Electron Microscopic Evidence for Externalization of the Transferrin Receptor in Vesicular Form in Sheep Reticulocytes

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ABSTRACT Using ferritin-labeled protein A and colloidal gold-labeled anti-rabbit IgG, the fate of the sheep transferrin receptor has been followed microscopically during reticulocyte maturation in vitro. After a few minutes of incubation at 37°C, the receptor is found on the cell surface or in simple vesicles of 100–200 nm, in which the receptor appears to line the limiting membrane of the vesicles. With time (60 min or longer), large multivesicular elements (MVEs) appear whose diameter may reach 1–1.5 μm. Inside these large MVEs are round bodies of ~50-nm diam that bear the receptor at their external surfaces. The limiting membrane of the large MVEs is relatively free from receptor. When the large MVEs fuse with the plasma membrane, their contents, the 50-nm bodies, are released into the medium. The 50-nm bodies appear to arise by budding from the limiting membrane of the intracellular vesicles. Removal of surface receptor with pronase does not prevent exocytosis of internalized receptor. It is proposed that the exocytosis of the ~50-nm bodies represents the mechanism by which the transferrin receptor is shed during reticulocyte maturation.

It is well known that the transferrin receptor is lost during the maturation of the reticulocyte into the erythrocyte (1–4). Recent studies have shown that the maturation process can be followed in vitro (4, 5–7). The loss of the transferrin receptor can be used as a marker of maturation (4–6), the transferrin receptor being released, undegraded, to the medium in vesicular form (6, 7). The transferrin receptor is known to recycle many times during the course of Fe^{3+} delivery without degradation of either the receptor or the natural ligand, transferrin (8–11). It has been shown in several systems (8, 12–20), using either labeled transferrin or antibody against the transferrin receptor, that the ligand and the receptor are internalized into vesicles (endosomes) during incubation at temperatures above 10°C and that this internalization may constitute part of the iron delivery mechanism.

Since the transferrin receptor is largely lost from sheep reticulocytes during 24 h of incubation in vitro (5–7), intermediate stages associated with vesicle externalization during receptor elimination might become apparent during the long-term incubation of reticulocytes. No studies to date have addressed the question of the processing that may occur to prepare the receptor for externalization during reticulocyte maturation. We have previously shown (6) that a polyclonal antibody against the transferrin receptor is internalized (as judged by resistance to acid treatment), and that during the course of long-term incubation (several hours at 37°C), this internalized antibody and the surface-bound antibody are lost from the cells along with the antibody-binding capacity of the cells. Since no degraded antibody was found, both internal and surface antibody appear to be eliminated from the cells without degradation.

The rate of loss of internalized ^{125}I-antibody as well as the antibody-binding capacity have a half-time >6 h, clearly different from the recycling time of the receptor during Fe^{3+} delivery, which varies from ~5 to ~20 min (8–11). The 6 h half-time is consistent with the half-time for maturation of the reticulocytes as measured by loss of RNA as well as the capacity to bind transferrin and anti-receptor antibody (5, 21). For this reason, an electron microscopic study was done to follow the fate of the transferrin receptor during in vitro maturation of sheep reticulocytes. The transferrin receptor was followed with an antibody directed against the receptor. The objective was to determine whether intracellular structures can be found that reflect the processing required to externalize vesicles bearing the transferrin receptor, during maturation of reticulocytes.

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MATERIALS AND METHODS

Isolation and Incubation of Sheep Reticulocytes

Reticulocyte production was induced by phlebotomy, and the reticulocytes were isolated by differential centrifugation (5, 22). Incubation of the reticulocytes was described earlier (5). In the present experiments, the reticulocyte count varied from 65–85%.

Antiserum Preparation

Rabbit anti-transferrin receptor antiserum was prepared as described earlier (5).

Ferritin–protein A was prepared according to the procedure described by Templeton et al. (23).

