Mannose-specific Endocytosis Receptor of Alveolar Macrophages: Demonstration of Two Functionally Distinct Intracellular Pools of Receptor and Their Roles in Receptor Recycling

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ABSTRACT Receptor-mediated endocytosis of rat preputial β-glucuronidase and the glycoconjugate mannose-BSA by rat alveolar macrophages is inhibited by chloroquine and ammonium chloride. We have previously reported that these drugs cause a loss of cell surface binding activity and that they do not inhibit internalization of receptor ligand complexes when incubated with cells at 37°C. In this report we more clearly delineate the intracellular site of weak base inhibition of receptor recycling and the mechanism of that inhibition. From our analysis of the kinetics of ligand transport we conclude that there are two functionally distinct intracellular pools of receptor. One of these, the cycling pool, is not sensitive to the presence of weak bases, and receptor-ligand complexes return from this pool to the cell surface intact. The second pool is responsible for the time-dependent intracellular delivery of ligand to acid vesicles, which is inhibited by weak bases. Chloroquine and ammonium chloride appear to inhibit the dissociation of receptor-ligand complexed in this second pool and thereby the production of free receptors for the continuation of receptor-mediated endocytosis. We examine the internalization and binding of ligand in normal and paraformaldehyde-treated cells and find that these are strongly affected by pH. In particular, the dissociation rate of receptor ligand complexes is enhanced >7.5 fold by lowering the medium pH from 7 to 6. From these results we propose that weak bases raise the pH of acid intracellular compartments, slowing the rate of receptor-ligand dissociation and thereby reducing the cellular pool of free receptors available for further uptake of ligand. In addition, we demonstrate that receptor-ligand complexes cannot return to the cell surface from the amine-sensitive (acid) intracellular pool that led us to call this the nonreleasable pool. This final observation indicates that receptor movements through these two pools are functionally distinct processes.

A number of macromolecules enter cells by receptor-mediated endocytosis; all involve binding to a specific receptor, and some have been shown to associate with coated pits on the plasma membrane and to be internalized in coated vesicles. Frequently, the ligand is deposited in lysosomes and degraded (for review, see reference 1).

This report describes some work done with the system that recognizes mannose-terminated glycoproteins. The mannose-receptor has been found on a number of cells of the reticuloendothelial system of the rat including Kupffer cells of the liver (2) and alveolar macrophages (3). Ligands for the receptor include certain lysosomal enzymes, mannose and L-fucose terminated neoglycoproteins (3, 4).

The fate of the receptor after internalization is not well understood in endocytic systems. We, and others, have proposed that receptors may be reused after a round of internalization (6–8). The evidence for recycling has been largely indirect, i.e., (*a*) the ability of cells to internalize ligand far exceeds the number of binding sites on their surfaces and (*b*) uptake proceeds in the absence of protein synthesis.

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We have attempted to learn something of the mechanism of receptor-mediated endocytosis by looking at the uptake of mannose-terminated glycoconjugates under normal conditions and when perturbed by various inhibitors, especially the lysosomotropic drugs, chloroquine and NH_4Cl. We have previously shown that mannose-specific endocytosis by alveolar macrophages demonstrates a similar inhibition by either of these weak bases (9). In the current work we have kinetically isolated several of the steps in receptor-mediated endocytosis and shown that the only demonstrable effect of ammonium chloride is to reduce the rate of receptor-ligand dissociation by raising the pH of acid intracellular vesicles. In addition, we have functionally defined two intracellular receptor/receptor-ligand pools that are distinct in their sensitivity to weak bases and in their physiologic function.

MATERIALS AND METHODS

Materials

Female Wistar rats were obtained from Harlan Industries (Cumberland, Ind.). Mannose-BSA with ~35 moles of sugar per mole of protein was prepared by the method of Lee, Stowell, and Krantz (10) and was kindly supplied by Y. C. Lee. Rat preputial β-glucuronidase was prepared by the method of Stahl et al. (11). Silicon oil (DC 550) was obtained from Accumetric (Elizabethtown, Ky.). Mineral oil and NH_4Cl were obtained from Taylor Chemical Co. (St. Louis, Mo.). Trypsin (type III), HEPES, chloroquine, yeast mannan, bovine serum albumin (BSA), Sephadex G100, EGTA, sodium phosphate, potassium iodide, and sodium metabisulfite were obtained from Sigma Chemical Co. (St. Louis, Mo.). Chloramine T was obtained from Eastman-Kodak (Rochester, N. Y.). MES was obtained from Boehringer-Mannheim (Indianapolis, Ind.), $Na^{125}I$ from Amersham (Arlington Heights, Ill.).

Ligand Labeling

Mannose-BSA was radio-iodinated as previously described, except that the first dialysis was against 0.005 M Tris-Cl pH 7.5, 1 M NaCl, 0.01 M KI, and the second dialysis was against 0.005 M Tris pH 7.5, 0.15 M NaCl (8). β-Glucuronidase was radioiodinated as described above, except that the mass of chloramine T used was equal to 0.1 of the mass of protein to be labeled, and the reaction was stopped after 5 min on ice by making the reaction mixture 1 mM in β-mercaptoethanol. After dialysis, ^{125}I-ligand was routinely tested by placing a small aliquot in 1 mg/ml BSA solution and bringing this to 10% TCA. The resulting precipitate was washed twice and counted. This could then be compared to later TCA precipitations (done in the same manner) on the ligand.

