Internalization and Processing of Transferrin and the Transferrin Receptor in Human Carcinoma A431 Cells

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ABSTRACT The binding and subsequent intracellular processing of transferrin and transferrin receptors was studied in A431 cells using ^{125}I-transferrin and a monoclonal antibody to the receptor (ATR) labeled with ^{125}I and gold colloid. Using ^{125}I-transferrin we have shown that, whereas at 37°C uptake proceeded linearly for up to 60 min, most of the ligand that was bound was internalized and then rapidly returned to the incubation medium undegraded. At 37°C, the intracellular half-life of the most rapidly recycled transferrin was 7.5 min. ^{125}I-ATR displayed the same kinetics of uptake but following its internalization at 37°C, it was partially degraded. At 22°C and below, the intracellular degradation of ^{125}I-ATR was selectively inhibited and as a result it accumulated intracellularly.

Electron microscopy of conventional thin sections and of whole-cell mounts was used to follow the uptake and processing of transferrin receptors labeled with ATR-gold colloid complexes. Using a pulse-chase protocol, the intracellular pathway followed by internalized ATR gold-receptor complexes was outlined in detail. Within 5 min at 22°C the internalized complexes were transferred from coated pits on the cell surface to a system of narrow, branching cisternae within the peripheral cytoplasm. By 15 min they reached larger, more dilated elements that, in thin section, appeared as irregular profiles containing small (30–50-nm diam) vesicles. By 30 min, the gold complexes were located predominantly within typical spherical multivesicular bodies lying in the peripheral cytoplasm, and by 40–60 min, they reached a system of cisternal and multivesicular body elements in the juxtanuclear area. At 22°C, no other compartments became labeled but if they were warmed to 37°C the gold complexes were transferred to lysosome-like elements. Extracting ATR-gold complexes with Triton X after a 30-min chase at 22°C and purifying them on Sepharose-transferrin indicated that the internalized complexes remained bound to the transferrin receptor during their intracellular processing.

Many of the proteins taken up at the cell surface for intracellular processing are taken up by receptor-mediated endocytosis. For some of these protein ligands, and most notably for low density lipoproteins (4, 10, 13), the uptake process has been shown to internalize both the protein ligand and its receptor. During the intracellular processing that follows uptake, the ligand is usually delivered to the lysosome, and in some systems it has been shown that the internalized receptor may then be returned to the cell surface for reuse (9, 17, 55). The membrane-bound endosome elements through which the internalized ligand is processed prior to its delivery to the lysosome have been identified (1, 2, 3, 61, 62) and it is clear that in many cells these elements represent a large and morphologically distinctive endosome compartment. In the hepatocyte some of the endosome elements concerned with the processing of the internalized receptor have also been identified (12, 36) although thus far only the pathway concerned with the internalization of the asialoglycoprotein receptor has been delineated morphologically (21). In other cell types the endosome compartment responsible for the differential processing of internalized receptors and their ligands has not yet been identified and the extent to which the hepatocyte pathways are representative of other uptake systems remains to be clarified.

In the study reported here, we examined the endosome compartment of an epithelial cell line, the epidermoid carci-

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noma A431 cell. We employed ^{125}I-transferrin as the physiological ligand and followed the kinetics of its uptake and intracellular processing. By radiolabeling a monoclonal antibody specific for the transferrin receptor (58) we also followed the kinetics of uptake and processing of the transferrin receptor independent of its physiological ligand. In these studies we found that the antibody-receptor complex formed on the cell surface was stable at pH 2.5 and reasoned that it should, therefore, survive the processing at low pH, which is believed to take place in transferrin-containing endosomes (51). Preliminary to studying the processing of transferrin and its receptor simultaneously, we used the antibody complexed to colloidal gold to identify in the electron microscope the pathway followed by the internalized antibody-receptor complexes.

For many cell types, transferrin is the major iron-carrying protein, and in the placenta and various tumor cell types it has been shown to bind with high affinity to a 180,000 M_r cell surface receptor (7, 18, 54, 58). Transferrin has a relatively long half-life in vivo (8–10 d) (6), and from physiological considerations (45) as well as from the kinetics of transferrin uptake measured in in vitro (38, 47) it has been proposed that, during iron uptake, transferrin is continuously internalized and then returned to the cell surface. The receptor for transferrin has also been shown to have a relatively long half-life (~60 h; [49]) and there is now preliminary evidence to suggest that it too is internalized and may be repeatedly recycled back to the cell surface (7, 30).

We have used epidermoid carcinoma cells for this investigation because these cells have been employed previously for the study of receptor-mediated uptake of several other ligands including epidermal growth factor (25, 27, 43) and low density lipoproteins (5). The nonabsorptive uptake of horseradish peroxidase in these cells has also been documented (28).

MATERIALS AND METHODS

Antisera, Reagents, and Radiochemicals: The B3/25 monoclonal antibody was raised against the human haemopoietic cell K562 and has been shown to be specific for the transferrin receptor (58). The transferrin antibody used for immunoprecipitation was a rabbit antihuman transferrin from Miles Laboratories Inc. (Elkhart, IN). The goat anti-rabbit IgG was raised against rabbit IgG. Human transferrin (essentially iron-free) was obtained from Sigma Chemical Co., London. Activated Sepharose 4B CNBr was obtained from Pharmacia, Uppsala, Sweden. I^{125} (carrier-free) was purchased from Amersham International, Amersham, United Kingdom.

Cell Maintenance and Incubation Procedures: A431 cells were grown in Dulbecco's modified minimum essential medium with 5% fetal calf serum and usually plated out at 10^6 cells per 3.5-cm petri dish 2 d before use. Before each experiment, cells were rinsed free of fetal calf serum and incubated for 2–4 h in medium supplemented with fetal calf serum that had been chromatographed on Biogel P60 to remove molecular weight species >60,000 M_r (transferrin-depleted medium).

For binding studies on intact cells, cells were incubated with PBS/10 mM PIPES/100 μg/ml BSA (binding buffer) containing ~500,000 cpm of radiolabel. After each incubation, they were given three thorough rinses in binding buffer at 5°C. For making Scatchard plot analysis (53), binding was carried out for 90 min at 5°C with the radiolabel supplemented with increasing concentrations of unlabeled ligand. In experiments requiring preincubation with transferrin or antitransferrin receptor (ATR)[1], cells were rinsed free of serum, preincubated for 2 h in transferrin-depleted medium, and then preincubated with Dulbecco's modified minimum essential medium supplements as appropriate for 4 h. Following the second incubation, the cells were rinsed six times with binding buffer at pH 7.0 before subsequent binding with ^{125}I-ligand was carried out.

For the removal of surface-bound label, cells were incubated at 5°C with either acetic acid saline (0.2 M acetic acid 0.5 M NaCl) pH 2.4 for 4 min, 50 μg/ml trypsin in PBS for 60 min, or 1 mM EDTA in calcium-free PBS for 60 min. Cell detachment was negligible and "displacement media" were readily decanted for counting. The attached cells were then rinsed briefly and finally digested in NaOH for 1 h at 5°C.

In pulse-chase procedures, cells were incubated for 30 min at 5°C with radiolabel, rinsed three times in PBS at 5°C, and then incubated in chase media at the appropriate temperature. Chase media normally contained 10 μg/ml transferrin. After the chase incubation, the cells were rinsed again with PBS at 5°C and then treated with displacement media at 5°C before digestion in NaOH. Chase media, displacement media, and the NaOH digest were finally taken for ^{125}I counting. Radioactivity in the chase media was precipitated either with an equal volume of ice-cold 20% trichloracetic acid or with transferrin antibody. Before trichloracetic acid precipitation, 0.1 μg/ml carrier BSA was added. After being left overnight at 5°C, the precipitates were washed by repeated centrifugation at 45,000 *g* and then counted. By this method, 80% of the ^{125}I-transferrin and 78% of the ^{125}I-ATR could be precipitated. Transferrin was also precipitated with rabbit antibody diluted 1:500 (incubated for 24 h at 5°C) followed by goat anti-rabbit IgG serum at 1:20 (incubated for 24 h at 5°C). The final immune precipitate was washed by repeated centrifugation for 45,000 *g*. In preliminary studies, this procedure was shown to precipitate up to 100 μg/ml transferrin with 55% efficiency.

Iodination: Transferrin and ATR were radiolabeled according to the method of Hunter and Greenwood (35) (10 μg of protein was labeled with 5 μCi (1 Ci = 3.7×10^{10} becquerels of ^{125}I). Free Na ^{125}I was removed by chromatography on Biogel P60 (Bio-Rad Labratories, Richmond, CA) in phosphate buffer. Assuming complete recovery of the protein, specific activity of the labeled proteins was 0.2–1 μCi/μg. In experiments reported here, background labeling as judged by binding for 10 min at 5°C was not usually >10%. No attempt was made to control the iron saturation of the transferrin since there is reason to believe that iron-free and iron-loaded ^{125}I-transferrin have similar binding characteristics (38, 47).

