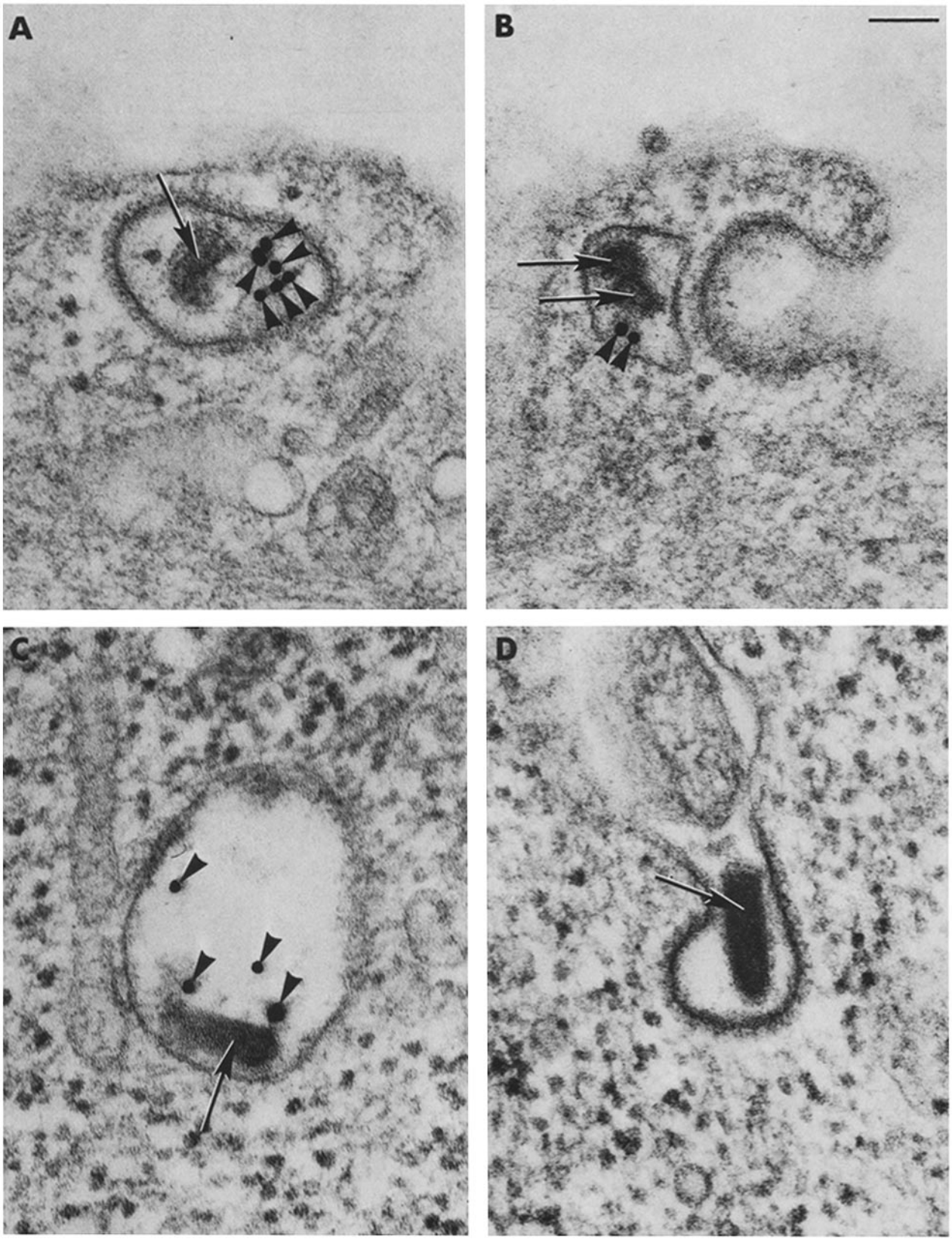


FIGURE 4 Co-internalization of $\alpha_2$M-gold and VSV. Cells incubated in the presence of both $\alpha_2$M-gold and VSV showed the presence of these two ligands together in the same coated pit (*A*), in the same uncoated endocytic receptosome adjacent to a coated pit (*B*), or in the same receptosome deeper in the cytoplasm (*C*). All of these possibilities exist simultaneously at 2 min after warming the cells from 4° to 37°C. If the cells were warmed for only a few seconds, only the image in *A* could be found; if warmed for 5 min, only the image in *C* could be found. Cells incubated with $\alpha_2$M-gold and VSV together with an excess of unlabeled $\alpha_2$M showed no gold labeling on the cells, and only VSV could be found in coated pits *D*. Uranyl acetate–lead citrate counterstain; arrows, VSV particles; arrowheads, $\alpha_2$M-gold particles. Bar, 0.1 μm. × 120,000.