Uptake and Binding Assays

Uptake and binding assays were run as previously described (8), buffered at pH 6 with 0.03 M sodium phosphate or 0.03 M 2-(*N*-Morpholino)ethanesulfonic acid (MES), and at pH 7 with 0.03 M HEPES or 0.3 M sodium phosphate. Uptake is defined as the total cell-associated radioactivity after incubation at 37°C. Binding is defined as the total cell-associated radioactivity after incubation for 90 min at 4°C (8). Cells that were to be used for binding studies were preincubated at 37°C in Eagle's minimum essential medium with Earle's salt solution (EMEM) with 30 mM phosphate (pH 6) and 10 mg/ml BSA, which insured optimal cell surface binding. Using this pretreatment gave us macrophage preparations that uniformly bound 3–4 ng of mannose-BSA per 500,000 cells at 4°C. This treatment had no effect upon uptake or receptor distribution if the cells were subsequently warmed to 37°C at pH 7. We presume that it serves merely to provide cells with a uniform and maximal compliment of available cell surface binding sites. In all cases, specific binding or uptake is that which can be eliminated by a large excess of a competitive ligand (e.g., yeast mannan) (8). Macrophages were obtained as previously described (3).

Paraformaldehyde Fixation

Cells (10^7/ml) were incubated on a tilt board in 0.15 M NaCl, 0.03 M $NaPO_4$ (pH 7.0) and 1% paraformaldehyde, for 30 min at 4°C. After 30 min the cells were diluted to 10 times their incubation volume and washed three times with buffered saline by centrifugation at 900 *g*. The supernatant was discarded and the cells were resuspended in fresh medium.

RESULTS

Weak Bases Do Not Inhibit Recovery of Cell Surface Binding After Mild Trypsinization

Previously, we have demonstrated that weak bases do not inhibit the internalization step of mannose-specific receptor-mediated endocytosis by alveolar macrophages and that they diminish cell surface binding after a round of internalization (9). The action of weak bases could be to trap the receptor-ligand complexes intracellularly or to prevent the return of dissociated free receptor to the cell surface. Both proposals are consistent with our previous observations. To explore the latter possibility we examined the effect of chloroquine on the recovery of binding activity after mild trypsinization at 4°C, conditions under which 80–90% of the surface binding activity can be eliminated (8). After trypsinization at 4°C, the cell's intracellular complement of receptors should include some free receptor that is available for repopulation of the cell surface and renewal of cell surface binding. If chloroquine inhibits return of free receptor to the cell surface, then warming these cells in the presence of chloroquine should result in the inhibition of the renewal of binding activity. Warming trypsinized cells to 37°C for a few minutes led to a rapid recovery of all the surface binding activity (Fig. 1 *A*). If trypsinized cells were warmed in the presence of chloroquine, surface binding activity was again increased (Fig. 1 *B*). The recovery, in this case, was not to control levels but rather to a level reflecting the level of binding activity one observes when untrypsinized cells are warmed for a similar period of time in the presence of chloroquine. This result demonstrates that chloroquine mediated the depletion of the intracellular pool of free receptor but not the actual movement of receptor from this pool to the cell surface. Therefore the weak base inhibition of endocytosis must occur before the formation of the free intracellular receptor pool.

pH Dependence of Binding and Uptake

Because it appeared that rapid receptor recycling must occur

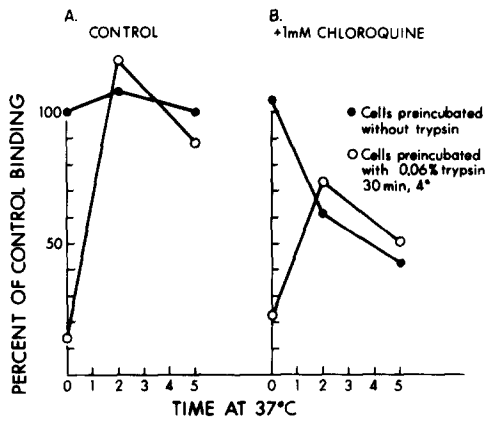

FIGURE 1 Effect of chloroquine on the recovery of surface binding after trypsinization. Cells (1.4×10^7/ml) were incubated at 4°, 30 min in MEM, pH 7 (controls) or MEM containing 0.06% trypsin, pH 7. The cells were then washed twice by centrifugation in 3 vol of MEM, 20% FCS at 4°C. Cells were then resuspended in MEM, 20% FCS (controls), or MEM, 20% FCS containing 1 mM chloroquine, and warmed at 37°C for various periods of time. Each sample was then cooled to 4°C, and tested for binding activity by incubating cells (5×10^6/ml) with ^{125}I-Man-BSA (1.7×10^6 cpm/μg, 4 μg/ml), for 90 min. The abscissa indicates minutes of incubation. Binding is expressed as percent of untrypsinized control cell binding before warm-up, and all values are corrected for nonspecific binding.

to allow prolonged uptake of ligand (8), some means of accelerating the dissociation of the receptor-ligand complex must exist to account for the uptake rates observed in alveolar macrophages. The ligand ends up in the lysosome and it seemed possible that the low pH found there, or in some other acidified compartment, might be sufficient to induce receptor-ligand dissociation. A study of the effect of pH on ligand binding and uptake was undertaken to explore this possibility.

A time-course of uptake at pH 7 and pH 6 is shown in Fig. 2. Uptake at 37°C is essentially completely inhibited by dropping the pH from 7 to 6. On the other hand, Fig. 2 *B* shows that binding to the cell surface at 4°C is essentially unaltered by reducing the pH from 7 to 6. Therefore either internalization is inhibited by pH 6 or the affinity constant at pH 6 is reduced so that essentially no uptake can occur at this pH. The observation that binding at 4°C is the same at pH 6 or 7 means that lowering the temperature overrides whatever effect the pH is having and low temperature binding does not serve as a satisfactory way to study the physiological binding and dissociation, although it still appears to be useful to quantitate cell surface receptor sites.

pH Dependence of Internalization of Prebound Ligand

In this experiment cells were incubated with ligand at 4°C for 90 min. Unbound radioactivity was removed by washing at 4°C and the cells were warmed to 37°C for various periods of time, as previously described (8). We monitored the radioactivity released into the medium during the warming period (referred to as 37°C-release), the cell-surface ligand radioactivity (released from the cells by subsequent treatment with trypsin-EGTA at 4°C), and the cell-associated ligand radioactivity (that remains with the cell pellet after treatment with trypsin-EGTA at 4°C).