Manufacture and Handling of Gold-ATR Complexes: 5- and 12-nm gold particles were made according to the methods of Faulk and Taylor (16) and Frens (19) as described by Tolson et al. (57). For stabilizing the gold, ATR was dialyzed overnight against distilled water, and then the pH optimum for stabilization of the gold was established as described by Geoghegan and Ackerman (22). The gold complexes were stored for up to 2 months. Before use, they were washed by centrifugation in PBS containing 0.1% carbowax on a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA) for 150,000 *g*. Using electron microscopy, the suspensions of particles were entirely monodisperse. For incubating with cells, the complexes were suspended in PBS containing 0.1% BSA, 0.1% carbowax.

Extraction of Transferrin Receptor Using Triton X-100 and Affinity Purification on Sepharose-Transferrin: To demonstrate that internalized gold-ATR complexes remained associated with transferrin receptors, internalized complexes were extracted in Triton X and purified on Sepharose-transferrin. Sepharose-transferrin was prepared by incubating 1 g of activated CH Sepharose 4B with 10 μg of transferrin according to the manufacturer's recommended procedure. Cells (10^7 cells/9-cm petri dish) were incubated with either ^{125}I-ATR or ^{125}I-ATR-gold as appropriate by incubating cells at 5°C with 1 ml of 0.2% Triton X-100, 50 μm phenymethylsulfonyl fluoride, 1 mM EDTA in 0.1 M citrate buffer pH 7.4 for 30 min at 5°C. The extract was decanted (<15% of the radioactivity remained on the dish) and centrifuged for 65,000 *g* (<5% of the radioactivity remained in the pellet). The supernatant was then added to 0.25 ml (packed volume) of Sepharose-transferrin beads and incubated for 2 h with gentle aggitation at 5°C. Following this incubation, the beads were washed exhaustively with 0.1 m citrate buffer at pH 5.0. When no further radioactivity could be removed (after about six rinses), the beads were counted and the bound material was extracted by heating for 2 min at 100°C with 0.5 ml, 0.1% SDS. The SDS sample was then run on a 4–10% gradient polyacrylamide gel, essentially as described by Laemmli (40). The gel was stained with silver nitrate (44).

Electron Microscopy: For prefixation in glutaraldehyde, cells were rinsed free of serum and then fixed for 10 min at room temperature in 0.5% glutaraldehyde in 0.1 M phosphate glycine buffer pH 7.4. They were then quenched in borohydride (41) before being incubated with the gold complexes. For whole-mount microscopy, cells were grown on coverslips, rinsed free of serum, incubated in transferrin-depleted medium for 4 h, rinsed in PBS incubated with gold-ATR complexes as appropriate, rinsed thoroughly, and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 30 min. They were then postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in ethanol, and critical point dried from amyl acetate. The dried cells were coated with carbon by evaporation, floated off the coverslips with hydrofluoric acid, rinsed in distilled water, and then mounted directly on copper grids.

[1] *Abbreviations used in this paper:* ATR, antitransferrin receptor (monoclonal B3/25); MVB, multivesicular bodies.

In some experiments, a conventional carbon replica was prepared by digesting the attached cells in 10 N NaOH for 3 h. By first shadowing with platinum carbon, this treatment could be shown to remove all cellular material without disturbing surface-located gold particles. For thin-section microscopy, cells were fixed in dilute Karnovsky fluid (39), postfixed in 2% osmium tetroxide, dehydrated, and embedded in EPON. Sections were stained with aqueous uranyl acetate and lead citrate (52). All attempts to introduce contrast into the preparation by staining en bloc with uranyl acetate and other heavy metals were unsuccessful because with these treatments the gold complexes lost their particulate form. Electron microscopy was carried out on a Philips 301 microscope at 100 kV.

RESULTS

Binding and Uptake of ^{125}I-transferrin by Intact Cells

The binding and uptake of ^{125}I-transferrin varied with temperature. As shown in Fig. 1, with continuous incubation, total cell-associated radioactivity began to reach a plateau level after 90 min at 5°C and 37°C. At 16°C, however, total cell-associated radioactivity was still increasing at 120 min. After 90 min at 5°C, ^{125}I-transferrin binding should be approaching equilibrium at the cell surface. A Scatchard plot (53) derived by incubating to saturation under these conditions indicated that, in log phase growth, A431 cells had on their surfaces $\sim 1.2 \times 10^5$ transferrin binding sites per cell (data not shown).

To identify surface-bound ^{125}I-transferrin, a series of treatments were evaluated for their ability to remove the radiolabel from cells incubated with ^{125}I-transferrin for 90 min at 5°C. These treatments included (*a*) brief incubation with acetic buffer pH 2.4 (26), (*b*) 50 μg/ml trypsin (shown to remove a 70,000-dalton protein fragment from the transferrin receptor in leukemic and HeLa cells [7, 48]), and (*c*) 1 mM EDTA. Low pH and trypsin removed >70% of the label bound at 5°C; 1 mM EDTA released <15% of the label under all conditions. A brief incubation at low pH was thus a most effective method for removing surface-bound transferrin. With these data, a pulse-chase protocol was designed so that the amounts of ^{125}I-transferrin bound to the surface (acid labile) could be measured, internalized (acid resistant), and released into the medium. Cells were preincubated in transferrin-depleted medium for 4 h, cooled to 5°C, and then incubated with ^{125}I-transferrin for 30 min at 5°C. They were given 3 × 2-min rinses at 5°C, and then Dulbecco's modified minimum essential medium (containing 10 μg/ml transferrin) chase medium at 37°C was added. After each chase interval, the chase medium was collected for assay and the cells were given a 4-min rinse in acetic acid buffer (pH 2.4) at 5°C. After the acid rinse, the cells were digested in NaOH. Chase media, acid rinses, and NaOH digests were then taken for ^{125}I-transferrin counting.

At the end of the 5°C pulse (Fig. 2), >80% of the ^{125}I-transferrin was acid displaceable (surface-bound) but within 5 min at 37°C >70% of this label became resistant to acid removal. By 10 min at 37°C >30% of the label was in the chase medium and <5% remained to be displaced by the acid rinse. At this time >70% of the radioactivity was cell-associated but resistant to the acid rinse. At later time points the label in the chase medium steadily increased to approach a plateau level at 20 min, and this accumulation was reflected in the steady decline of cell-associated label. 75% of the radioactivity present in the chase medium at the 10-min chase interval was acid precipitable and 46% was immunoprecipitable with transferrin antibody. Of the total ^{125}I-transferrin bound to the cell surface at the end of the 5°C pulse, <70% becomes inaccessible to the acid rinse within 5 min and <85% can be accounted for in the medium 15 min later. Fig. 3 shows the results obtained when the chase was carried out at 22°C. A pattern of radiolabel redistribution similar to that seen at 37°C was observed, but at 22°C there was a discrete 5

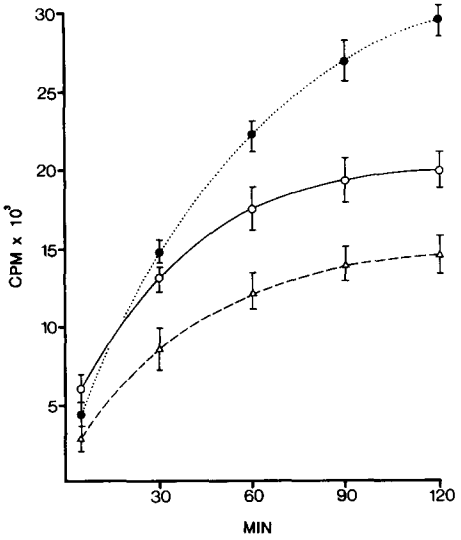


FIGURE 1 Incorporation of ^{125}I-transferrin. 10^6 cells/3.5-cm petri dish were preincubated for 4 h in transferrin-depleted serum, rinsed 3 times in PBS at 5°C, and then incubated at either 5, 16, or 37°C for the times indicated with 430,265 cpm ^{125}I-transferrin. They were then rinsed three times in PBS at 5°C, digested in NaOH, and counted. Each point represents data from triplicate dishes and the bars indicate SEM. Δ, 5°C; ●, 16°C; O, 37°C.