Incubation of Cells and Fixation for Electron Microscopy

DECORATION OF PREFIXED CELLS: In studies with prefixed cells, the reticulocytes were resuspended in 2.5% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4 and maintained at room temperature for 5 min. The fixed cells were washed several times with 0.1 M glycine-buffered isotonic saline, pH 8.0. A 10% hematocrit of the prefixed, washed cells, suspended in PBS was incubated with 75 μl/ml of rabbit antiserum against sheep reticulocytes (5) for 45 min at room temperature. The cells were washed and reincubated for 45 min with ferritin–protein A at room temperature. After washing away the unbound ferritin–protein A with PBS, the cells were prepared for thin sectioning (see below).

DECORATION OF NATIVE CELLS: (*a*) Ferritin label. Native, washed sheep reticulocytes were incubated with the specific anti-transferrin receptor antiserum at 0°C for 90 min which resulted in maximum labeling of the cells (5). After washing in PBS, the cells were reincubated at 0°C with ferritin–protein A for 45 min (23). The unbound antibody and protein A were washed away at 0°C, and the cells were transferred to 37°C and incubated for a further 30 min in culture medium (5). The cells were pelleted by centrifugation at 12,000 *g* for 5 min, washed, fixed overnight at 4°C in 2.5% glutaraldehyde with 0.1 M sodium phosphate buffer pH 7.4 and 0.1% sodium azide. (*b*) Colloidal gold label. For labeling with colloidal gold, the cells were incubated with antiserum for 90 min at 0°C, washed 2–3 times, and then reincubated at 0°C for a further 60 min with colloidal gold–labeled goat anti-rabbit IgG. After several washes at 0°C, the cells were introduced into culture medium and the temperature raised gradually to 37°C. Samples were taken throughout 18 h of incubation at 37°C as well as at initial times before warming to 37°C. Controls, in which cells were incubated throughout the experimental period with nonimmune rabbit serum, were also examined. Gold particles of 10-, 15-, and 20-nm diam were used. After incubation and before fixation, the cells were collected by centrifugation at 90,000 *g* for 60 min. Fixation in glutaraldehyde was as above except that 0.1 M Na cacodylate buffer pH 7.4 was used in the fixation. In those experiments where pronase was used, the controls and pronase-treated cells were fixed overnight at 4°C in a solution containing 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer.

For both native and prefixed cells, postfixation was in 1% OsO_4 for 1 h in Palade buffer (24) containing 5% sucrose. En bloc staining was done for 1 h in 2% uranyl acetate, 0.05 M sodium maleate buffer, pH 5.2. Thin sections were stained with 2% uranyl acetate in 50% alcohol for 3 min and Reynolds' lead citrate for 2 min. The micrographs were taken with a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Materials

Ferritin was purchased from Sigma Chemical Co. (St. Louis, MO). Protein A was obtained from Pharmacia Inc. (Uppsala, Sweden). Culture medium was obtained from Gibco Laboratories (Grand Island, NY). Goat anti-rabbit IgG labeled with colloidal gold was obtained from Janssen Life Sciences Products SPI Supplies (Toronto, Ontario). Pronase was purchased from Boehringer-Mannheim Canada Ltd. (Dorval, Canada).

RESULTS

Early Time Labeling with Ferritin Conjugates and Colloidal Gold IgG

Previous studies have shown that during prolonged incubation of sheep reticulocyte for 30–40 h at 37°C with ^{125}I-labeled anti-transferrin receptor antibody (ATRA),[1] there was no evidence for antibody degradation (6). Virtually all of the cell-associated antibody was recovered in externalized vesicles which contained the transferrin receptor. Evidence for vesicle release was also obtained using fluorescence and electron microscopy. No evidence for intermediate stages leading to vesicle formation and externalization was obtained in these studies, although it was apparent that the internalized antibody was lost during the course of incubation (6). To establish that the receptor is internalized, studies analogous to those done by other investigators with labeled transferrin or anti-receptor antibody (8–20) were executed with sheep reticulocytes and ATRA in culture.