When the experiment was done at pH 7, the prebound ligand rapidly disappeared from the cell surface upon warming (Fig. 3 *A*) and there was a concomitant rise in the cell-associated pool. After warming had continued for about 8 min, radioactivity began to appear in the medium. We have previously shown this to be degraded material having the molecular weight of monoiodotyrosine (8).

When the experiment was done at pH 6, the results were very different (Fig. 3 *B*). There was a rapid loss of material from the cell surface similar to that seen at pH 7, but very little of this entered the cell. Most of the material appeared in the medium. When a sample of this medium, taken from cells that had been warmed for 10 min, was analyzed by gel filtration chromatography (Sephadex G-100), virtually all of the radioactivity migrated exactly as intact ^{125}I-mannose-BSA. Thus, it appears that, at pH 6 and 37°C, rather than being internalized, the receptor ligand complexes rapidly dissociate, releasing intact ligand into the media.

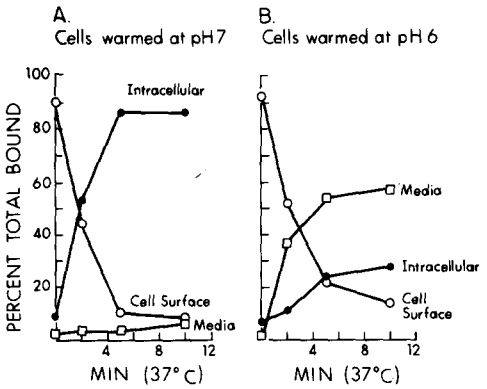

FIGURE 3 Internalization or prebound ligand at pH 6 and pH 7. Cells were warmed to 37°C for 30 min in MEM, 10 mg/ml BSA, 30 mM $NaPO_4$, pH 6, and cooled in an ice-bath. Then they were taken up in the same buffer containing ^{125}I-Man-BSA (4 μg/ml, 9.4 × 10^6 cpm/μg) at a concentration of 6.3 × 10^6 cells/ml. The cells were incubated at 4°C for 90 min and washed three times by centrifugation in 5 vol of the above buffer at 4°C. The cells were divided equally into two tubes, centrifuged, and taken up at a concentration 5 × 10^6/ml in the above buffer (panel *A*), or the above buffer adjusted to pH 7 (panel *B*). Samples from each tube were then warmed for the indicated periods of time, cooled, and the cells were separated from the media by centrifugation. Counts recovered in the supernatant are referred in the figure as "Media." The cell pellets were then taken up in CMF-HBSS, containing 10 mM EGTA and 1 mg/ml trypsin (3 × 10^6 cells ml), and incubated at 4°C for 15 min. The cells were then centrifuged through oil as previously described (8). Radioactivity recovered in the media under these conditions is referred to above as "Cell Surface." The remaining cell-associated radioactivity is referred to as "Intracellular." Radioactivity present in the three fractions is expressed as percent of the total radioactivity associated with the cells before warm-up.

Binding of Ligand to Paraformaldehyde-treated Cells

Receptor-ligand dissociation can be studied only under conditions that prevent internalization of the ligand or receptor-ligand complex. No internalization occurs at 4°C; however, prebound ligand does not dissociate to any significant extent at either pH 6 or 7 at 4°C, even after long periods of incubation (data not shown). To observe cell surface receptor-ligand interactions at 37°C, we pretreated cells with paraformaldehyde at 4°C. Using cells pretreated with paraformaldehyde, we measured the concentration dependence of ^{125}I-Man-BSA binding (4°C). The binding in treated cells was essentially identical to that in untreated controls (Fig. 4, inset). However, treatment of cells with paraformaldehyde produced a complete inhibition of internalization (data not shown).

Paraformaldehyde-treated cells were allowed to bind ligand at 4°C, and unbound ligand was removed. The cells were transferred to medium at pH 6 or 7 and warmed to 37°C for

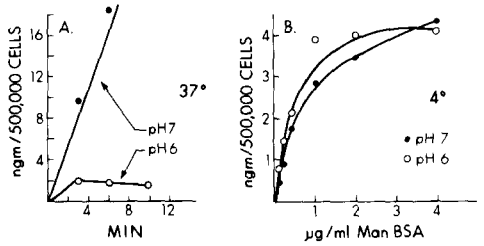

FIGURE 2 Uptake and binding of Man-BSA at pH 6 and pH 7. Panel *A*: Cells (3.4 × 10^6/ml) were incubated with ^{125}I-Man-BSA, (2.9 × 10^5 cpm/μg, 8 μg/ml), for the indicated periods of time, at 37°C in MEM, containing BSA (10 mg/ml) and 30 mM $NaPO_4$ adjusted to pH 7.0 (●) or pH 6.0 (○). Specific uptake per 500,000 cells, as a function of time at 37°C, is shown for both buffers. Panel *B*: Cells (5 × 10^6/ml) were incubated in MEM, 20% CS, buffered with either 30 mM $NaPO_4$, pH 6 (○), or 10 mM HEPES, 10 mM BES, 10 mM PIPES, pH 7 (●), with ^{125}I-Man BSA (5.2 × 10^6 cpm/μg) at the indicated concentrations for 90 min at 4°C. Specific ligand binding per 500,000 cells as a function of ligand concentration is shown for both buffers.

