Characterization of Endocytic Compartments Using the Horseradish Peroxidase–Diaminobenzidine Density Shift Technique

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Abstract. We have employed a modification of the horseradish peroxidase (HRP)–diaminobenzidine density shift technique of Courtoy et al. (J. Cell Biol., 1984, 98:870–876) to examine the biochemical properties of the endosome. This organelle is involved in receptor recycling and the sorting of internalized receptor ligand complexes. Transferrin covalently bound to HRP was used to place peroxidase activity specifically within the endosome. The peroxidase-catalyzed polymerization of diaminobenzidine within these vesicles causes an increase in buoyant density, thus allowing them to be separated from other membranes. Using this technique we demonstrate that 125I-low density lipoprotein, 131I-epidermal growth factor, and Tf-HRP are internalized into the same endosome. We discovered that the diaminobenzidine reaction product “cross-links” the lumen of the vesicle, rendering vesicular components detergent insoluble. Furthermore, the reaction inactivates enzymatic activities associated with the endosome. Thus, the diaminobenzidine density shift procedure has limited usefulness in studies designed to isolate endosomal constituents. Nonetheless, we have found that the inactivation of enzymatic activities is confined to those endosomes that contain peroxidase. This selectivity allows us to define endosome-specific activities.

During the process of receptor-mediated endocytosis, newly internalized receptor–ligand complexes are found in a nonlysosomal, acidic, low density compartment called the endosome (7, 19, 20). Studies suggest that dissociation of receptor–ligand complexes occurs within the endosome and that the endosome is involved in receptor recycling (5). The endosome may also be the organelle in which the sorting of internalized molecules occurs. For example, some internalized ligands are transferred to lysosomes while others are recycled to the cell surface. The fact that molecules may have different eventual fates raises the question of whether these receptor–ligand complexes are internalized into the same endosome. Morphological studies suggest this possibility (6) although rigorous biochemical evidence is lacking. Little is known about the biochemical characteristics of the endosome beyond the fact that it maintains an acidic pH.

Similarly, it is unclear which subcellular organelle(s) constitutes the endocytic pathway. Morphological studies suggest that the endocytic pathway is associated with, and may be part of, the Golgi system (16, 26). To resolve this issue, and to define the constituents of the endosome, it is necessary to purify or otherwise separate the endosome from other membrane compartments.

We have modified the horseradish peroxidase (HRP)1–3,3′-diaminobenzidine (DAB) density-shift procedure developed by Courtoy et al. (3) to specifically increase the buoyant density of the endosome. The peroxidase–H2O2-catalyzed oxidation of DAB within vesicles causes a dense polymer of DAB to form within the lumen which increases the buoyant density of the vesicle. Thus, peroxidase-containing vesicles can be separated from other vesicles by density gradient centrifugation. Diferric transferrin (TF) covalently attached to HRP can be used to place peroxidase activity within the endocytic pathway. TF is particularly useful in studying this pathway because it traverses the complete endocytosis-recycling route without being transferred to the lysosome. Diferric TF binds to receptors on the cell surface and the receptor–ligand complexes are internalized via coated pits. These complexes are internalized into endosomes, where iron is released from TF. The apo-TF–TF receptor complex is recycled back to the cell surface where apo-TF dissociates from the receptor and is free to bind iron again (4, 9, 10, 15). The use of the TF–HRP conjugate allows us to specifically mark the endocytic pathway.

The density shift approach is extremely useful for determining whether different receptor–ligand complexes are internalized into the same endosome. In this paper we show that at least three different receptor–ligand complexes are internalized into the same endosome.

In an attempt to purify endosomes using the density-shift procedure we have discovered a severe limitation of the peroxidase–DAB technique. The DAB polymer formed during

1 Abbreviations used in this paper: DAB, 3,3′-diaminobenzidine; EGF, epidermal growth factor; HRP, horseradish peroxidase; TF, diferric transferrin.
the peroxidase–H₂O₂ reaction apparently cross-links and/or oxidizes the luminal contents of the endosome, rendering these vesicles insoluble in detergent. The inability to extract protein from DAB-treated vesicles reduces the usefulness of this approach to purify and analyze peroxidase-containing compartments. However, the ability to specifically inactivate endosomal constituents can be used to define activities that are included within the endosome. Using this approach we demonstrate that the enzyme leucyl-β-naphthylamidase is highly enriched in the endosome.