## Localization of $\alpha_2$M-Gold in Cells

When Swiss 3T3 cells were incubated at 0°C for 5 h with $\alpha_2$M-gold (Fig. 3 *A*), the gold was visualized primarily concentrated in coated pit regions. Some gold particles were also seen distributed diffusely over the cell surface. In parallel incubations containing 2 mg/ml $\alpha_2$M (data not shown), both classes of surface-bound gold particles were virtually absent. A few gold particles remained associated with fibronectin or the plastic culture dish (<1%). Previous data using peroxidase-labeled, anti-$\alpha_2$M antibodies are in agreement with the present study (20–23): after exposure to $\alpha_2$M, binding to cells is concentrated in coated pits and also diffusely spread over other regions of the cell surface, depending on the concentration of $\alpha_2$M employed. At low concentrations of $\alpha_2$M, most of the ligand clusters in coated regions, whereas at close to saturating levels the diffuse component is also observed and represents the majority of the bound ligand.

After binding $\alpha_2$M-gold to cell surfaces at 0°C for 4 h and then washing away unbound $\alpha_2$M-gold, we warmed cell monolayers to 37°C for various times to determine the fate of internalized $\alpha_2$M-gold-receptor complexes. After cells were incubated for 10 min at 37°C, the cell surface was entirely cleared and the gold was localized exclusively in uncoated, cytoplasmic vesicles (Fig. 3 *B*). These were easily visualized because of the intense contrast of the gold particles. The vesicles had a membrane-associated proteinaceous layer on their inner surface and are identical to those previously described by use of peroxidase-labeled antibodies to $\alpha_2$M and termed receptosomes (20–23). After 30 min of incubation at 37°C, the gold was found mostly in lysosomes (Fig. 3 *C*). These lysosomes were small and homogeneous and probably represent "primary lysosomes" derived from the GERL system. After 2 h of incubation at 37°C, the gold was localized in larger, heterogeneous lysosomes (Fig. 3 *D*). In addition to being localized in lysosomes, the gold was also localized in very small vesicles in the vicinity of the Golgi apparatus after 2 h at 37°C (Fig. 3 *E*). Although previous studies (20–23) have localized $\alpha_2$M in lysosomes after 30 min or more of incubation at 37°C, the small vesicles in the region of the Golgi containing $\alpha_2$M after 2 h of incubation were not noted. Their function is unknown, but they could represent a step in the exocytosis of intracellular $\alpha_2$M, a process that is known to occur in $\alpha_2$M-free medium (15). If cells were incubated in the continuous presence of $\alpha_2$M-gold for 4 h at 37°C (Fig. 3 *F*), the lysosome was the primary site of concentration of gold. This is in agreement with previous studies using either peroxidase-labeled $\alpha_2$M antibodies (20–23) and electron microscopy or fluorescence-labeled $\alpha_2$M (11, 12) and fluorescence microscopy. The same sequence of localizations of gold was observed when NRK-2T cells were used instead of Swiss 3T3 cells (data not shown).

## Cointernalization of $\alpha_2$M-Gold and VSV

The simultaneous internalization of VSV and $\alpha_2$M-gold was studied. Cells were incubated at 4°C with the two ligands. After washing away unbound ligands, cells were warmed to 37°C for 2 min (Fig. 4 *A* and *B*). $\alpha_2$M-gold and VSV were often concentrated in coated pits together (Fig. 4 *A*). Some uncoated vesicles containing $\alpha_2$M-gold and VSV in the immediate vicinity of coated pits were also observed (4 *B*). This suggested that the transition of coated pits to uncoated vesicles occurs very rapidly. Fig. 4 *D* shows that, in the presence of an excess of unlabeled $\alpha_2$M, virus particles but not $\alpha_2$M-gold were observed concentrated in coated pits. This demonstrates that the presence of $\alpha_2$M-gold did not somehow facilitate the concentration of VSV in coated pits. Cells incubated with the two ligands and warmed to 37°C for 2 min also showed $\alpha_2$M-gold and VSV within uncoated vesicles previously termed receptosomes (Fig. 4 *C*). These receptosomes had a characteristic size and appearance which have been previously described (19, 21, 23). A previous study has demonstrated at the electron microscope level that $\alpha_2$M-gold and ferritin–epidermal growth factor are clustered and internalized together by this same pathway (19). Other studies (10, 19, 21, 23) have shown a clear progression of the clustered ligands present in coated pits in the 1st or 2nd min after warming to 37°C into receptosomes. This is an endocytic process that is complete over the entire cell surface within 5 min after warming to 37°C. The present study is the first clear demonstration that viruses and receptor-bound proteins and hormones share a common pathway of cellular internalization.

In summary, we have developed a new technique for the study of receptor-mediated endocytosis of $\alpha_2$M. $\alpha_2$M–colloidal gold is easy to prepare and use, and its visualization by electron microscopy is extremely clear. This technique may prove to be of general use in the study of other ligands, including hormones, which bind to membrane receptors. Because colloidal gold may be prepared in many different sizes (3, 9), the use of gold-labeled ligands may also allow simultaneous study of the binding and internalization of several proteins, hormones, and viruses.

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