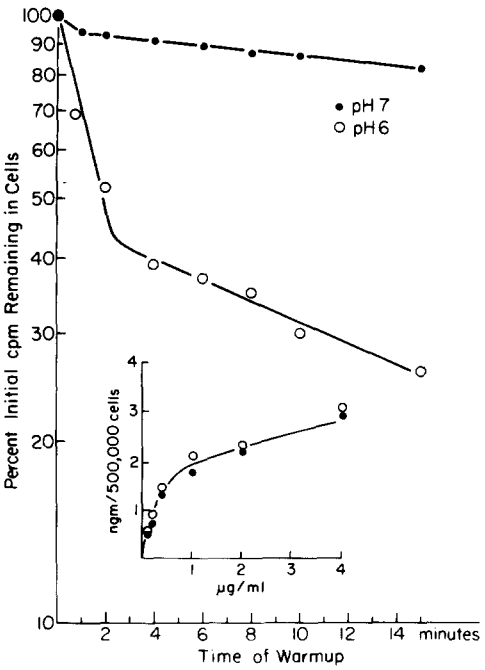

FIGURE 4 Release of prebound ligand from paraformaldehyde-treated cells. Cells were warmed to 37°C in MEM, containing 10 mg/ml BSA, 30 mM $NaPO_4$, pH 6 for 30 min, cooled, collected by centrifugation, and taken up in 150 mM NaCl, 30 mM $NaPO_4$ (pH 7.1), 1% paraformaldehyde, and incubated at 4°C for 30 min. Excess paraformaldehyde was removed by washing the cells three times by centrifugation in 4 vol of cold MEM, pH 6, buffered as described above. The final wash was divided equally into two tubes, centrifuged, and each cell pellet was taken up in the same buffer containing ^{125}I-Man-BSA (6.4×10^6 cpm/μg, 4 μg/ml), at a cell concentration of 1.1×10^7/ml. One tube also contained mannan (1.3 mg/ml). Tubes were incubated for 90 min at 4°C, washed three times with 12 vol of the above buffer, and cells were taken up at a concentration of 10^7/ml in $NaPO_4$ buffered MEM, pH 7 (●), or $NaPO_4$-buffered MEM, pH 6 (○). Aliquots of cells (0.1 ml) were then warmed for the indicated times in microfuge tubes, and the cell pellet and the supernatant were counted as previously described (8). The radioactivity remaining with the cells is corrected for nonspecific binding and is expressed as percent of the total present before warmup. The inset shows 4°C binding of ^{125}I-man-BSA (1.9×10^6 cpm/μg) to normal cells and cells treated as described above with paraformaldehyde. Conditions were as described for Fig. 2, panel *B*, in pH 6 buffer. Normal cells (●); paraformaldehyde-treated cells (○).

various periods of time. The results in Fig. 4 show that dissociation at pH 7, 37°C is slow, with $t_{1/2} > 15$ min. Dissociation at pH 6 is both rapid and biphasic, and >50% of the ligand dissociated from the cells in <2 min. At both pH's the specifically bound ligand could be removed by treatment with EGTA or α-methyl-mannoside (data not shown).

Return of Receptor-Ligand Complexes to the Cell Surface

It appeared that the high lysosomal pH generated by the weak base could prevent ligand and receptors from dissociating. We considered the possibility that, even though dissociation might be reduced, receptor-ligand complexes might still be cycling from intracellular to cell surface locations. If this proved to be the case, ligand could effectively label the receptor and could be used to monitor receptor movement.

^{125}I-β-Glucuronidase was the ligand used for the remaining experiments. This ligand was chosen because it is degraded very slowly whereas ^{125}I-mannose-BSA is rapidly degraded. Although qualitatively similar results were obtained using ^{125}I-mannose-BSA as a ligand, the much smaller degradation component obtained using ^{125}I-β-glucuronidase simplified interpretation of the results.

Cells were preloaded with ^{125}I-β-glucuronidase by incubation for 8 min at 37°C. The loading was done in the presence or absence of NH_4Cl. Because the inhibition of uptake by NH_4Cl is time-dependent, during the 8-min incubation, a sizable amount of ligand accumulates inside the cells in the presence of this inhibitor. Unbound ligand was removed by washing and centrifugation at 4°C in buffer. Ligand was determined to be inside the cell by the criterion that treatment with 0.1% trypsin and 10 mM EGTA at 4°C for 15 min released <10% of the total radioactivity. This treatment has been previously shown to release >90% of surface bound ligand (8).

The preloaded cells were then taken up in various buffers (referred to as stripping media) that were known to accelerate the release of receptor-bound ligand. These included (*a*) Ca^{++}-, Mg^{++}-free Hank's balanced salt solution (CMF-HBSS) pH 7.0, containing 10 mM EGTA, (*b*) HBSS adjusted to pH 6, and (*c*) 0.1 M α-methyl-mannoside containing 80 mM NaCl. The cells were allowed to warm to 37°C in stripping medium for various periods of time. At each time-point, the radioactivity released into the medium and that still cell-associated were counted. A sample of the radioactivity released into the medium was placed in 10% TCA and tested for precipitability. The accuracy of TCA precipitation in determining the fraction of low-molecular-weight material in a sample of partially degraded ligand was confirmed by Sephadex G25 chromatography of representative samples. In all of the results shown, the TCA-soluble radioactivity-degraded ligand released into the medium has been subtracted. Normally this component represented <10% of the total amount of ligand originally cell-associated.

The results of one of these experiments is shown in Fig. 5. In Panel *A*), 30 mM NH_4Cl was present in the loading, washing, and stripping buffers. In panel *B*, NH_4Cl was absent. In the presence of NH_4Cl, ~10% of the total ligand was released into the medium as high-molecular-weight material when the stripping buffer was HBSS medium, i.e. buffer that normally does not accelerate the dissociation of ligand from its receptor. The magnitude of this component is approximately equivalent to nonspecific uptake, and could be ligand that was adhering nonspecifically to cells. If the stripping buffer contained additions that are known to accelerate receptor-ligand dissociation, considerably more intact ligand was released into the medium. Using α-methyl-mannoside as the stripping reagent, 40% of the total ligand was released as intact ligand upon warming. It is unclear whether the amount of intact ligand released by the various stripping reagents simply reflects their differing capability of accelerating receptor ligand dissociation, or whether more complicated effects are involved. In the absence of NH_4Cl, only a small portion of the total intracellular ligand was released by HBSS. α-Methylmannoside- and EGTA-containing buffers produced a small, but significant, release of the total internalized ligand as intact ligand. In the absence of NH_4Cl the fraction of cell-associated ligand that can be released by the stripping buffers is small but it represents approximately the same number of nanograms per cell as is released in the presence of NH_4Cl.