Materials and Methods

Cells

HeLa cells were grown on plastic culture dishes in MEM containing 10% newborn calf serum (Flow Laboratories, Inc., McLean, VA), penicillin (200 U/ml), and streptomycin (0.2 mg/ml). Cells were maintained at 37°C in a 5% CO₂ atmosphere and were subcultured by trypsinization. For some experiments, cells were incubated in serum-free media for 12 h before use.

Preparation of ¹²⁵I-Tf(Fe) and ¹²⁵I-EGF

Transferrin was saturated with iron (24). Tf and epidermal growth factor (EGF) were radiolabeled using IodoGen (Pierce Chemical Co., Rockford, IL) as described by Wiley and Cunningham (25). EGF and radiolabeled EGF were generous gifts from Dr. Steven Wiley. ¹²⁵I-LDL was a kind gift from Dr. R. G. W. Anderson.

Conjugation of HRP to Tf

The HRP-Tf conjugate was prepared by the method of Nakane and Kawaoi (14), except that the final reaction was carried out using sodium cyanoborohydride (60 min, room temperature). The final preparation was stored in PBS in the presence of 10 mg/ml BSA at −20°C.

Binding of ¹²⁵I-Tf or Tf-HRP

The binding of ¹²⁵I-Tf or Tf-HRP to cells was performed as described elsewhere (2). Removal of surface bound ligand was achieved by washing cells at 0°C with a citric acid-phosphate buffer (pH 3.8), containing 150 mM NaCl for 3 min followed by PBS (pH 7.2) for 5 min. This cycle was repeated three times.

Subcellular Fractionation

All operations were performed at 0°C. Monolayers were washed with PBS and cells removed using a rubber policeman. Cell pellets were resuspended in 0.25 M STE buffer (0.25 M sucrose in 10 mM Tris HCl [pH 7.2] and 1 mM EDTA). Cells were homogenized in a precooled, tight-fitting Dounce homogenizer using 25–30 strokes or until 80–90% of the cells were disrupted as monitored by phase-contrast microscopy. The homogenate was centrifuged at 400 g for 15 min, and the supernatant applied to 12% Percoll gradients. Unlike most internalized ligands that are directed to the lysosome, Tf and its receptor are cycled back to the cell surface. Incubation of cells at 37°C in the presence of ¹²⁵I-Tf results in distribution of the radiolabeled ligand as peak A (Figure 4A), the more dense peak as peak B. If endosomes were in equilibrium by buoyant density, the gradient profiles should be the same for all methods of loading. We found instead that the distribution of radioactivity differed depending on the method of loading. The bimodal distribution of the endosomal marker, however, was the same for each method of loading, with the peaks of radioactivity occurring in the same positions on the gradients. The acid wash procedure re-

Enzyme Analyses

Hexosaminidase (EC 3.2.1.30), galactosyltransferase (EC 2.4.1.38), and leucyl-β-naphthylamidase (EC 3.4.99) were assayed as described by Lamb et al. (12). Because Percoll interferes with colorimetric and absorbance readings, the smallest possible sample volumes were used in these reactions (usually 50 μl). In some cases, enzyme analysis was performed on samples that had been detergent solubilized and cleared of Percoll by centrifugation (see soluble receptor assays).

Soluble Receptor Assays

The method of Lamb et al. (12) was used for quantifying soluble Tf receptors. Membranes were solubilized by adding 1% Triton X-100 to a final concentration of 0.1% and BSA (20 mg/ml) to 1 mg/ml. Percoll was removed from detergent lysates by centrifugation of samples at 105,000 g for 60 min over a cushion of 35% sucrose in TE buffer.

Protein Determination in the Presence of Percoll

Protein determinations were made by the method of Vincent and Nadeau (21) using BSA (Fraction V, Sigma Chemical Co., St. Louis, MO) as a protein standard.

Results

Subcellular Fractionation

We first evaluated the separation of subcellular organelles on Percoll gradients. Unlike most internalized ligands that are directed to the lysosome, Tf and its receptor are cycled back to the cell surface. Incubation of cells at 37°C in the presence of ¹²⁵I-Tf results in distribution of the radiolabeled ligand between the cell surface and the endo-
distribution was that lysosomes occurred in the most dense region of the gradient and were well separated from both endosome peaks. The distribution of the lysosomal enzyme hexosaminidase was similar regardless of whether gradients were top- or bottom-loaded (data not shown). The trans-Golgi marker galactosyltransferase (17) coincided with endosome peak A, and plasma membrane showed a similar distribution compared with endosome peak B. The enzyme leucyl-β-naphthylamidase had the same distribution as endosomes, supporting the suggestion that this enzyme represents an endosomal marker in HeLa cells (12). Even though endosomal markers do not reach density equilibrium under these centrifugation conditions, a reasonable degree of separation among various subcellular organelles could be achieved.