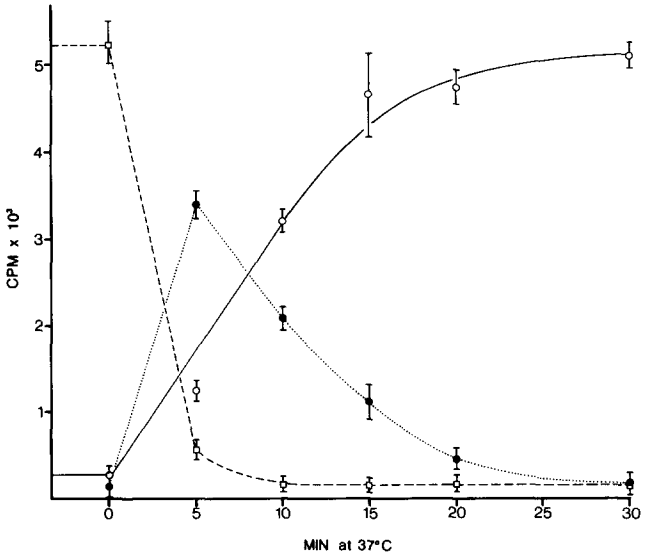


FIGURE 2 Pulse-chase experiment with ^{125}I-transferrin; chase at 37°C. 10^6 cells/3.5-cm petri dish were preincubated for 4 h in transferrin-depleted serum, rinsed three times in PBS at 5°C, and then incubated for 30 min at 5°C with 560,300 cpm ^{125}I-transferrin. Following a further rinsing (three times in PBS at 5°C), cells were incubated at 37°C for 5 to 30 min as shown in chase medium containing 10 μg/ml transferrin. At each time point the chase medium was collected and the cells were incubated with acetic-saline (pH 2.4) at 5°C for 4 min. This medium was collected and the cells were digested in NaOH. The graph shows results ± SEM from triplicate dishes. □, counts displaced by acetic saline; ●, counts in NaOH digest; O, counts in chase medium.

min postpulse interval before the level of radioactivity in the chase medium increased significantly. Clearance of radioactivity from the cell was also considerably prolonged at 22°C.

Together, these pulse-chase experiments indicated that ^{125}I-transferrin bound to the cell surface at 5°C was rapidly internalized when the temperature was raised to 22°C and above. Once internalized, most of the labeled transferrin was rapidly returned, undegraded, to the medium. The capacity of the transferrin uptake process is large. As shown in Table I, preincubation of the cells for 4 h with 1 μm cold transferrin (a concentration sufficient to displace 93% of the ^{125}I-transferrin bound at 5°C) reduced the binding by only 7%.

Binding and Processing of ^{125}Antitransferrin Receptor Antibody

The incorporation of ^{125}I-ATR increased linearly at 5, 16, and 37°C over 20 min (Fig. 4). At 5 and 37°C it approached steady state after 60 min and 30 min, respectively. Incubation at 5°C for 90 min with saturating concentrations of ^{125}I-ATR indicated a density of 1.8×10^5 surface binding sites/cell (data not shown). ^{125}I-ATR bound to the cells at 5°C resisted removal by acetic acid buffer pH 2.5 (<4%) and 1 mM EDTA (<3%) but was susceptible to protease. Trypsin (50 μg/ml at 5°C for 60 min) was most effective, removing >70% of the counts. Using trypsin to discriminate between surface and internalized ^{125}I-ATR in a pulse-chased protocol similar to that employed for ^{125}I-transferrin showed that the kinetics of uptake for ATR and transferrin were essentially the same (Fig. 5) with a half-time of 2.5 min. 30% of the radiolabel taken up was released again into the medium within 30 min at 37°C, and 30% of this released label was acid precipitable. With a chase at 16°C the release of acid-soluble label into the medium was inhibited by >90% (Table II).

Preincubation of cells for 4 h at 5°C with 1.5 μg/ml ATR reduced subsequent binding of ^{125}I-ATR at 5°C by 90% (Table I). Following a similar incubation with ATR, the binding of ^{125}I-transferrin at 5°C was, however, reduced by only 3%. Preincubation of cells for 4 h at 37°C with 1 μM transferrin did not reduce significantly the subsequent binding of ^{125}I-ATR at 5°C. However, preincubation at 37°C with 1.5 μg/ml ATR reduced subsequent binding of ^{125}I-transferrin by 73%. These results confirm earlier studies (8) that the B3/25 monoclonal (ATR) does not bind competitively with transferrin and they show that transferrin did not significantly down-regulate the transferrin receptor. After 4 h with ATR, however, the receptor was significantly down-regulated.

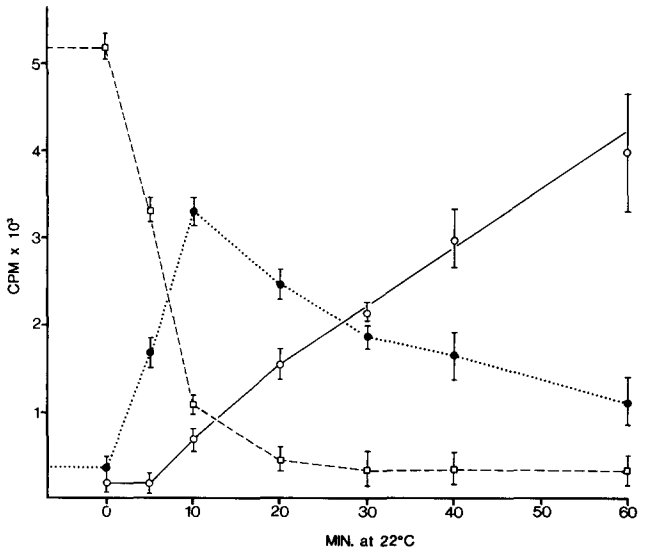


FIGURE 3 Pulse-chase experiment with ^{125}I-transferrin; chase at 22°C. Procedure as in Fig. 2 except that chase extended to 60 min and was carried out at 22°C. Graph symbols are also the same.

TABLE I

Effect of Preincubation with Transferrin or Antibody on Subsequent Binding of ^{125}I-ligands

Preincubation for 4 h	Binding for 90 min at 5°C	CPM bound per 10^6 cells	* Percent change
1 μM transferrin at 37°C	^{125}I-transferrin	3,205 ± 24	7
No preincubation	^{125}I-transferrin	2,983 ± 121	
1 μM transferrin at 37°C	^{125}I-ATR	17,503 ± 562	8
No preincubation	^{125}I-ATR	19,042 ± 834	
1.5 μg/ml ATR at 5°C	^{125}I-transferrin	3,882 ± 65	3
1.5 μg/ml ATR at 16°C	^{125}I-transferrin	957 ± 183	76
1.5 μg/ml ATR at 37°C	^{125}I-transferrin	1,101 ± 206	73
No preincubation	^{125}I-transferrin	4,007 ± 58	
1.5 μg/ml ATR at 5°C	^{125}I-ATR	1,604 ± 242	89
1.5 μg/ml ATR at 16°C	^{125}I-ATR	6,843 ± 542	52
1.5 μg/ml ATR at 37°C	^{125}I-ATR	6,574 ± 211	54
No preincubation	^{125}I-ATR	14,243 ± 1023	
1.5 μg/ml ATR at 5°C	^{125}I-ATR gold	828 ± 212	92
No preincubation	^{125}I-ATR gold	10,348 ± 672	

5×10^5 cells per 3.5-cm dish were preincubated for 2 h in transferrin or antibody as indicated. Following the second preincubation, dishes were rinsed 6 × 2 min at 5°C before the incubation with radiolabel was carried out. For ^{125}I-transferrin, 464,000 cpm, ^{125}I-ATR, 583,000 cpm, and for ^{125}I-ATR 5 nm gold, 397,000 cpm added/dish. Dishes were then rinsed 3 × 2 min at 5°C and the cells digested in in NaOH. All data shown are averages of triplicate determinations and are corrected for nonspecific binding cpm ± SEM.

* Percent change relative to cpm bound without preincubation.

Binding and Uptake of ^{125}I-ATR-Gold Complexes

Preincubation of cells with 1.5 μg/ml ATR reduced subsequent binding of ^{125}I-ATR-gold at 5°C by >90% (Table I). To demonstrate that gold-ATR complexes remain bound to the transferrin receptor following internalization, radiolabeled gold-ATR complexes were chased into the juxtanuclear endosome compartment (see below), and then extracted with detergent and analyzed. The analysis of the extracted gold complexes was based on previous studies that have shown that whereas the transferrin-transferrin receptor complex is unaffected by the presence of detergent (46) its dissociation is markedly reduced at pH 5.0 (60). Extracted gold complexes bound to the transferrin receptor could thus be removed from the detergent extract with Sepharose transferrin and then analyzed by gel electrophoresis.