The released component appears to have a number of char-

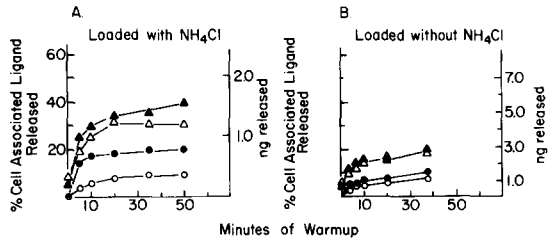

FIGURE 5 Release of ligand from cells loaded in the presence or absence of NH_4Cl. Panel *A*: Cells (1.2×10^7/ml) were incubated with β-glucuronidase (10 μg/ml, 4.2×10^6 cpm/μg) for 8 min at 37°C in buffer containing 30 mM NH_4Cl. Cells were washed three times in chilled buffer and divided equally into four tubes. The cells in each tube were taken up in a different stripping buffer, and the samples were then warmed to 37°C for various periods of time. After warmup, the radioactivity in the media was separated from that which was still cell-associated by centrifugation through oil. A sample of the supernatant was tested for precipitability in 10% TCA. Stripping buffers: HBSS containing 30 mM NH_4Cl, pH 7, (○); HBSS containing 30 mM NH_4Cl, pH 6, (●); CMF-HBSS containing 10 mM EGTA, 30 mM NH_4Cl, pH 7, (△); 100 mM α-methyl mannoside, 82 mM NaCl, 5 mM $CaCl_2$, 5 mM $NaPO_4$, 30 mM NH_4Cl, pH 7, (△). Panel *B*: Cells were treated as described above except that β-glucuronidase concentration was 8 μg/ml, (11.6×10^6 cpm/μg), and the buffer contained no NH_4Cl. Stripping buffers ○, ●, △, were as described above except that no NH_4Cl was present. Stripping buffer △ was 0.1 M α-methyl mannoside, 90 mM NaCl, 10 mM $CaCl_2$, 5 mM HEPES, pH 7. The left ordinate in each panel is the percent of total radioactivity that is cell associated after loading and washing that is recovered as TCA-precipitable ligand in the media. The right ordinate is the same value but expressed as nanograms of ligand.

acteristics: (*a*) it is released rapidly (i.e., in 5–10 min the majority of this component has been released), (*b*) ~50% of the ligand appears to be unavailable for release by any of these conditions.

Time-dependence of Ligand Entry into Different Cellular Pools

Preliminary experiments indicated that the percentage of total cell-associated ligand that was released undegraded into the medium depended upon the duration of the loading phase of the experiment. This suggested that we should carefully study the kinetics of ligand entry into the intracellular pools.

To examine this, we loaded cells for various periods of time (up to 32 min) with ^{125}I-β-glucuronidase. As before, unbound ligand was removed. The cells were loaded and washed in a concentration of NH_4Cl (30 mM) that inhibits, but never completely stops, uptake. At each time-point, the surface component was estimated by determining the percentage of total ligand removed by treatment at 4°C with EDTA/trypsin. Each sample was tested for total releasable ligand by incubation with 10 mM EGTA for 30 min at 37°C. The precipitable radioactivity released into the medium minus the surface component gave the component that was returning to the cell surface in complex with receptor when the cells were warmed, and it will be referred to as the cycling pool. The remaining cell-associated ligand was designated the nonreleasable pool.

The results of this experiment are given in Fig. 6. Both the cycling and the surface pools appear to saturate rapidly and then remain constant for the duration of the experiment. The cycling pool appears to be about twice as large as the surface pool. The nonreleasable pool continues to accumulate ligand during the course of the experiment. The accumulation of ligand in this pool accounts for the bulk of the time-dependent uptake of ligand by cells. This readily explains the difference in the percentage of total uptake found in the cycling pool in uninhibited verses inhibited cells (see Figs. 5 and 7). Finally, there does not appear to be any lag in the entrance of ligand into the nonreleasable pool, even at the earliest time-points.

The Role of NH_4Cl in the Release of Intact Ligand from Cells

To determine whether NH_4Cl plays any direct role in the release of intact ligand from cells, release of ligand from cells

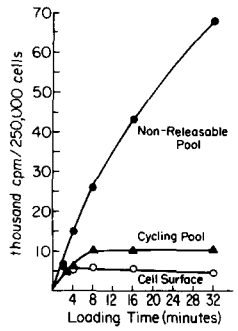

FIGURE 6 Time dependence of ligand entry into different cellular pools. Cells (1.3×10^7/ml) were incubated with ^{125}I-β-glucuronidase (12 μg/ml, 11.6×10^6 cpm/μg) in buffer containing 30 mM NH_4Cl for various periods of time. They were then washed three times with the above cold buffer and taken up in CMF-HBSS containing 10 mM EGTA, 30 mM NH_4Cl, pH 7, and warmed for 30 min. The cells were centrifuged through oil, and the cell-associated and supernatant radioactivities were determined. Samples of the supernatant were tested for TCA precipitability. Cell samples from each time-point were also tested for release of surface-bound radioactivity by treatment at 4°C with 0.1% trypsin, 10 mM EGTA. From this test, a percentage of total radioactivity released by trypsin/EGTA was obtained. This factor was multiplied by the total radioactivity present at each time-point to arrive at the cell surface-specific radioactivity. To estimate the cycling pool, the radioactivity associated with the cell surface was subtracted from the TCA-precipitable counts released by warming cells in the presence of EGTA. The nonreleasable pool is that radiolabel which was not released by any of these procedures.