**Preparation of Tf-HRP Conjugates**

Internalized 125I-Tf in peak A and the Golgi enzyme galactosyltransferase exhibited a similar distribution on 12% Percoll (Fig. 2 b). Similarly, a plasma membrane marker (vesicles from cells labeled at 0°C) had a similar centrifugation pattern to endosome peak B. These activities might merely moved >85% of the surface-bound ligand (data not shown), so neither peak represented 125I-Tf bound to plasma membrane receptors. These results suggest that there are two physically different populations of endosomes that also differ in some characteristic besides density. Pulse-chase experiments using 125I-Tf to label endosomes and chase times ranging from 1 to 15 min resulted in the same relative distributions between peaks A and B (data not shown). This suggests that within this time frame radioligand is not being processed from one compartment to the other.

Because of the relatively short centrifugation times used in these experiments, initial separation of membrane vesicles may be due to differences in size. Increasing the centrifugation time to bring the gradients to equilibrium produced a single peak of radioactivity for both top- and bottom-loaded gradients (Fig. 1 b). Although endosomes appeared to reach density equilibrium by 40 min, resolution between cellular compartments was poor. Radiolabeled plasma membrane vesicles (surface-bound radioactivity) could not be separated from endosomes (internalized radioactivity) under these conditions.

Centrifugation in 12% Percoll for 27 min, which yielded
have similar sedimentation properties or they might actually be in the same compartment. To distinguish between these possibilities we used a modification of the density shift technique developed by Courtoy et al. (3). This technique permits placement of HRP into specific intracellular compartments by covalently coupling the enzyme to an appropriate ligand. HRP-containing vesicles can then be reacted with \(\text{H}_2\text{O}_2\) and DAB. The HRP-\(\text{H}_2\text{O}_2\) oxidation reaction causes DAB to polymerize into a dense complex within the vesicle. Thus, any vesicle containing HRP will increase its buoyant density under these conditions. HRP was conjugated with Tf in order to specifically increase the density of the Tf-containing compartment and to compare the sedimentation properties of the more dense compartment with markers for other organelles.

The protein conjugation procedure of Nakane and Kawai (14) was used to couple HRP to Tf. The conjugate was prepared using an initial 3:1 molar ratio of HRP/Tf. The final ratio based on protein concentration and enzyme activity was \(\approx 1:1.5\). This value represents an approximation since the conjugation procedure affected peroxidase activity (using \(o\)-dianisidine as a substrate [18]) causing the reaction rate to slow with time (data not shown). Further analysis revealed that neither \(\text{H}_2\text{O}_2\) nor substrate was limiting during the reaction. We conclude that one of the steps used in preparing the HRP for conjugation alters enzymatic activity. We have not pursued this issue further since the enzyme retained enough activity for use. Analysis of the conjugated material by column chromatography and SDS polyacrylamide gel electrophoresis revealed products of various molecular weights. High molecular weight material was characterized by altered recycling kinetics. Presumably this material represents multimers of Tf-HRP-Tf. Conjugates with molecular weights below 200,000 were used for most experiments. Electrophoresis data suggested that a significant proportion of this material consisted of one to three molecules of HRP per molecule of Tf. Less than 5% of the protein migrated in a region corresponding to unconjugated HRP. Unconjugated HRP would be internalized by fluid phase pinocytosis. As demonstrated elsewhere (1), at the concentration used in this study, the amount of HRP taken up by pinocytosis is insignificant and has no measurable effect on these experiments.

The most critical test of the conjugate was to demonstrate that it not only bound to the Tf receptor, but participated in the normal Tf cycle. Cell-associated HRP activity was drastically reduced when cells were incubated with the conjugate and excess Tf (Fig. 3a). Furthermore the rate of loss of cell-associated peroxidase activity was similar to that for the radiolabeled ligand (Fig. 3b). This result indicated that peroxidase accumulated during incubation at 37°C in the normal Tf recycling pathway. The kinetics of loss of peroxidase activity suggest a process of exocytosis rather than degradation (\(t_a\) for degradation of HRP in HeLa cells is 14 h). Peroxidase activity was recovered in the chase media, suggesting that the conjugate was not degraded during incubation with cells.