Cells were incubated for 60 min at 22°C with gold-ATR,

rinsed, and chased for 30 min at the same temperature. They were then extracted with Triton X at 5°C as described in Materials and Methods, and the extract was centrifuged for 60,000 *g* on a microfuge to remove cell debris. The supernatant was then centrifuged for 300,000 *g* to sediment the gold complexes, and only 25% of the radioactivity in the extract remained in the supernatant. The pellet was resuspended in extract buffer and incubated for 2 h with Sepharose-transferrin beads. Following this incubation, 43% of the counts bound to the beads withstood exhaustive washing at pH 5.0. Finally, the material bound to the Sepharose beads was extracted with 0.1% SDS and separated by gel electrophoresis. Bands corresponding to gold, ATR, and a band of ~180,000 M_r were the major components identifiable under nonreducing conditions. Under reducing conditions the 180,000 M_r, band was no longer present and an ~80,000 M_r, band appeared (Fig. 6). The 180,000 M_r, band thus corresponds with the receptor proteins identified in previous studies (7, 15, 18, 54). In a control experiment, cells were incubated with ATR-gold for 60 min at 22°C and chased for 30 min at 22°C, rinsed at 5°C, and then incubated for 90 min with ^{125}I-ATR at 5°C before being extracted and processed as described above. In this control preparation the radioactivity sedimenting with the gold gave some indication of the extent to which the internalized gold binds to "free" receptors during the detergent extraction (<18%). Together, these results demonstrated that a substantial proportion of the gold-ATR complexes remaining within the cell after a chase of 30 min at 22°C were bound to the transferrin receptor.

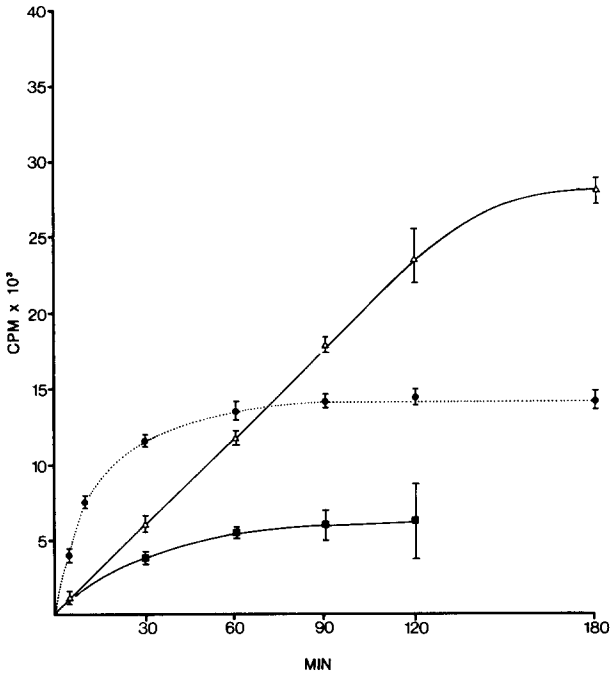


FIGURE 4 Incorporation of ^{125}I-ATR. 10^6 cells/3.5-cm petri dish were preincubated for 4 h in transferrin-depleted medium, rinsed three times in PBS at 5°C, and incubated at 5, 16, and 37°C for the times indicated, with 436, 500 cpm ^{125}I-ATR. They were then rinsed three times in PBS at 5°C, digested in NaOH, and counted. Each point is derived from triplicate dishes, background at 0 min (<15%) subtracted. Bars represent ± SEM. ■, 5°C; △, 16°C; ●, 37°C.

Electron Microscopy of Thin Sections

On cells prefixed with 0.5% glutaraldehyde, quenched, and incubated with gold-ATR, gold particles are localized almost exclusively (>75%) within coated pits of the plasma membrane. The pits were seen as both shallow depressions and as deeper, flask-shaped structures. In cells incubated for 30 min at 5°C with gold complexes before fixation the distribution of gold particles was essentially the same as after prefixation. In some areas of the cell surface, however, gold particles were found in deeply indented invaginations. The lumena of these

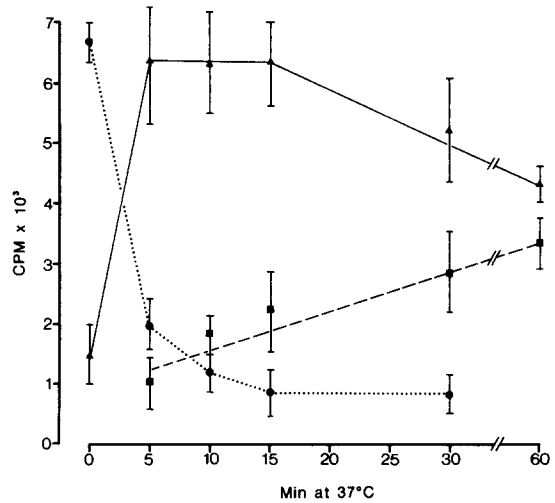


FIGURE 5 Pulse-chase experiment with ^{125}I-ATR. 10^6 cells/3.5-cm petri dish were preincubated for 4 h with transferrin-depleted serum, rinsed three times in PBS at 5°C, and incubated for 30 min with 497,595 cpm ^{125}I-ATR. After further rinsing at 5°C, cells were incubated at 37°C in chase medium containing 10 μg/ml transferrin. At each time point the chase medium was collected and the cells were incubated with 50 μg/ml trypsin for 60 min at 5°C. The trypsin digest was taken for counting and the cells were then digested in NaOH. Results from triplicate dishes expressed as ± SEM. ●, counts removed by trypsin; ▲, counts resistant to trypsin; ■, counts in the chase medium.

TABLE II

Redistribution of ^{125}I-ATR after Chases at 5, 16, and 37°C

	Trypsin digest	NaOH digest	Chase medium	Percent acid precipitate label in chase medium
30-min pulse at 5°C with ^{125}I-ATR	4,560 ± 65	343 ± 25	—	—
30-min ATR pulse at 5°C plus 60 min chase at 37°C	336 ± 35	863 ± 120	3,246 ± 541	30
30-min ATR pulse at 5°C plus 60 min chase at 16°C	423 ± 65	2,163 ± 341	1,400 ± 125	76
30-min ATR pulse at 5°C plus 60 min chase at 5°C	2,993 ± 410	647 ± 25	483 ± 63	76
30-min ATR pulse at 5°C plus 60-min chase at 16°C plus 30-min chase at 37°C	285 ± 46	857 ± 122	2,872 ± 350	40

Triplicate dishes containing 5 × 10^5 cells were incubated with 47.8 × 10^5 cpm ^{125}I-ATR. Procedure was as described in Materials and Methods. cpm ± SEM

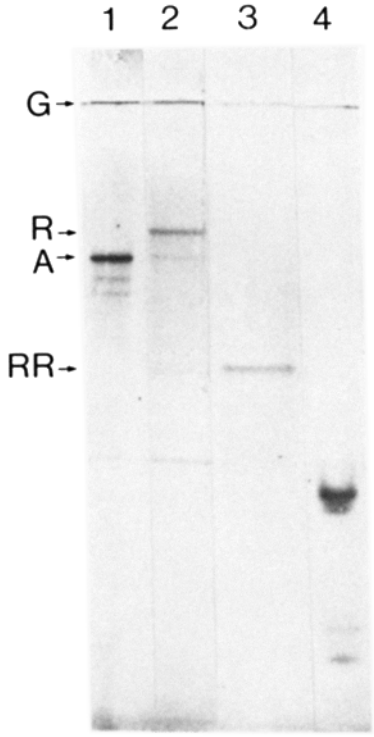


Figure 6 Polyacrylamide gel showing components associated with gold ATR complexes extracted in Triton X. Lane *1*, gold-ATR complex alone; gold (*G*) and major ATR component (*A*) shown. Lane *2*, complexes from cells inubated with 12 nm of gold-ATR for 60 min at 22°C, then chased without gold-ATR for 30 min at the same temperature. Bands corresponding to gold, ATR, and ~180,000 M_r component (*R*) are present. Lane *3*, as sample as lane *2* but reduced with mercaptoethanol; a ~80,000 M_r component (*RR*) appears. Lane *4*, as lane *1* but reduced with mercaptoethanol. 9×10^6 cells were incubated with ^{125}I-ATR-gold for 60 min at 22°C, rinsed, and chased for 30 min at 22°C before extracted as described in Materials and Methods.

invaginations were always electron-lucent and in appropriate section planes their limiting membranes always displayed a clear, three-leaflet morphology.

In cells preincubated with gold-ATR complexes at 5°C for 30 min and then transferred to medium at 22°C containing 1 μg/ml transferrin for 5 min, gold particles were distributed as shown in Fig. 7*a*. At this time, in addition to the distribution seen after a 5°C incubation, gold particles were also distributed within another, morphologically distinct group of membrane-limited elements. These elements lay in the peripheral cytoplasm within the immediate vicinity of the plasma membrane. They have a finely granular, moderately dense content and a limiting membrane that appeared thinner than the plasma membrane. In our preparations the limiting membrane of these structures was difficult to resolve and did not display a clear, three-leaflet morphology. No continuity between the limiting membrane of these peripheral elements and the cell surface (plasma) membrane was observed. The lumena of the peripheral elements were occasionally sac-like (Fig. 7) but usually the intralumenal diameter was restricted to between 20 and 50 nm so that the 12-nm gold complexes that they contained were closely enveloped. After a 15-min chase interval, gold particles were no longer observed at the plasma membrane and were less commonly observed within peripheral elements. At this time the majority of the gold particles were located in larger (200–800-nm diam), irregularly-shaped structures with electron-lucent lumena (Fig. 8*a*). Within these structures one or more 30–50-nm diam vesicles were often present.