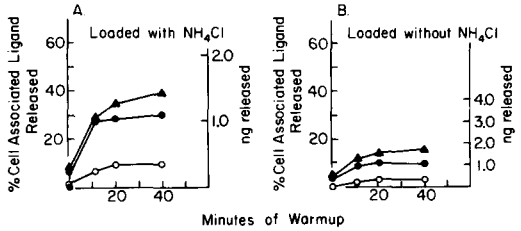

FIGURE 7 Release of ligand from cells by EGTA in the presence or absence of NH_4Cl. Cells were loaded with ^{125}I β-glucuronidase by incubating cells (4.1×10^7/ml) with ligand (20 μg/ml, 4×10^6 cpm/μg) for 18 min at 37°C. For panel *A* the cells were loaded in MEM containing 10 mg/ml BSA, 0.03 M $NaPO_4$ and 0.045 M NH_4Cl, pH 7.5. Control cells (panel *B*), were loaded in MEM containing 10 mg/ml BSA, 0.03 M $NaPO_4$, pH 7.5. Cells were then washed three times in their respective cold buffers. Cells from each tube were then divided equally into three different tubes and taken up in one of the following buffers, each of which contained 5 mM glucose, 5 mg/ml BSA, pH 7.5. Additions to this were HBSS (○); CMF-HBSS plus 10 mM EGTA, (●); CMF-HBSS plus 0.045 M NH_4Cl and 10 mM EGTA, (▲). Cells were then warmed for the indicated periods of time, after which cell-associated radioactivity and supernatant radioactivity were separated by centrifugation. The ordinates have the same meaning as in Fig. 5.

was studied in the presence or absence of NH_4Cl. A concentration of NH_4Cl (0.045 M, pH 7.5) was chosen that gave complete inhibition of uptake after 10 min of warming in the presence of the drug. Because we were concerned about the potential cytotoxicity of NH_4Cl, cells were incubated for 20 min in 0.03–0.1 M NH_4Cl at 37°C and subsequently washed with NH_4Cl-free buffer. Uptake recovered nicely (85% of control cells treated similarly but without the NH_4Cl) and was linear with time (data not shown).

Cells were loaded with ^{125}I-β-glucuronidase as previously described, in either the presence or absence of 0.045 M NH_4Cl, pH 7.5. The cells were then cooled, washed, and transferred to one of the following stripping media: (*a*) control buffer, pH 7.5; (*b*) EGTA alone; (*c*) EGTA and NH_4Cl. Cells were then warmed for various periods of time and the TCA-precipitable radioactivity that was released into the media was measured (Fig. 7). With cells loaded in the presence of NH_4Cl (Fig. 7*A*), the presence of NH_4Cl in the release phase of the experiment made little difference in the percentage of total ligand that was released. With cells loaded in the absence of NH_4Cl, the presence of NH_4Cl in the release phase of the experiment, again, made little difference in the percentage of total ligand that was released. Obviously, NH_4Cl in the loading phase of the experiment has a large effect on the percentage of total ligand that will be released in a subsequent 37°C incubation. In all cases the absolute amount of ligand that could be stripped from the cells did not vary greatly (see Fig. 7). This suggests that NH_4Cl primarily inhibits accumulation of ligand in the nonreleasable pool.

The effect of NH_4Cl on ligand accumulation in the various pools can be seen by comparing the distribution of ligand in cells loaded in the presence or absence of NH_4Cl. Therefore, we repeated the preceding experiment, keeping cell concentration, ligand concentration, ligand specific activity, and cell sample size identical for the two loading conditions, so a comparison could be meaningful. The surface pool is defined as the amount of radioactivity released by incubation with trypsin/EGTA as previously described and with no warmup. The cycling pool is the TCA-precipitable radioactivity released by warming to 37°C in the presence of EGTA, with the surface pool subtracted. The nonreleasable pool is the remaining cell-associated radioactivity.

The results are shown in Fig. 8. Loading in the presence of NH_4Cl results in a decrease in ligand in all three pools, but the reductions in the surface and cycling pools are small compared with the reduction in the nonreleasable pool.

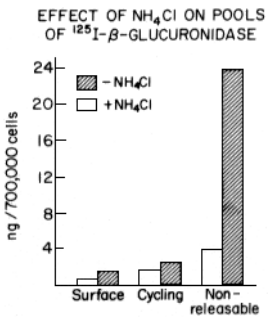

FIGURE 8 Effect of NH_4Cl on the sizes of intracellular ligand pools. The experiment in Fig. 7 was repeated, with careful attention to keeping the aliquots and cell concentrations precisely the same for all conditions. After loading the cells, they were rapidly cooled by dilution with 10 ml of cold buffer and placed in an ice-bath before washing. The stripping buffer used was CMF-HBSS plus 10 mM EGTA at pH 7.5. Cells were incubated for 30 min at 37°C in this buffer. In addition, an aliquot of cells was treated with 1 mg/ml trypsin at 4°C as previously described to determine the cell surface component. Under these conditions we determined the amount of ligand (nanograms) in each pool in cells loaded without NH_4Cl (hatched) and those loaded in 0.045 M NH_4Cl (not hatched).

DISCUSSION

We have previously proposed receptor reuse as a means of repopulating the cell surface with receptors after a round of internalization, and have supplied indirect evidence for this in mannose-specific glycoconjugate endocytosis by alveolar macrophages (8, 9). In this report we provide direct evidence for receptor cycling between the cell surface and intracellular compartments. We also offer a possible explanation of how receptor and ligand might get separated inside the cell, and of the mechanism of endocytosis inhibition by the weak bases chloroquine and NH_3. In our hands, chloroquine and NH_4Cl have qualitatively similar, if not identical, effects.