**Density Shifting of Endosomes**

The following experiments were performed to confirm that the buoyant density of endosomes could be affected by the peroxidase reaction product and that the Tf-HRP conjugate and \(^{125}\text{I}-\text{Tf}\) were internalized into the same compartment. The most critical test of the conjugate was to demonstrate that neither \(\text{H}_2\text{O}_2\) nor substrate was limiting during the reaction. We conclude that one of the steps used in preparing the HRP for conjugation alters enzymatic activity. We have not pursued this issue further since the enzyme retained enough activity for use. Analysis of the conjugated material by column chromatography and SDS polyacrylamide gel electrophoresis revealed products of various molecular weights. High molecular weight material was characterized by altered recycling kinetics. Presumably this material represents multimers of Tf-HRP-Tf. Conjugates with molecular weights below 200,000 were used for most experiments. Electrophoresis data suggested that a significant proportion of this material consisted of one to three molecules of HRP per molecule of Tf. Less than 5% of the protein migrated in a region corresponding to unconjugated HRP. Unconjugated HRP would be internalized by fluid phase pinocytosis. As demonstrated elsewhere (1), at the concentration used in this study, the amount of HRP taken up by pinocytosis is insignificant and has no measurable effect on these experiments.

The following experiments were performed to confirm that the buoyant density of endosomes could be affected by the peroxidase reaction product and that the Tf-HRP conjugate and \(^{125}\text{I}-\text{Tf}\) were internalized into the same compartment. To determine whether different ligands were localized in the same compartment, we used a modification of the density shift technique described by Courtoy et al. (3). This technique permits placement of HRP into specific intracellular compartments by covalently coupling the enzyme to an appropriate ligand. HRP-containing vesicles can then be reacted with \(\text{H}_2\text{O}_2\) and DAB. The HRP-\(\text{H}_2\text{O}_2\) oxidation reaction causes DAB to polymerize into a dense complex within the vesicle. Thus, any vesicle containing HRP will increase its buoyant density under these conditions. HRP was conjugated with Tf in order to specifically increase the density of the Tf-containing compartment and to compare the sedimentation properties of the more dense compartment with markers for other organelles.

The following experiments were performed to confirm that the buoyant density of endosomes could be affected by the peroxidase reaction product and that the Tf-HRP conjugate and \(^{125}\text{I}-\text{Tf}\) were internalized into the same compartment.
Figure 4. Density shift of endosomes. (A) Cells were incubated at 37°C with 125I-Tf (3 × 10⁻⁹ M) in the presence or absence of Tf-HRP (4 × 10⁻⁹ M). Cells were placed at 0°C, surface-bound ligand was removed, and cells were homogenized. Homogenates were applied to 12% Percoll and centrifuged at 59,000 g for 27 min, the gradients were fractionated, and radioactivity determined. (Solid circles) Control cells; (open circles) cells incubated with Tf-HRP. (B) Fractions from the more dense peak (peak A) were pooled and incubated with DAB and H₂O₂ as described in Materials and Methods. This sample was applied to 27% Percoll and centrifuged as described above. The symbols represent samples obtained from control cells (closed circles) or cells incubated with Tf-HRP (open circles). (Solid triangles) Density. (C) Samples obtained as described above were treated in the absence of DAB, applied to 27% Percoll, centrifuged, and the distribution of radioactivity determined. (Solid circles) Control vesicles; (open circles) Tf-HRP vesicles.

Figure 5. Internalized Tf and EGF are in the same endocytic compartment. Cells were incubated at 37°C for 30 min in the presence of 125I-Tf. 131I-EGF was added for 10 min and the cells placed at 0°C. Surface ligand was removed and cells were homogenized. Homogenates were applied to 12% Percoll, centrifuged as described in Fig. 2, and radioactivity was determined (A). Material from peak A was pooled and divided into aliquots. One aliquot was incubated with DAB in the presence of H₂O₂ and centrifuged in 27% Percoll (B). The other aliquot was incubated with DAB in the absence of H₂O₂ and centrifuged as in B (C).

Figure 6. Internalized EGF, LDL, and Tf-HRP enter the same endocytic compartment. Cells were incubated for 30 min at 37°C in the presence of Tf-HRP (5 × 10⁻⁹ M). 125I-LDL, 131I-EGF, and Tf-HRP. Surface-bound ligand was removed at 0°C as previously described and the cells were homogenized. Endosomal fractions were isolated from 12% gradients. The distribution of 125I and 131I was bimodal and essentially identical (Fig. 5 a). Each peak was reacted separately with DAB and H₂O₂, and applied to a 27% Percoll gradient. The distributions of both Tf and EGF were affected by the DAB reaction, indicating that the two ligands were internalized into the same compartment (Fig. 5 b). A control experiment where H₂O₂ was left out of the reaction was also performed (Fig. 5 c). The peak of 131I is slightly more dense than the peak of 125I. It is possible that this material represents EGF in vesicles that have begun fusion with secondary lysosomes but have not separated totally from Tf. The time-dependent transition of internalized ligand from low density vesicles to vesicles with a higher buoyant density has been observed before (13).
Percoll gradients and reacted with DAB-H$_2$O$_2$. The radioactivity profiles of 35% Percoll gradients indicate that low density lipoprotein was internalized into the same compartment as both EGF and Tf (Fig. 6). Slightly higher Percoll concentrations were used in this experiment to insure that we could differentiate density shifted material from that which might have accumulated in lysosomes.