At 30-min chase, gold particles were present in typical (1–1.5-μm diam) multivesicular bodies (MVB). In these structures the majority of the gold particles could be seen to lie within 5 nm of the limiting membrane (Fig. 8*b*). After chases of 40 and 60 min most of the gold particles were within MVB or within vesicular or tubular elements (~150-nm diam) that lay within their immediate vicinity. The number of gold particles within each MVB was greater than at earlier time points. However, the close association of these particles with the outer limiting membrane remains. At this time most of the MVB-containing gold particles were concentrated within the juxtanuclear area (Fig. 8*c*). At 22°C no gold particles were observed within stacked Golgi cisternae or within the many secondary lysosomes that lay within the juxtanuclear area.

With a continuous incubation in ATR-gold at 16°C for up to 120 min, all of the compartments seen to contain gold complexes following a chase at 22°C were again labeled (Fig. 8*d*). MVB in the Golgi area contained particles but neither flattened Golgi cisternae nor lysosomal elements were labeled. However, if, after 60 min at 16°C cells were incubated for 10 min at 37°C, gold complexes could be found within the lysosomal elements in the juxtanuclear area. These elements rarely contained internal vesicles and the gold particles were no longer associated with the limiting membrane.

Electronmicroscopy of Whole-mount Preparations

On the surface of cells prefixed in 0.5% glutaraldehyde 5-nm gold complexes identified transferrin receptors in clusters of four to eight and usually as a circular group. Occasionally, and particularly at the edge of the cell, broader bands of gold particles occurred (Fig. 9). The number of monodisperse particles on the cell surface in these preparations was <5%. Preincubation of the prefixed preparation with 10 μg/ml ATR reduced the binding to negligible levels.

When cells were incubated continuously for 60 min at 22°C with 12-nm gold complexes, >80% of all cells present in the preparation were labeled. Within the cells the pattern of labeling was very complex and various elements that often appeared to be extensively interconnected were visible. To obtain a clearer indication of the steps involved in the pathway followed by gold-ATR complexes, a pulse-chase protocol based upon that used to follow ^{125}I-transferrin uptake was again adopted. After 30 min at 5°C with gold-ATR and a 10-min chase incubation at 22°C, gold complexes were no longer distributed as circular clusters; they were, instead, arranged in elaborate branching arrays. Some of these arrays contained groups of tightly-packed and often overlapping particles but many were made up of a single line of particles. Branching of these linear arrays was common and in some areas they formed an elaborate reticulum (Fig. 10*a*).

Taking into account the results obtained with ^{125}I-transferrin and the observations made on thin sections, it was probable that the majority of gold particles observed in the whole-mount preparations at the 10-min chase interval had been transferred across the plasma membrane into the cell. Additional support for this view was obtained by digesting the carbon-supported whole-mounts with NaOH. This treatment leaves the particle clusters seen on the cell surface (e.g., in prefixed preparations) undisturbed. However, from 10-min-chase preparations all gold particles, including those in linear array, were removed. At 20-min post pulse, linear arrays of particles were less common and broader, particle-containing elements of irregular outline predominated (Fig. 10*b*). In some of these elements, particle-free areas, similar to those seen in MVB at later time points (Fig. 10*a*) were present. At 30-min chase the gold complexes were concentrated within approximately spherical bodies (Fig. 10*c*). As noted earlier, sectioned material obtained at this time point indicated that these structures were MVB. At 40- and 60-min time points

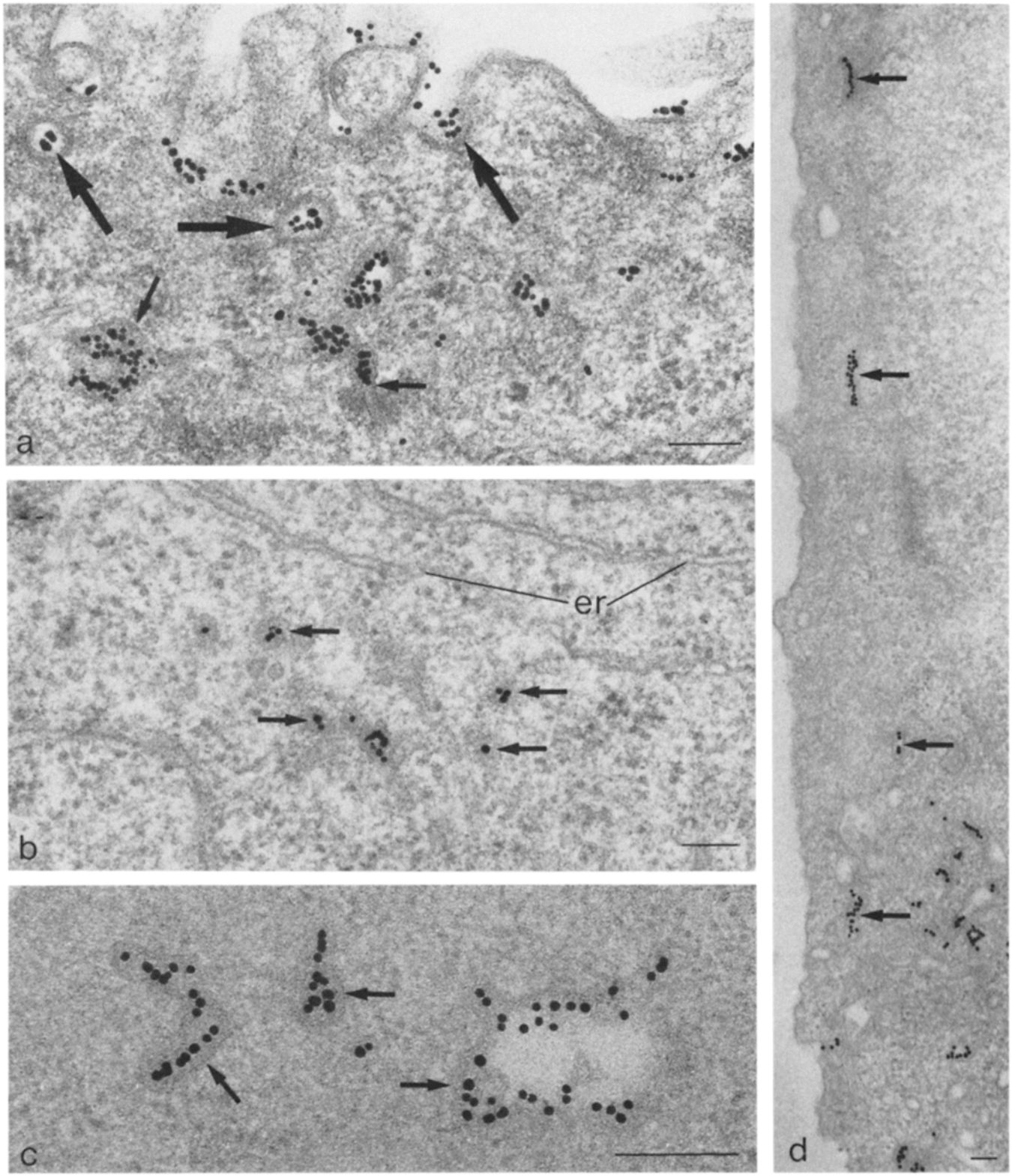


FIGURE 7 Cells were incubated for 30 min at 5°C, and chased for 5 min at 22°C. (*a*) 12-nm gold-ATR particles have bound to the plasma membrane and entered peripheral cisternae (small arrows). Large arrows indicate coated regions of the plasma membrane. *b*, *c*, and *d* are the same as *a* except that chase was for 10 min at 22°C. *er*, endoplasmic reticulum. Bars, 50 nm. *a*, × 120,000; *b* and *c*, × 200,000; *d*, × 28,000.

the majority of gold particles remained associated with MVB. However, whereas at 30 min most labeled MVB were distributed throughout the peripheral cytoplasm, the majority at 40- and 60-min time points were in the juxtanuclear area. Within the full thickness of the whole-mount preparation the superimposition of profiles was extensive but there were images that indicated that MVB had numerous cisternal extensions (Fig. 11*b*). In this juxtanuclear area the density of the sur-