Preliminary studies with reticulocytes coated with ATRA and ferritin-labeled protein A showed that, in prefixed cells, there was no internalization of the ferritin, and the label was fairly uniformly distributed on the cell surface (Fig. 1). In both native and prefixed cells, label appeared in surface pits (Table I), some of which appeared to be coated (Fig. 1). In cells incubated with ferritin-labeled protein A but without the specific antibody, no ferritin was found on the cell surface. The appearance of label in pits in prefixed cells is consistent with reports by Harding et al. (15), Hopkins and Trowbridge (12), and Sullivan et al. (17), which also showed that antibody and/or transferrin were found in pits on the cell surface in prefixed cells or at 4°C, suggesting that these pits exist on the cell surface before the addition of ligand. Our earlier study has already shown that incubation of the antibody-decorated cells for a few minutes at 37°C resulted in the clustering of the surface receptors as well as the internalization of the label (18). Although the studies reported were after 15–30 min of incubation, surface clustering was apparent by 5 min. The internalized label was found lining the limiting membrane of simple vesicles of ~100 nm in diameter (18). These observations are consistent with earlier reports on transferrin receptor internalization using microscopic techniques (12–20).

In the present studies using colloidal gold IgG as a marker, the grains are seen at the cell surface or lining the limiting membrane of simple vesicles at early times (up to 30 min) (Fig. 2). It should be pointed out that only cells treated with immune serum bind the colloidal gold–labeled IgG and that at 0°C all the label is bound at the surface. Thus, an average of 5 grains per cell were found with gold-labeled IgG in cells treated with nonimmune serum (total number of cells counted was 1,300); in contrast, 66 grains per cell were found after immune serum treatment (500 cells counted). The present data are analogous to those using ferritin-labeled protein A described above as well as those reported by others with other markers (8, 12–20).

Since receptor recycling for transferrin processing in reticulocytes (and other cells) is very rapid (8–16, 25) and a steady state uptake of transferrin in reticulocytes is usually reached within 30 min at 37°C (8, 10, 20, 25, 26), structures associated with receptor recycling should be evident during the first half hour of incubation at 37°C. The simple vesicles evident at 15–30 min of incubation are the likely candidates for the structures associated with receptor internalization during iron processing. In contrast, receptor loss (which is probably continuous in cultured reticulocytes) should become prominent at latter times of incubation when followed with the labeled

[1] *Abbreviations used in this paper:* ATRA, anti-transferrin receptor antibody; MVEs, multivesicular elements.