The use of two separate isotopes allowed us to further determine the specificity of the density shift procedure. Cells were incubated in the presence of $^{125}$I-EGF alone or $^{125}$I-Tf plus Tf-HRP. Vesiicles isolated from each culture were mixed, reacted with DAB-H$_2$O$_2$ and applied to 27% Percoll gradients. Under these conditions some Tf-containing vesicles exhibited a shift in density while EGF-containing vesicles did not (Fig. 7a). Cells incubated with $^{125}$I-EGF were mixed and homogenized with cells that had been incubated with $^{125}$I-Tf and Tf-HRP. Peak A was isolated and subjected to the DAB reaction. Again, there was a change in the distribution of $^{125}$I-Tf without a concomitant change in the radioactivity profile of $^{125}$I-EGF (Fig. 7b). Normally >80% of the endosomal marker shifts after the DAB treatment. In these experiments only ~50% of the label was found in the high density region of the gradient. The degree of density shift is somewhat variable. This experiment represents one of the lower degrees of shift. It is also possible that the manipulations involved inactivated some of the peroxidase. Finally, cells were incubated with $^{125}$I-Tf for 10 min at 37°C, shifted to 0°C, and incubated with Tf-HRP for 60 min. Vesicles obtained from homogenates of these cells showed no density shift when subjected to the DAB treatment (data not shown). These data indicate that only vesicles that contain Tf-HRP can be density shifted and neither the homogenization of cells nor the various reaction conditions induced vesicle fusion or a mixing of vesicular contents.

**Limitations of the Density Shift Procedure**

One of the original goals of this study was to use the density shifting technique to isolate and purify endosomal populations. Density shifted endosomal fractions from 27% Percoll gradients were analyzed on SDS-polyacrylamide gels. The results from such gels were inconclusive because little or no protein could be detected by Coomassie Brilliant Blue staining. To determine whether the density-shifted fractions actually contained significant amounts of protein we assayed trichloroacetic acid precipitable radioactivity from cells that had been labeled with [$^{35}$S]methionine. We were able to detect 3-4% of the total acid precipitable $^{35}$S in the density-shifted region of the gradient. Most of the $^{35}$S-label in these samples was found in a precipitate that formed when samples were boiled in preparation for electrophoresis. Although Percoll will precipitate under these conditions, we determined that protein was not being trapped by the Percoll precipitate by adding $^{125}$I-Tf to Percoll and boiling using the same conditions used for electrophoresis samples. We next analyzed the ability of detergent to solubilize $^{125}$I-Tf from shifted and nonshifted vesicles. Table I illustrates the ability of Triton X-100 to release internalized $^{125}$I-Tf into a high speed supernatant after incubation of vesicles with DAB-H$_2$O$_2$. Similar results were obtained if the vesicles were extracted with SDS (data not shown). The data suggest that the peroxidase-DAB reaction cross-links the luminal contents of the vesicles such that $^{125}$I-Tf cannot be detergent solubilized from the complex.

To determine whether a shift in density could be obtained without cross-linking, the amount of DAB used in the reaction was titrated. Cells were incubated to steady state at 37°C in the presence of $^{125}$I-Tf and Tf-HRP. Surface ligand was removed at 0°C, cells homogenized, and the endosomal fractions pooled from 12% Percoll gradients. Vesicles were reacted with concentrations of DAB ranging from 1.9 to 450 µg/ml. The amount of radiolabeled ligand shifted to the lower half of the gradient was compared with the amount of radioactivity that was insoluble in detergent (Table II). The effect for both density shifting and detergent solubility were concentration dependent. However, a concentration of DAB (17 µg/ml) that was still effective in producing a density shift caused a major proportion of the radiolabel to become detergent insoluble. Thus, the level of reaction sufficient to