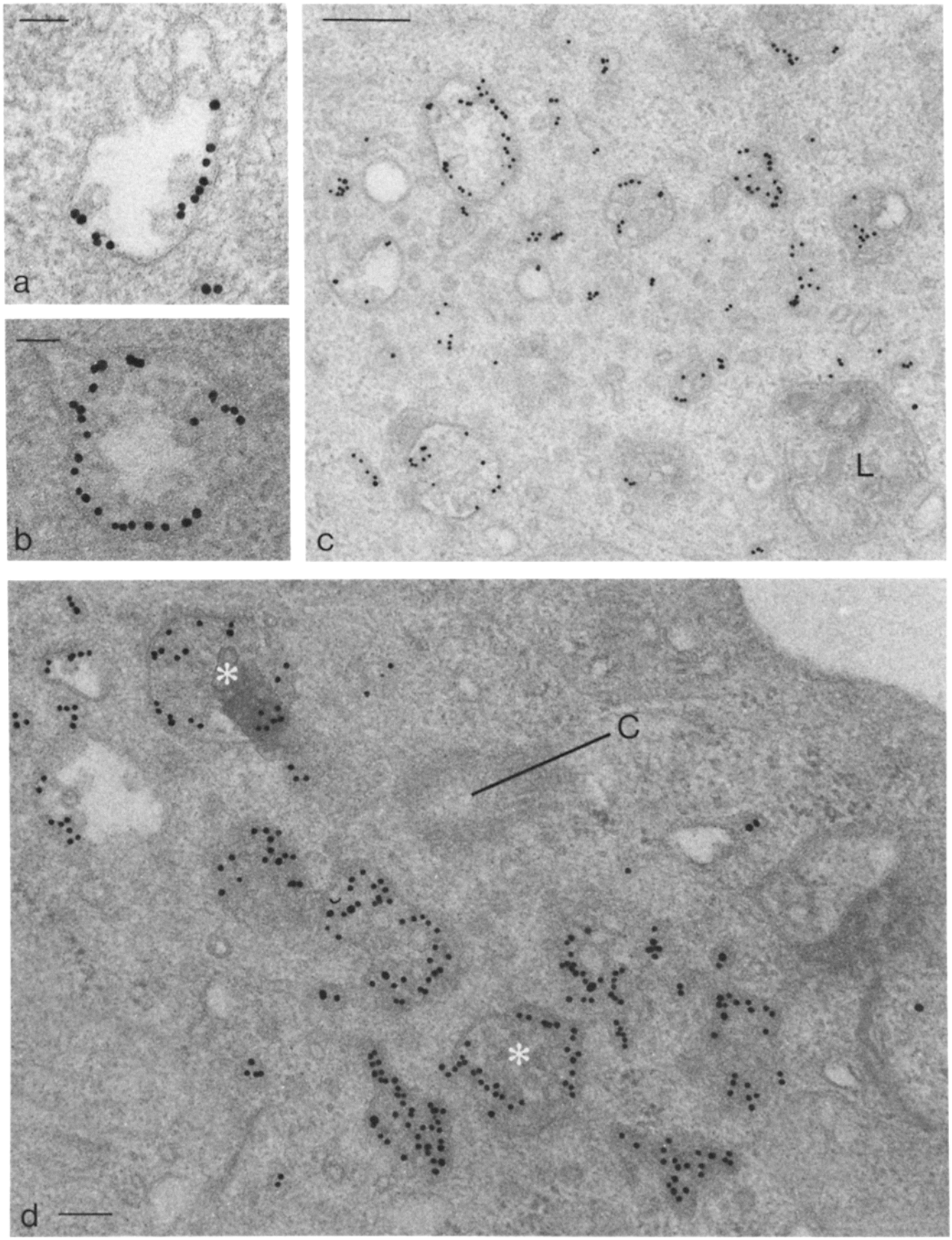
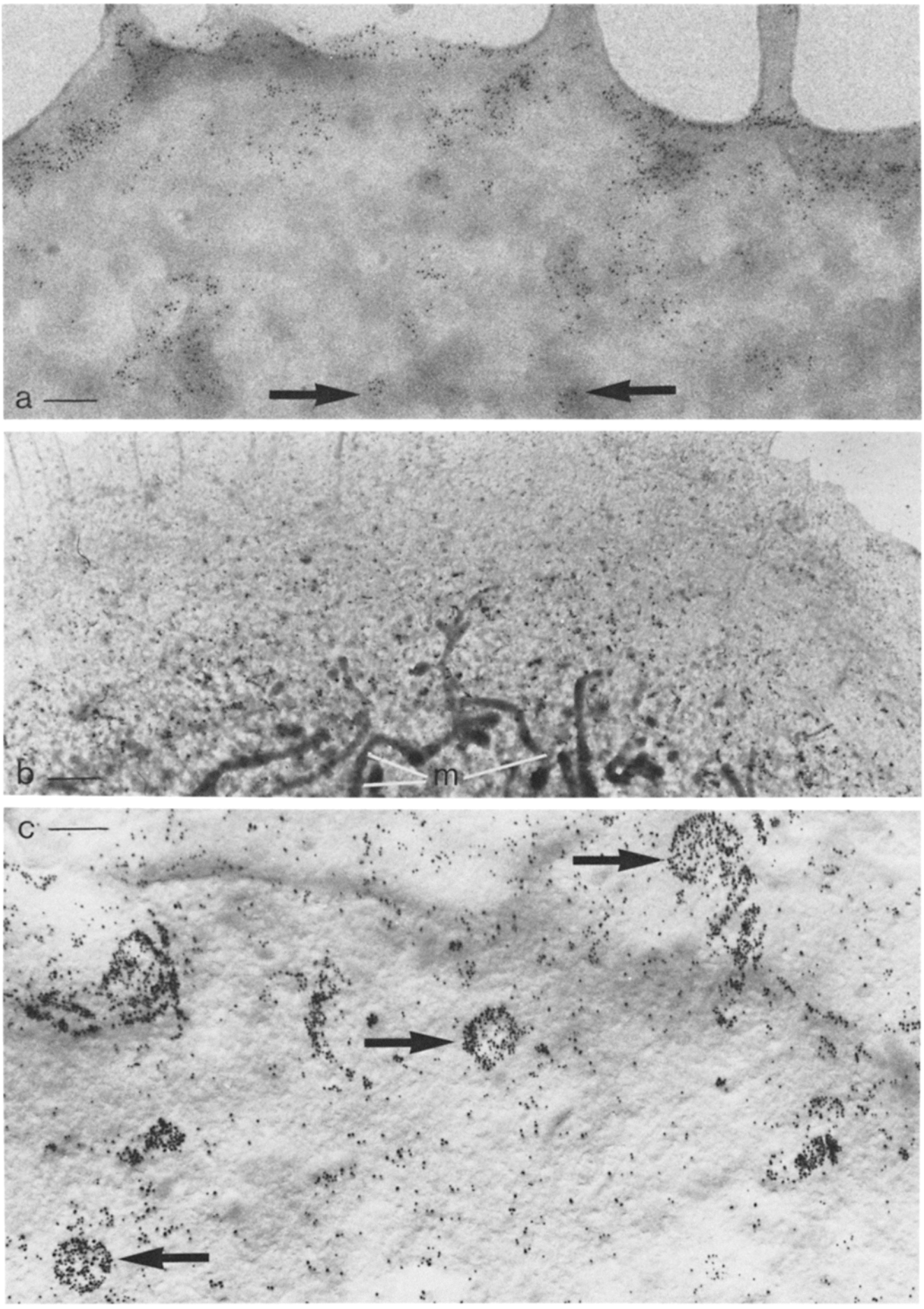


FIGURE 8 (*a*) Cells were incubated for 30 min at 5°C and chased for 15 min at 22°C; peripheral component with irregular profile containing vesicles and 12-nm gold-ATR complexes. *b* is the same as *a* except that a 30-min chase was performed at 22°C. MVB with 12 nm gold-ATR complexes bound to limiting membrane. *c* is the same as *a* except that a 40-min chase at 22°C in the juxtanuclear area was performed. Gold particles are within narrow vesicular-tubular cisternal elements and MVB; lysosome (*L*), free of gold particles. (*d*) Cells were incubated for 60 min at 16°C with 12 nm gold-ATR; juxtanuclear area is shown (*C*; contriole). MVB contains large numbers of gold particles. When section plane passes close to MVB equator (*), it is clear that gold particles remain close to the limiting membranes. Bar 50 nm. × 160,000 (*a* and *b*); bar, 0.1 μm. × 70,000 (*c*); bar, 50 nm. × 100,000 (*d*).


a
b
m
c

rounding cytoplasmic components made the pulse-labeled components difficult to identify, and to explore the possibility that further transfers to other compartments in this area may have also subsequently occurred a prolonged pulse of 60 min at 16°C, followed by a chase of 30 min at the same temperature, was carried out. After this prolonged treatment, groups of up to 20 gold-loaded MVB were identifiable in the juxtanuclear area (Fig. 11 *a*) but no other large cisternal or vacuolar compartments became labeled.