A reasonable explanation for the inhibition of receptor-mediated endocytosis by weak bases such as NH_3 can be developed from their effects upon intracellular pH. These drugs have been demonstrated to raise the pH of acid intracellular compartments (12, 13), including at least some elements of the pinosome-lysosome complex. The effect of these drugs on the lysosomal pH is rapid, taking <30 s to occur (14). Gonzalez-Noreiga (15) and co-workers have demonstrated with the mannose-6-phosphate-specific endocytosis receptor that lowering the pH enhances the rate of receptor-ligand dissociation. Their results using cultured fibroblasts in the presence of weak bases were consistent with the intracellular accumulation of the receptor-ligand complexes and the subsequent inhibition of net ligand transport into cells. They proposed that raising the lysosomal pH with weak bases was preventing dissociation of receptor-ligand complexes. This inhibited recycling of the free receptor and consequently inhibited endocytosis. Others have emphasized the potential role of lysosomal pH in ligand delivery by the galactose-specific receptor of hepatocytes (16, 17). Previous work from our laboratory has shown that weak bases (*a*) produce a time-dependent inhibition of uptake, (*b*) do not inhibit the internalization step, and (*c*) cause a reversible loss of cell surface binding by the mannose receptor of alveolar macrophages (9). Taken together, these results indicate a general mechanism for weak base action. Fig. 9 shows the minimal mechanism for a recycling receptor system such as the mannose receptor. We have shown the site of weak base action in segment III where it inhibits receptor-ligand dissociation and the return of free receptor to the cell surface. Weak bases are presumed to do this by raising the pH of acid intracellular vesicles. In the present report we have attempted to demonstrate that the dissociation of receptor-ligand complexes is pH dependent and that the return of free receptor to the cell surface is not affected by weak bases. We suspected that segment III really consisted of at least two significant intermediates as shown in Fig. 10. By measuring the recovery of cell surface binding at 37°C in the presence and absence of weak base after trypsinization at 4°C, it is possible to distinguish between the models in Figs. 9 and 10.

We have presented a number of observations on the mannose-specific receptor of alveolar macrophages demonstrating the enhancement of receptor-ligand dissociation by acid pH. In paraformaldehyde-treated cells the rapid dissociation at pH 6 and 37°C could be demonstrated directly (Fig. 4). The

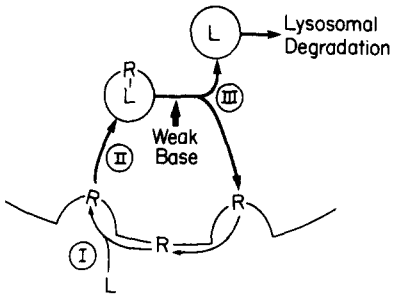

FIGURE 9 Minimal model for recycling receptor mechanism. This figure shows what we believe to be the minimal number of steps and intermediates we consider necessary for a recycling receptor mechanism. The portions of the pathway labeled with roman numerals indicate segments of a pathway and may include many individual steps.

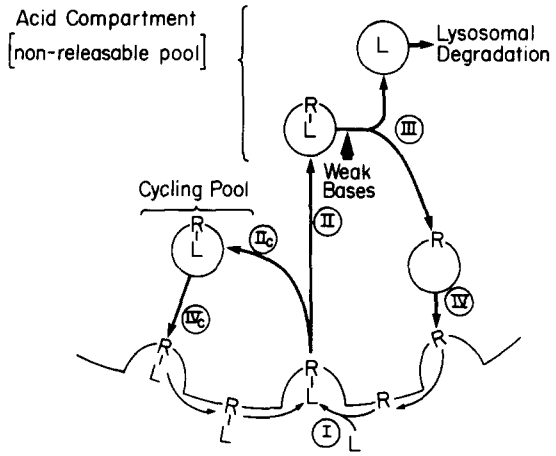

FIGURE 10 Expanded mechanism for the mannose-specific endocytosis receptor. This figure includes the free receptor pool and the cycling pool of receptors (subscript c). Once again, the labeled segments probably include many individual steps.

biphasic dissociation in the paraformaldehyde-treated cells is unexplained. But a number of observations (binding quantitatively similar to that of untreated cells, α-methylmannoside and EGTA removal of specifically bound ligand) lead us to conclude that the receptor in paraformaldehyde-treated cells is binding ligand in the normal mannose-specific fashion. In untreated cells at pH 6, the pH-dependent dissociation was demonstrated by the release of bound ligand into the media (Fig. 3). The time-course of the release at pH 6 is fast enough to permit the rate of receptor recycling required during extended periods of uptake.

Our evidence is very clear that weak bases exert their effect after the internalization step. Therefore, in Fig. 10 we have indicated the site of weak base action in the segment of the mechanism labeled III. With this simple mechanism, after trypsinization at 4°C the recovery of cell surface binding in the presence of high doses of NH_4Cl should be inhibited. Our data indicate that the rate of binding recovery is rapid in the presence of chloroquine and does not account for the observed inhibition of uptake by weak bases (see Fig. 4). Previously, we have demonstrated that incubation in NH_4Cl reversibly reduces cell surface binding by 70–80% (9). In Fig. 4 the effect of 1 mM chloroquine is to reduce cell surface binding by 40% in 2 min. If the cells were trypsinized before warming, the cell surface binding at 2 min is the same as that of the untrypsinized control and thereafter declines the same as that of the control (right panel in Fig. 4). This result is consistent with the mechanism shown in Fig. 10. In this case there is an intracellular pool of unoccupied receptors that return to the cell surface, producing the recovery of binding after trypsinization. In the presence of weak bases the movement of receptors to the cell surface from this pool is unimpeded but this pool appears to get progressively smaller upon incubation of the cells with weak base. Therefore we put the site of weak base inhibition before the formation of this pool (see Fig. 10).