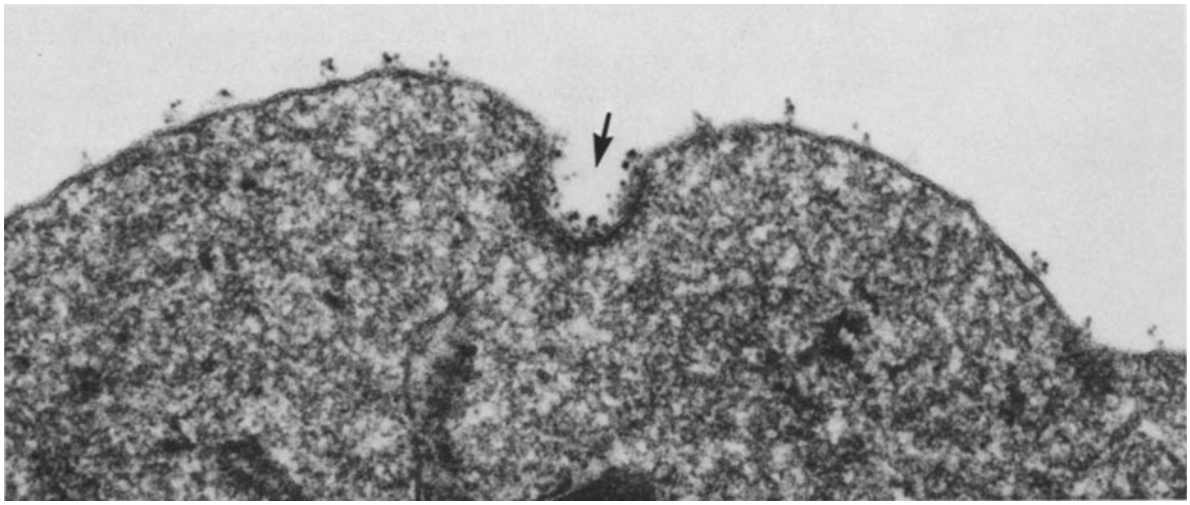

FIGURE 1 Prefixed reticulocytes, surface-labeled with ATRA. A suspension of reticulocytes containing at least 65% reticulocytes (by methylene blue staining) were fixed for 5 min in 2.5% glutaraldehyde in PBS, pH 7.4. The cells were washed three times with 0.1 glycine-buffered saline (pH 8). The washed cells were suspended in PBS, pH 7.4 and incubated for 45 min at room temperature with 75 μl/ml of rabbit antiserum against the sheep transferrin receptor followed by ferritin–protein A as described (23). Note that the ferritin grains are evenly distributed on the cell surface. The arrow shows invaginations with ferritin clusters in a coated pit. × 98,625.

TABLE I. *Distribution of Immunoferritin in Nonincubated and Incubated Cells*

Treatment of cells	No. of Reticulocytes counted	No. of cells with ferritin label	No. of surface invaginations with ferritin	No. of ferritin-containing endocytic vesicles
Incubation at 37°C for 30 min	265	98	62	90
Prefixed cells (not incubated)	180	55	30	3

A 10% sheep reticulocyte suspension in saline was decorated with the immune and nonimmune serum for 90 min at 0°C. The cells were washed in PBS at 0°C and then incubated at 0°C with ferritin–protein A for 45 min. Unbound ligand was removed by washing. Cells with visible intracellular structures were counted as reticulocytes. Cells with ferritin-labeled surfaces, invaginations with ferritin, and/or endocytic vesicles with ferritin clusters were counted. The total number of cells counted is given. Prefixed and native cells were treated as described in Materials and Methods. Preparation of cells for microscopy was described in Materials and Methods.

antibody. Therefore, experiments of several hours duration were also executed to see if antibody becomes associated with different cellular structures during the course of incubation for longer periods.

Patterns of Receptor Labeling during Long-Term Incubation

In the first hour, the internalized grains start to appear in multivesicular elements (MVEs) (Fig. 3). Inside these MVEs, which have a diameter of 200–400 nm in the larger dimension, are small round bodies with an average diameter of 30–50 nm. The gold grains, which first appeared to line the limiting membrane of the simple vesicles, now appear closer to the surface of these small bodies. The MVEs reported here are similar to those reported by several other investigators (12, 13, 15, 20). Harding et al. (15) also noted that in these MVEs the gold-labeled transferrin becomes associated with the internal, small bodies rather than the limiting membranes of the MVEs. After ~60 min, these MVEs are prominent intracellularly, and fusion of the MVEs with the plasma membrane also becomes apparent. By 3 h, fusion of large MVEs with the plasma membrane (Fig. 4) is clearly evident. The limiting membrane of the MVE is now devoid of the label. By 18 h of incubation (data not given), the majority of gold grains are found in MVEs that are fusing with the plasma membrane and are releasing their contents into the medium as noted in Fig. 4. The large vesicles fusing with the membrane may approach a diameter of 1–1.5 μm. It is now very clear that the small, exocytosed bodies contain the gold grains at their external surfaces, while the limiting membrane of the main vesicle is devoid of gold label (Fig. 4). No simple vesicles of ~200-nm diam with gold label at the limiting vesicular membrane are seen by 18 h of incubation at 37°C.

The studies show that at the time of externalization of the antibody from the cell, there is little surface-bound antibody (compare Figs. 2 and 4). Such data suggest that the externalized receptor comes from the internal receptor and not through budding at the cell surface. To substantiate this conclusion, cells were decorated with colloidal gold–IgG at 0°C and incubated for 30 min at 37°C to allow some label to become internalized. The remaining surface label was removed by digesting the cell surface with pronase at 0°C. The cells were washed free from pronase and incubated at 37°C. The surface-digested cells released antibody-bearing vesicles during the incubation at 37°C (Fig. 5, *A* and *B*) in a manner analogous to that of control cells. Control experiments have shown that surface-bound antibody was removed by pronase digestion since all ^{125}I-antibody bound at 0°C was released into the cell-free medium by pronase.