**Figure 7. Endocytic compartments do not mix during preparation.** (A) Cells were incubated with $^{125}$I-Tf and Tf-HRP for 30 min at 37°C. An equivalent set of cells was incubated with $^{125}$I-EGF for 10 min. Surface ligand was removed at 0°C and cells were homogenized. Homogenates were applied to 12% Percoll and centrifuged as described in Fig. 2. Peak A from each gradient was pooled and the two pools mixed. This mixture was then incubated with DAB-H$_2$O$_2$ and centrifuged in 27% Percoll. (Solid circles) $^{125}$I-Tf; (open circles) $^{125}$I-EGF. (B) Cells were prepared as described in A. Cells from the two cultures were combined and homogenized together. The homogenate was incubated with DAB-H$_2$O$_2$ and applied to 27% Percoll. The figure illustrates the radioactivity profiles from the gradient. (Solid circles) $^{125}$I-Tf; (open circles) $^{125}$I-EGF.

**Table I. The DAB Reaction Affects the Detergent Solubility of Membrane Vesicle Contents**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Detergent-extractable $^{125}$I-Tf %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (−DAB)</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>Control (+DAB)</td>
<td>69.8 ± 2</td>
</tr>
<tr>
<td>Tf-HRP (−DAB)</td>
<td>68 ± 1</td>
</tr>
<tr>
<td>Tf-HRP (+DAB)</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Control/Tf-HRP mix</td>
<td>49 ± 2</td>
</tr>
</tbody>
</table>

Cells were incubated at 37°C with $^{125}$I-Tf ($1 \times 10^{-8}$ M) for 40 min in the presence or absence (control) of Tf-HRP ($5 \times 10^{-9}$ M). The cells were placed at 0°C, surface ligand was removed, and the cells homogenized. Vesicles were obtained by centrifugation over a step gradient of sucrose (see Materials and Methods). DAB reactions were carried out using control and Tf-HRP vesicles alone, or with an equal mixture of control and Tf-HRP vesicles. The DAB reaction within vesicles was stopped by the addition of Triton X-100 (final concentration 0.1%). The sample was layered over a cushion of 35% sucrose in TE buffer and centrifuged for 60 min at 100,000 g. The distribution of radioactivity was determined in the top layer and in the sucrose layer (including pellet). The data in the table represent the percentage of total radioactivity from each sample remaining in the top layer.
produce an increase in buoyant density also prevents the contents of the vesicle from being released by detergent extraction. Titrations of Tf-HRP yielded essentially the same result, i.e., concentrations of the conjugate in the media corresponding to receptor occupancies as low as 6% caused endosomal $^{125}$I-Tf to become detergent insoluble after DAB treatment (1).

Inactivation Occurs Only within Peroxidase-containing Endosomes

The fact that the cross-linking is confined to vesicles containing peroxidase was shown by mixing vesicles containing $^{125}$I-Tf and Tf-HRP with control vesicles before the addition of DAB-H$_2$O$_2$. The resultant detergent-soluble radioactivity is approximately equal to half of the sum of control and Tf-HRP values and indicates that only the contents of the peroxidase-containing vesicle become resistant to detergent solubilization (Table I [Mix]). Furthermore, if Tf-HRP vesicles were lysed by detergent before DAB treatment, all of the radioactivity remained in the high-speed supernatant (see below).

To determine whether other intracellular organelles were affected by the DAB–H$_2$O$_2$ reaction, we assayed both Tf receptor activity and selected enzymatic activities (Table III). Tf receptor activity (measured in a detergent extract of cell membranes) was significantly diminished after treatment with DAB. This measure is probably an overestimate of endosomal receptor activity since there is some background binding due to receptors from plasma membrane. The other activity that diminished after this treatment was leucyl-$\beta$-naphthylamide, a putative endosomal marker in this cell type (12). The lysosomal enzyme hexosaminidase was unaffected by DAB treatment. While these results suggest that the peroxidase–DAB inactivation is limited to endosomes, the possibility exists that naphthylamide and the Tf receptor are sensitive to oxidation or the DAB reaction product. To test this possibility, vesicles were lysed before reaction with DAB-H$_2$O$_2$. This procedure resulted in a negligible loss of Tf receptor activity. Although some naphthylamidase activity was lost by detergent lysis, lysis of Tf-HRP vesicles before DAB treatment resulted in little loss of activity. Thus, neither Tf receptors nor naphthylamidase are selectively sensitive to the DAB treatment, and the inactivation observed is confined to intact vesicles. This observation supports the suggestion that in HeLa cells leucyl-$\beta$-naphthylamidase is concentrated in the endosome.