Because we believe that we have good evidence that weak bases are inhibiting the intracellular dissociation of receptor complexes, we now have the opportunity to effectively label the receptor during its intracellular transit. If cells are exposed to ligand at 37°C in the presence of high concentrations of NH_4Cl, endocytosis ceases in a short period of time. When these cells are cooled and treated with trypsin-EGTA at 4°C (previously shown to remove >90% of cell surface ligand [8]), the bulk of the cell-associated ligand is not released and therefore we presume it to have been internalized. If preloaded cells are cooled and washed, and then warmed back up to 37°C, only conditions that enhance receptor-ligand dissociation (i.e. medium pH at 6, EGTA, or α-methylmannoside) cause release of intact ligand into the media (Fig. 6). This is not a consequence of general cell death. Cells appear morphologically normal after such treatment and >90% still exclude trypan blue. If the cells were subsequently washed free of NH_4Cl and/or the various stripping buffers, warmed to 37°C for a few minutes, and then tested for ligand uptake, only slight reductions in uptake were observed. Moreover, the release of ligand is not a general release of lysosomal contents because cells treated with the stripping buffers release virtually none of their β-glucuronidase or *N*-acetyl-hexosaminidase activity into the medium (data not shown).

From these results we make two conclusions: (*a*) the ligand returned to the media intact involves the return of receptor-ligand complexes to the cell surface and (*b*) receptor-ligand complexes must be continuously cycling between the cell surface and intracellular vesicles. The second conclusion also requires the observation that there is no time-dependent increase of cell surface receptor-bound ligand in the presence of NH_4Cl once the system has equilibrated (Fig. 6). In fact the cell surface pool of binding sites is depleted by 50–80% (Fig. 8) by weak bases, as we have previously shown in the absence of added ligand (9). This depletion could result from the trapping of receptor ligand complexes in the nonreleasable pool and may indicate that segregation of receptors occurs at the cell surface.

In addition, our data clearly demonstrate that not all of the ligand is returned to the media by 37°C incubation in the dissociative media. At best, ~40% of the ligand can be released in the presence of NH_4Cl. Because our data indicate that only a small fraction of receptor-ligand complexes will dissociate in 15 min at neutral pH (Fig. 4), we believe that there must be two functionally distinct intracellular pools of receptor-ligand complexes. One pool involves an acid intracellular vesicle that, in the absence of weak base, is responsible for the accumulation of ligand in excess of the cellular complement of receptors (see Fig. 6). In the absence of weak bases, ligand alone accumulates in this pool because their acid pH promotes dissociation and the resulting free receptor returns to the cell surface to participate in further endocytosis. In the presence of weak bases, dissociation in this pool is minimal and the receptor-ligand complexes in this pool cannot return to the cell surface. The

second intracellular pool of receptor-ligand complexes is not directly affected in any measurable way by the addition of weak bases. Most importantly, receptor-ligand complexes readily return to the cell surface from this pool, and we have chosen to call this the cycling pool.

Our observations are consistent with the following model (see Fig. 10) for mannose-specific receptor-mediated endocytosis in alveolar macrophages: (*a*) Receptor-ligand complexes are formed on the cell surface and internalized. (*b*) Intracellular receptor-ligand complexes are found in two functionally distinct pools. (*c*) From the cycling intracellular pool, receptor-ligand complexes can return to the cell surface intact. (*d*) Receptor-ligand complexes in the nonreleasable pool are dissociated by a mechanism requiring acid intracellular compartments. (*e*) Free receptor may return to the cell surface to participate in subsequent endocytic events. (*f*) Free ligand is retained intracellularly and is subject to proteolytic digestion.

According to this model, the addition of NH_4Cl raises the lysosomal pH, inhibiting receptor-ligand dissociation in this compartment. After a short exposure to ligand in the presence of a high concentration of weak base, the cells' entire receptor population is in the form of receptor-ligand complexes. In this situation, the cell-associated (specifically bound) ligand also approximates the cell receptor population. At this point, uptake will cease, but our data indicate that under these conditions a fraction of the receptor-ligand complexes continue to move between the surface and the cycling pools in a normal fashion (Figs. 5 and 7). After removal of this ligand in dissociating media, the remaining cell-associated receptor-ligand complexes must represent receptor-ligand complexes that do not return to the cell surface. Therefore, we propose that normally the acid pH of the nonreleasable pool is crucial (perhaps by dissociating the receptor-ligand complex) in allowing the receptor to return to the cell surface. This is the essential functional distinction between the two intracellular pools of receptor.

The biochemical basis for the two functionally distinct intracellular pools is unknown. The data we present demonstrate that two pools exist. They could be the result of anything from a random partitioning of receptors upon internalization to molecular heterogeneity in the mannose receptor population or associated proteins. We can say that there is probably little equilibration among the intracellular receptor pools. The observations that receptor-ligand complexes enter the nonreleasable pool as fast or faster than they enter the cycling pool (Fig. 6) and that release of ligand from the cycling pool does not deplete the nonreleasable pool imply that there is no significant sequential movement between these pools. Therefore we believe that the segregation must occur during or before the internalization process or in a subsequent compartment whose lifetime is very short (such as coated vesicles).

The physiologic role of mannose-specific glycoconjugate endocytosis is at present unclear and therefore the importance of this kind of regulation to cellular physiology or biochemistry is not apparent. However, the potential relevance of a similar cycling pool of receptor-ligand complexes in other receptor-mediated processes is perhaps more clear. Many of the consequences of altering the size of the cycling pool are strikingly similar to phenomena (e.g., receptor down-regulation and negative cooperativity) seen in hormone receptor and growth-factor receptor systems. It has recently been reported that down-regulation of the insulin receptor in chicken hepatocytes is actually an intracellular sequestration of receptors and includes no change in the total cellular receptor content (18). In addition, the ability to alter the rate of ligand and/or receptor degradation and the lifetime of receptor-ligand complexes is clearly relevant to these systems. We believe that the concept of a cycling pool of receptor-ligand complexes is an exciting and important one to be pursued in this context.

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