DISCUSSION

Surface Receptors

The ^{125}I-transferrin and ^{125}I-ATR binding assays employed identified a population of high-affinity receptors that were saturable at 5°C. Recent studies (60) have shown that each 180,000 M_r receptor complex binds two molecules of transferrin and thus we estimated that there were $\sim 5 \times 10^4$ surface receptor sites/A431 cell. At the present time it is not clear why ^{125}I-ATR binding indicated more binding sites per cell than ^{125}I-transferrin. The number of binding sites identified by ^{125}I-transferrin is lower than those reported from other cell lines (29) but in these previous studies the detergent extraction procedure employed would also have measured intracellular binding sites.

Uptake and Recycling of Transferrin

The half-time for the uptake process at 37°C was 2.5 min. Using similar methods with teratocarcinoma cells, Karin and Mintz (38) found that the uptake half-time was 2 min, and in HeLa cells Bleil and Bretscher (7) estimate that half of the labeled receptors are internalized in 5 min. The pulse-chase protocol demonstrated that >80% of the ^{125}I-transferrin internalized at 37°C was subsequently returned to the cell surface and released into the medium. Since a significant proportion of the released radiolabel remained precipitable by trichloracetic acid and transferrin antibody, we concluded that most of the transferrin was internalized and released without being degraded. Essentially the same results were obtained by Karin and Mintz (38) and by Octave et al. (47) in their studies of transferrin processing in teratocarcinoma cells and rat embryo fibroblasts. However, our studies also indicated that whereas the half-time for a pulse of ^{125}I-transferrin to transit through the intracellular compartments of the A431 cell was 7.5 min at 37°C, a continuous incubation with the label at this temperature resulted in a linear increase in cell-associated activity for up to 60 min. From these date we calculated that there was probably more than one intracellular pathway involved in the processing of transferrin: a short circuit identifiable by pulse chase and a larger capacity circuit that became loaded after prolonged incubation with the tracer.

Receptor Recycling

To date, recycling of a surface receptor has been most clearly demonstrated for the low density lipoprotein receptor (4, 9, 10, 13). In other systems, where the receptor population may include a pool of internal receptors, the recycling of the relatively small proportion of surface receptors is more difficult to demonstrate. In A431 cells a 4-h preincubation with saturating levels of transferrin reduced the number of surface receptors available by <10% and, since our pulse-chase experiments indicated that the total surface population of transferrin receptors was internalized every ~5 min, this observation indicates either that the A431 cell must have an internal reserve of receptors an order of magnitude greater than the number available on the cell surface or that extensive recycling occurs. In HeLa cells, Bleil and Bretscher (7) have estimated that only ~75% of the transferrin receptors are internal. In our studies, we have not measured the total number of transferrin receptors directly. However, we have some indication of the total that can be internalized from experiments in which cells were incubated with ^{125}I-ATR for prolonged periods at 16°C. In these circumstances, whereas internalization proceeded to saturation, internal degradation was largely prevented. After 3-h incubation at 16°C with a saturating concentration of ^{125}I-ATR, there was fivefold more cell-associated radioactivity than after a similar incubation at 5°C. These experiments indicated that, as in HeLa cells, 75–80% of the transferrin receptors in A431 cells were intracellular. We concluded that, in these cells, recycling of the transferrin receptor was extensive.

Incubating the cells at 37°C with ^{125}I-ATR indicated that some intracellular degradation of the antibody did occur. The rate of degradation was similar to that observed for low density lipoproteins by fibroblasts (8) and for mannosylated serum albumin by macrophages (55). At 16°C, whereas ^{125}I-ATR uptake continued, its degradation was markedly reduced and, as a result, radiolabel accumulated within the cell. This temperature-sensitive block was essentially the same as that identified in the asialoglycoprotein processing pathway of the hepatocyte (14). The ability of ATR to down-regulate the transferrin surface receptors also suggested that some of the internalized antibody-receptor complexes were selectively removed from the supply normally available to the cell surface. However, since the down-regulation reached 50% only after 4 h, it is likely that a substantial proportion of receptor antibody complexes also recycled. Antibodies to the LDL receptor that either allow recycling or, alternatively, cause degradation have also been described (24).

Morphological Studies with ATR-Gold Complexes

The distribution of transferrin receptors identified by ATR-gold complexes on the cell surface was nonrandom. On prefixed cells in transferrin-free medium, the receptors were aggregated into clusters of six to eight over most of the cell surface. The prefixation method employed 0.5% glutaraldehyde. This fixative has been shown to prevent the ligand-induced clustering of epidermal growth factor receptors in granulosa cells (33), and in A431 cells we have shown (unpublished results) that it will prevent the redistribution of surface-bound β-microglobulin antibody induced by second antibody (34).

In aggregating spontaneously, transferrin receptors are therefore similar to low density lipoprotein (23) and α_2-macroglobulin-protease (32) receptors and they are different

FIGURE 9 Prefixed whole-cell mount labeled with 5 nm gold-ATR. Bands of particles at the edge of the cell and isolated clusters, typical of the distribution over the cell surface as a whole are shown (arrows). Bar, 50 nm. × 100,000.

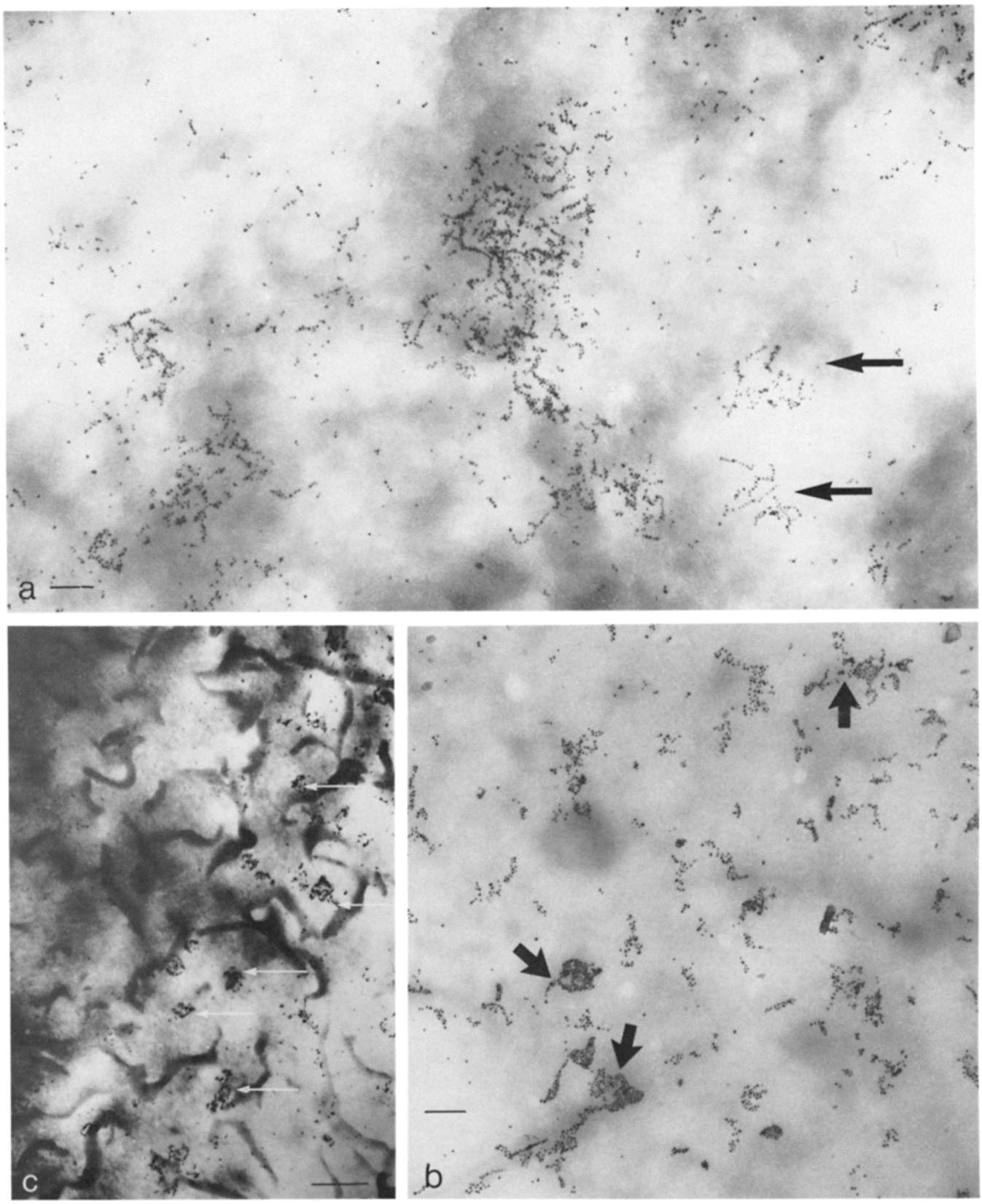


FIGURE 10 Pulse-chase experiment. Whole-mounts derived from cells were incubated for 30 min at 5°C and then chased for 10, 20, and 30 min at 22°C. (*a*) 10-min chase gold particles in elaborate branching patterns; arrows indicate clearly displayed linear arrays of particles. (*b*) 20-min chase particles in broader bands that are often continuous (arrows) with dilated, roughly spherical bodies containing particle-free areas. (*c*) 30-min chase. Particles are now more concentrated within roughly spherical bodies (arrows), but the bodies themselves remain distributed throughout peripheral cytoplasm. Bar, 100 nm. ×40,000 (*a* and *b*); bar, 0.75 μm. × 15,000 (*c*).