The fact that at early times the labeled antibody lies on the limiting membrane of the internal vesicles, whereas in the MVE the receptor appears at the outer surface of the small inner bodies, suggests that these inner 50-nm bodies may be formed by a budding process at the internal surface of an early vesicle. Since the receptor in externalized vesicles shed

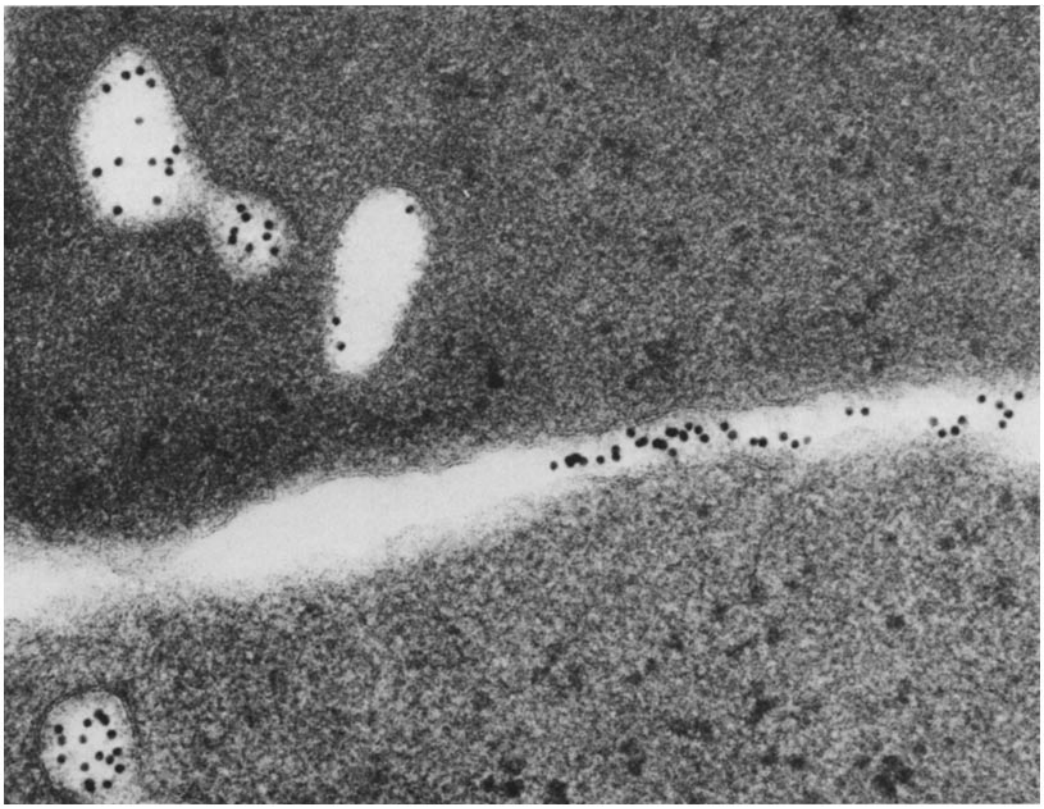

FIGURE 2 Internalization of colloidal gold-labeled IgG at 37°C. Samples treated with 10-nm colloidal gold-labeled goat anti-rabbit IgG were incubated at 37°C for 15 min. The micrograph shows gold grain clusters remaining at the cell surface and grains lining the walls of simple vesicles. × 98,625.