To test the hypothesis that the DAB reaction will inactivate luminal contents of any vesicle that contains peroxidase, we performed the following experiment. HeLa cells were incubated at 37°C in media containing 2 mg/ml HRP for 12–18 h. Under these conditions, peroxidase should accumulate in lysosomes. Cells were washed extensively with cold PBS and incubated in media without HRP at 37°C for 60 min. Homogenates of cells were obtained and membrane vesicles collected. DAB reactions were performed either on intact vesicles or vesicles that had been lysed with detergent. After DAB treatment, detergent was added to all samples and hexosaminidase, as well as soluble Tf receptor activity, were assayed. The results of this experiment are shown in Table IV. Lysosomal enzyme activity was significantly diminished while endosomal Tf receptor activity was unaffected. These results confirm the hypothesis that the DAB reaction will abrogate activities associated with the luminal side of compartments containing peroxidase.

**Table III. Effect of the DAB Reaction on Selected Activities**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Control</th>
<th>Control (lysed)</th>
<th>Tf-HRP</th>
<th>Tf-HRP (lysed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfn receptor</td>
<td>95 ± 3</td>
<td>100</td>
<td>51 ± 2</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>Hexosaminidase</td>
<td>96 ± 4</td>
<td>92 ± 1</td>
<td>98 ± 1</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>Naphthylamidase</td>
<td>95 ± 5</td>
<td>53 ± 5</td>
<td>9 ± 3</td>
<td>38 ± 3</td>
</tr>
</tbody>
</table>

Cells were treated in a manner similar to those in Table I. The enzyme activities of hexosaminidase and leucyl-$\beta$-naphthylamidase were then assayed. The ability of detergent-solubilized transferrin receptors to bind ligand was also analyzed. To determine if the peroxidase–DAB reaction itself would affect activities, vesicles were lysed with detergent (0.1% Triton X-100) before the reaction (lysed). Addition of detergent did not inhibit the DAB reaction.

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Endosomes, Diaminobenzidine Density Shift

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Table IV. Localization of HRP in Lysosomes Affects Lysosomal, but Not Endosomal, Activities

<table>
<thead>
<tr>
<th>Activity</th>
<th>Control %</th>
<th>Control (lysed) %</th>
<th>HRP %</th>
<th>HRP (lysed) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexosaminidase</td>
<td>98 ± 3</td>
<td>95 ± 1</td>
<td>30 ± 3</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>Tf receptor</td>
<td>98 ± 2</td>
<td>97 ± 3</td>
<td>95 ± 3</td>
<td>97 ± 2</td>
</tr>
</tbody>
</table>

Cells were incubated at 37°C with HRP (2 mg/ml) for 12-18 h. Cells were washed extensively with PBS at 0°C and incubated for 60 min at 37°C in media without HRP. Cells were homogenized, vesicles prepared as described elsewhere, and the vesicles either lysed by the addition of Triton X-100 to a final concentration of 0.1% or left intact. These preparations were then reacted with DAB-H2O2. The DAB reaction within vesicles was stopped by adding Triton X-100 to all samples to yield a final concentration of 0.2%. Hexosaminidase and soluble transferrin receptor activity were measured in these lysates. Data are presented as the percent of maximum (control) activity.

Cells were incubated at 37°C in the presence or absence of Tf-HRP until steady state binding was reached. Cultures were then pulsed with 125I-EGF and 131I-Tf for 3 min, placed at 0°C, and washed free of unbound ligand. Cultures were then returned to 37°C in the presence or absence of Tf-HRP. At various times cultures were placed at 0°C, surface bound ligand removed, and homogenized. Endosome peak A was isolated from 12% Percoll gradients and treated with DAB-H2O2. Detergent extractable 125I and 131I were then measured (Table V). The data are presented as maximum detergent-extractable radioactivity from control samples. The results indicate that within the 3-min pulse of radioligand, some separation of Tf and EGF had already occurred. This separation continued until a maximum was reached at ~20 min. However, the degree of lysosomal transfer of EGF was less than expected based on studies with fibroblasts (13). Recycling of 125I-EGF would result in a cellular distribution similar to that of 131I-Tf and Tf-HRP. This recycling would manifest itself by the inability to detergent extract 100% of the radiolabeled EGF. Independent experiments demonstrate that in HeLa cells a significant amount of EGF is capable of recycling (data not shown). Recycling of EGF has been observed in at least one other cell type (1). This approach can therefore be used not only to observe the separation of ligands, but also to demonstrate ligand recycling.