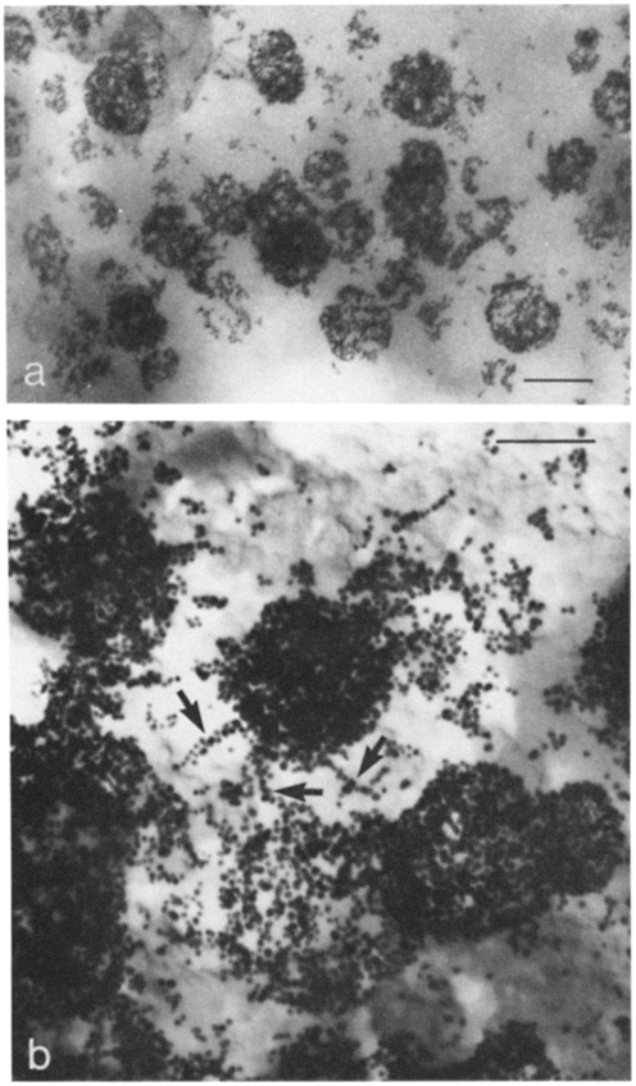


FIGURE 11 (*a*) Whole-mount incubated 60 min at 16°C with 12 nm gold-ATR showing gold-loaded MVB in the juxtanuclear area. (*b*) Whole-cell mount. Cells were incubated for 30 min at 5°C with 12 nm gold-ATR and then chased for 60 min at 22°C. Micrograph shows densely-labeled MVB in juxtanuclear area. Arrows indicate linear arrays that are probably tubular cisternae connecting directly with the limiting membrane of MVB. Bar, 0.3 μm. × 30,000 (*a*); bar, 0.2 μm. × 65,000 (*b*).

from those for epidermal growth factor (33). Thin sections demonstrated that the clustered receptors were almost exclusively in coated invaginations of the plasma membrane. Epidermal growth factor has been shown to induce bulk phase endocytosis in A431 cells; however, we have found no evidence that either transferrin or ATR induces this form of uptake (unpublished results).

At 22°C the gold particle complexes were rapidly transferred to a membrane-bounded compartment in the peripheral cytoplasm. This compartment consisted primarily of narrow tubular cisternae that, as seen in whole-mounts, often formed branching networks. The lumena of the tubular cisternae was moderately electron dense and distinctly different from the electron-lucent extracellular space contained within invaginations of the plasma membrane. Gold complexes were not observed within the tubular cisternae in prefixed cells or in cells incubated at 5°C. After a chase at 22°C when most gold complexes were seen within the tubular cisternae, >85% of ^{125}I-transferrin was resistant to displacement by pH 2.5 rinsing (Fig. 3). We believe therefore that the tubular elements represented the first endosomal compartment of the A431 cell. Internalized epidermal growth factor-ferritin complexes have been identified within similar vesicular profiles in a previous thin-section study on the A431 cell (27). In other cell types, particularly hepatocytes (21, 61) and monocytes (2), peripheral tubular components containing ligand endocytosed within the previous few minutes have also been reported.

After 15 min at 22°C, ATR-gold complexes were found within variously distended cisternal components distributed throughout the peripheral cytoplasm. In thin section these components appeared as irregular profiles in which the gold complexes were closely associated with the limiting membrane. Within their lumena, these elements often contained small vesicles and they were similar therefore to the receptosomes described by Willingham et al. (62). In fibroblasts various endocytosed ligands have been shown to be transported to these endosomal structures, and in the hepatocyte endosomal vacuoles containing variable numbers of small vesicles have also been shown to be involved in the earlier stages of asialoglycoprotein (21, 61) uptake.

After 40 min at 22°C, ATR-gold reached the MVB population concentrated in the juxtanuclear area. These MVB were more spherical and contained more vesicles than the similar elements seen in the peripheral cytoplasm. From our studies, the mode of transfer from the peripheral elements to the juxtanuclear MVB was not apparent. Within the MVB, the ATR-gold complexes remained closely associated with the outer limiting membrane. They differ, therefore, from the epidermal growth factor-ferritin complexes, which in A431 cells have been shown to be associated with the internal vesicles of the MVB (43). When physiological ligand complexes rather than receptor antibodies were followed, the label was invariably in the lumen of the endosome by this stage. These different distributions of the ligands and their receptors were in keeping with the evidence that suggested that (*a*) during the early stages of endocytic processing the internal pH of the endosome was lowered (20, 31, 42, 59,), and (*b*) many ligand-receptor interactions involved in endocytosis were Ca^{++} dependent (31 for review). For ATR we have shown that binding to the receptor remained stable at pH 2.4 and in the presence of 1 mM EDTA.

Within the juxtanuclear area narrow, tubular cisternae containing ATR-gold complexes were common. These structures were similar to the peripheral elements described above, except that they had a more clearly defined limiting membrane and they were often seen as tubular profiles. In sections the limiting membrane of these elements was often continuous with the membrane boundary of MVB, and in whole-cell mounts there were frequent profiles suggesting that these cisternae were extensive and may even form connections interlinking neighboring MVB. In a recent study, Willingham and Pastan (63) have identified epidermal growth factor-horseradish peroxidase tracer within tubular elements of the Golgi system. In the present study, gold particles were not observed within flattened Golgi stacks even after prolonged incubation with ATR complexes. It is probable, therefore, that the MVB and the related cisternal elements to which the ATR-gold complexes were transported represented a functionally distinctive component of the juxanuclear area that included the Golgi complex.

Prolonged incubations at 16°C with gold-ATR indicated that the step in the pathway that was inhibited at this temperature was later than the MVB stage. This step either may involve transfer of the ATR-receptor complex out of the MVB to a degradative compartment or it may require the fusion of lysosomal elements with the MVB. If cells incubated with ATR at 16°C were warmed to 37°C, gold particles were found in lysosome-like structures, and the gold complexes were no longer associated with the limiting membrane. No other elements became labeled and we concluded therefore that these lysosome-like vesicles were the sites at which ^{125}I-ATR, and probably the transferrin receptor, were degraded. After 30 min at 22°C, the gold-ATR reached the MVB in the peripheral cytoplasm but had not reached MVB in the Golgi area. By this time, the pulse-chase ^{125}I-transferrin experiments showed that 50% of the internalized ^{125}I-transferrin had been returned to the medium. It is probable, therefore, that internalized transferrin could be recycled back to the cell surface from the peripheral MVB stage of the pathway outlined by gold-ATR.

In conclusion, our results indicated that transferrin was taken up into A431 cells by receptor-mediated endocytosis via receptors that aggregated spontaneously in coated pits. When bound to antibody, the receptors were then transferred to an intracellular system of compartments, which includes a peripheral cisternal network and a various MVB. In being transported through these endosomal compartments, the transferrin receptor remained closely associated with the limiting membrane boundary. Since many of these compartments were cisternal rather than vesicular, receptor movement through the endosomal system may have depended largely upon transfers in the plane of the cisternal membrane boundaries. At the present time, evidence for only two discontinuous steps exists: The first, inhibited at 5°C concerns uptake at the plasma membrane; the second, inhibited at 16°C, is prelysosomal, in the juxtanuclear area.

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