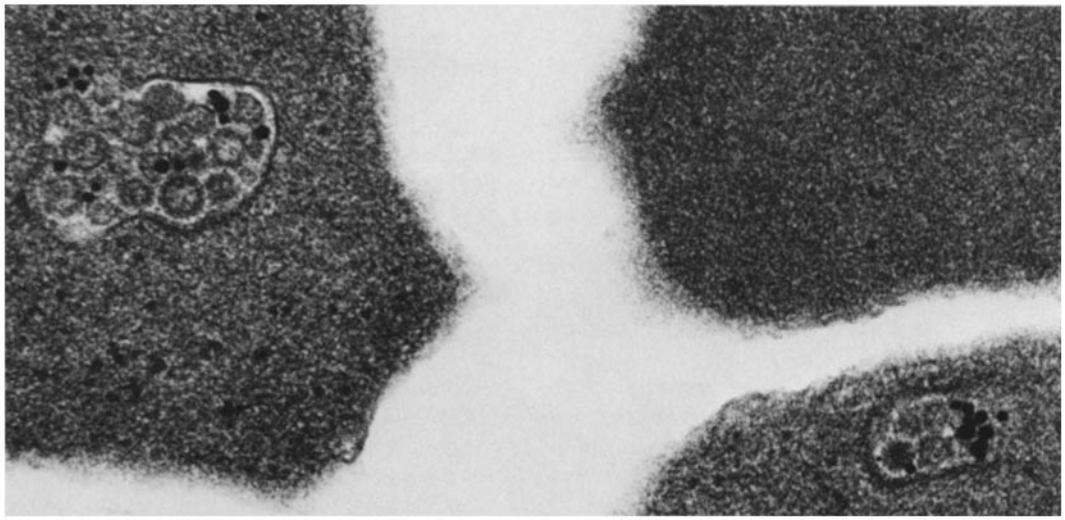

FIGURE 3 Intracellular localization of MVEs and their fusion with the plasma membrane. In the MVEs, the gold label is close to the small dense bodies of ~50 nm which are present inside larger vesicles of 300–500 nm. Note the absence of surface label. Colloidal gold of 20 nm was used. Incubation was for 60 min at 37°C. × 61,500.

from the cell have the receptor (and antibody) exposed at the external surface (6, 7), some inversion must occur to restore the receptor to its original orientation.

DISCUSSION

The mammalian red blood cell undergoes extensive modification during the course of its lifetime (27). Little is known about the mechanisms by which the cell loses its structural components (28). Few cells undergo the extensive remodeling of the cellular contents and surface components that take place in mammalian red blood cells. In the final stages of development and after its release into the circulation, the reticulocyte will lose all vestigial evidence of internal structures in 24–48 h (29). One of the cell surface components lost during this last phase is the transferrin receptor (1–4). Since the transferrin receptor can be detected by immunological

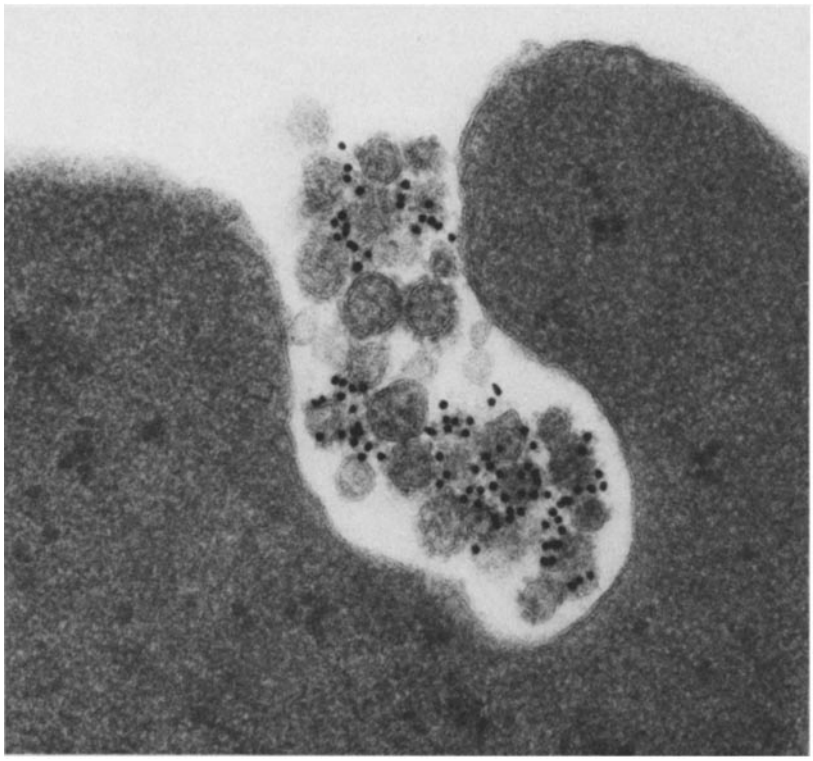

FIGURE 4 Fusion of MVEs with the plasma membrane and release of round bodies. The figure shows exocytosis into the medium of small dense bodies labeled with gold label after a 3-h incubation at 37°C. The gold label is only present on the 50-nm bodies which are inside vesicles of 300–800 nm in diameter. The limiting membrane of the MVE is devoid of label. Other conditions are as in Fig. 2. × 98,625.