Discussion

Morphological approaches used to define the endocytic compartment have been restricted by the lack of suitable cytochemical markers and the absence of absolute landmarks. Morphological data are difficult to quantify and suffer from artifacts associated with fixation and embedding. Subcellular fractionation techniques have also been used to characterize the endocytic compartment. Although activities associated with various cellular organelles can be quantified, fractionation techniques cannot distinguish between activities that are truly confined to the same compartment from those that merely have similar physical properties. We have modified a technique developed by Courtoy et al. (3) to specifically increase the buoyant density of the endosome. A conjugate of Tf-HRP was used to direct peroxidase activity to the endocytic compartment. Control experiments validated that the conjugate behaved as Tf. The peroxidase-catalyzed oxidation of DAB within intact vesicles resulted in a dense polymer that was restricted to those vesicles that contained peroxidase. We demonstrated that the reaction is limited to endosomes and that mixing of vesicular contents does not occur. Thus, only material that is in the same compartment with internalized Tf-HRP will exhibit an increase in buoyant density.

Percoll gradients were used to fractionate cellular homogenates. As reported by other investigators, the endocytic compartment was found in the low density region of the gradients (7, 20). The endosome was defined by internalized 125I-Tf and two distinct peaks of internalized ligand were found. The differences in centrifugation properties between the two peaks was not due solely to density differences, since different methods of loading gradients yielded different ratios between these peaks. Some separation on the gradients may be due to size and not density. We have not pursued this possibility further.

We used the density shift technique to determine whether different receptor–ligand complexes were internalized into the same compartment. Experiments using cells that had been incubated simultaneously with 125I-low density lipoprotein, 131I-EGF, and Tf-HRP revealed a concomitant increase in buoyant density for all three ligands after treatment with DAB. This result indicates that all three ligands are internalized into the same endocytic vesicle. The finding that internalized receptor–ligand complexes that have different eventual fates are internalized into the same compartment adds compelling evidence to the notion that the endosome is responsible for sorting these complexes (5, 13). The density shift technique can also be used to measure the rate at which different receptor–ligand complexes leave the endosome. Receptor–ligand complexes that become separated from the endocytic apparatus (e.g., those entering lysosomes) will no longer be affected by the DAB–H2O2 reaction.

Although useful for demonstrating whether different internalized complexes are in the same compartment, the density shift technique suffers from a major limitation. Material that has been shifted cannot be easily analyzed. Our results suggest that oxidation of DAB within vesicles causes cross-linking of the luminal contents. The DAB molecule contains four reactive amino groups. Under oxidizing conditions these groups would not be expected to limit their interactions solely to other DAB molecules. Thus, the DAB polymer is likely to include many molecules associated with the endosome. The inability to extract protein from peroxidase–DAB-treated vesicles, even after boiling in 1% SDS under...
reducing conditions, supports the idea of cross-linking. Although the technique is limited by this fact, it remains useful for analysis of the contents of the endosomal compartment. For example, one can use a method of subtraction to analyze the membrane components in density shifted vs. nonshifted gradients. An alternative method for density shifting was recently reported (8). This technique utilizes the ability to place acetylcholinesterase activity into endocytic compartments. Reaction with the modified Karnovsky–Roots incubation medium results in a dense copper- and iron-containing precipitate which increases the buoyant density of the compartment. This chemical reaction appears not to cross-link luminal contents and thus may represent a feasible approach to purifying endosomal compartments.

Perhaps the most powerful use of this technique is the ability to distinguish what proteins or activities are contained within the endosomal compartment by determining specific losses after DAB treatment. For example, the rate at which Tf and EGF become separated was measured by the ability to detergent extract 125I-EGF after incubation with DAB. Activity for the enzyme leucyl-β-naphthylamide was lost after DAB treatment, whereas the lysosomal enzyme hexosaminidase was unaltered in its activity. This result indicates that in this cell type naphthylamide has a definite endosomal association, and the lysosome is not included in the Tf-endocytosis–recycling pathway. We have also used the peroxidase–DAB technique to inactivate endosomal contents and demonstrate that unoccupied Tf receptors are internalized in HeLa cells (1). These types of studies can be easily expanded to localize activities associated with other organelles. For example, when peroxidase was placed specifically in the lysosome, only lysosomal enzyme activity was reduced after reaction with DAB-H2O2. These approaches can be combined to determine whether newly synthesized lysosomal enzymes are targeted to lysosomes via an endocytic mechanism or if fluid phase uptake is dependent upon receptor-mediated endocytosis. We are currently carrying out experiments of this nature.

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