means, it seemed worthwhile to get some insight into the mechanism by which the receptor is lost by examining the fate of the receptor using immunocytochemistry. Few other systems provide an opportunity to follow the mechanism by which a cell can rid itself of proteins and structures that are no longer required for its survival.

The transferrin receptor released in vesicular form is apparently unchanged in its molecular weight, peptide map, and the capacity to bind transferrin (5, 6). In this study, using an immunocytological approach, the movement of the transferrin receptor from the surface of the cell into internal vesicles, followed by receptor release into the medium in an organized structure, have been examined over a period of 18 h, during which most of the receptor is known to be lost from the cell (5, 6).

No information has heretofore been available on the cellular events that lead to vesicle release. In recent studies, Harding et al. (15) and Iacopetta et al. (20) noted the presence of MVEs in rat and rabbit reticulocytes, respectively, which they suggested might be involved in receptor processing during iron delivery. However, Harding et al. (15), using gold-labeled transferrin to follow the receptor, pointed out that these MVEs were unlikely to be the sole route by which iron is delivered since the time frame for receptor processing through MVEs was much longer than was the average time for transferrin recycling. They considered the possibility that these multivesicular structures might be involved in ridding the cells of their transferrin receptors, and in recent publications (15, 30), have shown evidence for the fusion of MVEs with the plasma membrane and release of their contents into the medium.

In the present study using an immunological approach, we have obtained visual evidence that the MVEs are the penultimate structures from which the transferrin receptor appears to be released during long-term incubation of sheep reticulocytes. The data show the changing nature of the intracellular structures that contain transferrin receptors during long-term incubation and that ultimately, small bodies with the transferrin receptor at the external surface are released.

The present data suggest that the small intravesicular bodies are formed by an evagination process from the inner membrane of the larger vesicles. The size of these bodies (50 nm) is consistent with previous observations on the size of the vesicles formed during maturation of reticulocytes (6, 30), as is the disposition of the receptor at the surface of the released vesicles (6). As in the present work, Harding et al. (15, 30) also noted that initially the labeled marker (transferrin in their case, antibody in our experiments) was associated with the limiting membrane of a simple vesicle, whereas with time the label appeared at the external surface of the 50-nm bodies. Whether the maturing reticulocyte is the only type of cell to undergo receptor loss in this manner is not known.

That the externalized transferrin receptor originates from the internal receptor pool and not directly from the surface receptor is shown by the studies with pronase-treated cells. These cells, despite the absence of surface label or surface receptor, release internalized ATRA in a manner identical to that of control cells (Fig. 5, *A* and *B*).

Zweig et al. (31) suggested that specific areas of membrane could be lost from maturing reticulocytes by membrane budding at the cell surface. Earlier, we also suggested a budding from the surface (6), a conclusion that is no longer tenable. The current observations suggest that the budding may occur from the inner membrane of intracellular vesicles, followed by formation of a population of intravesicular, free buds.

Since indirect immunocytochemistry has been used in these studies, it is possible that the MVEs bodies are formed in response to these complex ligands and that the ligands are also degraded through the lysosomal system. To address the former question, we have probed fixed sections of reticulocytes with ^{125}I-antibody followed by radioautography (results not given). These studies too showed the presence of MVEs

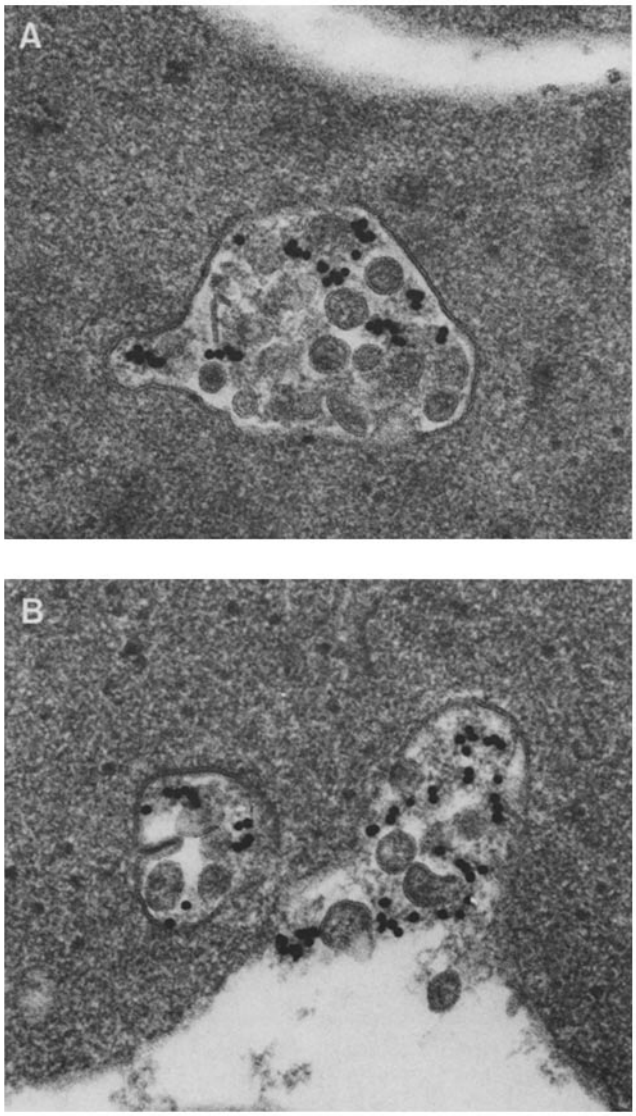

FIGURE 5 Release of labeled vesicles in pronase-treated cells. For *A* and *B*, the cells were labeled with ATRA and colloidal gold IgG and washed free from excess antibody as described in Materials and Methods. After a 30-min incubation at 37°C, an aliquot of the cells was removed, cooled to 0°C, and treated on ice with 1 mg/ml of pronase. At the end of 30 min, while keeping all reagents and the cell suspension cold, 1% bovine serum albumin was added, and the cells were washed three times in ice cold PBS containing 1% albumin. After the third wash, the cells were resuspended in fresh culture medium, brought gradually to 37°C, and incubated for periods up to 18 h. The figures given are from a 3-h (*A*) and 18-h (*B*) culture, respectively. 15-nm colloidal gold was used. × 85,000.

in which the internal small bodies bound labeled antibody against the receptor. Moreover, Harding et al. (15, 30), using direct labeling with transferrin, demonstrated similar bodies. In addition, in the presence or absence of ligands in the medium, a population of free vesicles can be harvested that contain undegraded receptor (6, 7). Therefore, it is unlikely that these bodies arise as a consequence of the presence of complex polyvalent ligands. It also appears unlikely that the gold-labeled antibody complex is degraded by lysosomes. Reticulocytes do not degrade ^{125}I-ATRA in culture periods up to 40 h (6, 7). If the gold complex were directed to lysosomes, structures other than the MVEs would appear during a 24-h incubation period in vitro. In our experiments, the gold label appeared to accumulate only in the multivesicular bodies. By 24 h of incubation, when most of the receptor is shed from the cell (6–8), the only gold-bearing structures visible are multivesicular bodies fusing with the plasma membrane.

During the final stages of development, the reticulocytes face a dilemma. They must maintain the ability to internalize transferrin for iron delivery and at the same time lose transferrin receptors as hemoglobin synthesis wanes. There is no evidence for receptor degradation (6, 7), and it may be presumed that all the transferrin receptor is eventually externalized (30). How these events are regulated and programmed is still unknown. It would be interesting to determine whether agents that could prevent the formation of the multivesicular structures would slow down or prevent the maturation of the reticulocyte into the erythrocyte.

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