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)–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
the peroxidase--H$_2$O$_2$ 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% CO$_2$ atmosphere and were subcultured by trypsinization. For some experiments, cells were incubated in serum-free media for 12 h before use.

**Preparation of $^{125}$I-Tf(Fe) and $^{125}$I-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. $^{125}$I-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 Kawai (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 $^{125}$I-Tf or Tf-HRP**

The binding of $^{125}$I-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 3 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 600 g for 10 min, and the supernatant applied to a 12% Percoll gradient centrifuged at 59,000 g~ for 27 min. The distribution of internalized $^{125}$I-Tf on Percoll gradients is shown in Fig. 1 a. The homogenate was either bottom loaded with the peaks of radioactivity occurring in the gradient with a bimodal distribution. For simplicity, we refer to the more dense peak as peak A, and the lighter peak as peak B.

**Diaminobenzidine Treatment**

The DAB treatment described by Courtoy et al. (3) was used with minor modifications. DAB solutions were prepared at a concentration of 3 mg/ml in 0.25 M STE and the pH was carefully adjusted to 7.2 with 30 N NaOH. This solution was filtered through a 0.45-μm filter (Millipore/Continental Water Systems, Asby, MA) and protected from light at 0°C. The final reaction mixture contained 0.45 mg/ml DAB and 0.003% H$_2$O$_2$. Reactions were performed by adding the DAB solution to vesicles and incubating at room temperature for 15 min. H$_2$O$_2$ (0.3%) was then added and the mixture incubated for 15 min. Reaction mixtures were gently rocked in plastic snap-cap tubes covered with aluminum foil. Some DAB-endosomal inactivation studies were carried out on crude membrane preparations obtained by centrifuging the 800 g supernatant over a sucrose step gradient consisting of the supernatant underlayered with 13% sucrose (wt/vol) in 10 mM Tris HCl, pH 7.2, 1 mM EDTA (TE buffer) and finally with 35% sucrose in the same buffer. These gradients were centrifuged at 70,000 g~ for 40 min and membranes were collected at the 13--35% sucrose interface.

**Enzyme Analyses**

Hexosaminidase (EC 3.2.1.30), galactosyltransferase (EC 2.4.1.38), and leucyl-β-naphthylamidase (EC 3.4.9.99) 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% (wt/vol) 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 $^{125}$I-Tf results in distribution of the radiolabeled ligand between the cell surface and the endocytic pathway. Surface bound ligand can be selectively removed by washing cells at 0°C with isotonic citric acid--phosphate buffer (pH 3.8) alternating with PBS (see Materials and Methods), leaving internalized ligand as the only source of cell-associated radioactivity. Greater than 95% of surface-bound ligand can be removed using this procedure (data not shown). Endosomes were marked by internalized $^{125}$I-Tf and separated from other subcellular organelles by applying cellular homogenates to Percoll gradients. The distribution of internalized $^{125}$I-Tf on a 12% Percoll gradient centrifuged at 59,000 g~, for 27 min is shown in Fig. 1 a. The homogenate was either bottom loaded in 12.5% (wt/vol) sucrose, top loaded in 9% Percoll, or brought to 12% Percoll and mixed throughout the centrifuge tube before centrifugation. All of these procedures gave rise to similar gradients of Percoll as measured by refractive index. Under these conditions internalized ligand was distributed in the gradient with a bimodal distribution. For simplicity, we refer to the more dense peak as peak A, and the less 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-
Figure 1. Distribution of internalized $^{125}$I-Tf in 12% Percoll gradients. (A) Cells were incubated at 37°C for 30 min in media containing $5 \times 10^{-5}$ M $^{125}$I-Tf. Surface-bound ligand was removed at 0°C and the cells homogenized. Homogenates were either top loaded in 9% Percoll, bottom loaded in 12.5% sucrose, or brought to 12% Percoll and mixed throughout the Percoll before centrifugation. Samples were applied to 12% Percoll and gradients were prepared by centrifuging at 59,000 g for 27 min. Gradients were formed by centrifuging at 59,000 g for 27 min. Gradients were collected by pumping from the bottom of the centrifuge tube. Bottom load (open circles); top load (open triangles); mixed (solid circles); density (solid squares). (B) Cell homogenates were prepared as described above and applied to 12% Percoll. Samples were applied either to the top or the bottom of the tube and centrifuged at the same g-force as above, but for a period of 40 min. Plasma membrane was labeled by incubating cells at 0°C for 60 min with $^{125}$I-Tf. Homogenates of these cells were applied to the bottom of the tube before centrifugation. 37°C label, bottom load; 0°C label, top load (solid triangles); 0°C label, bottom load (open triangles).

moved >85% of the surface-bound ligand (data not shown), so neither peak represented $^{125}$I-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 $^{125}$I-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 a bimodal endosome distribution, was also effective in separating other organelles. Several marker enzymes were used to localize lysosomes and trans-Golgi elements across the gradient (Fig. 2, a and b). The most notable feature of these gradients 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-$\beta$-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 $^{125}$I-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...
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 H$_2$O$_2$ and DAB. The HRP-H$_2$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 Kawaoi (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 ~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 H$_2$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. 3 a). Furthermore the rate of loss of cell-associated peroxidase activity was similar to that for the radiolabeled ligand (Fig. 3 b). This result indicated that peroxidase accumulated during incubation at 37°C participated 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 $^{125}$I-Tf were internalized into the same compartment. Cells were incubated at 37°C in the presence of both $^{125}$I-Tf and TF-HRP. Surface-bound ligand was removed at 0°C, cells were homogenized, and the homogenate applied to 12% Percoll gradients (Fig. 4 a). Peak A was collected and incubated with DAB in the presence or absence of H$_2$O$_2$. This sample was then applied to a 27% Percoll gradient. There was a significant increase in the buoyant density of TF-HRP containing vesicles incubated with H$_2$O$_2$ and DAB (Fig. 4 b). The buoyant density of vesicles from cells not incubated with TF-HRP was unaffected by the DAB treatment, and TF-HRP-containing vesicles did not increase their density if H$_2$O$_2$ was left out of the reaction mixture (Fig. 4 c). If vesicles containing TF-HRP were mixed with vesicles containing only $^{125}$I-Tf, there was no increase in density of the $^{125}$I-Tf-containing vesicles after DAB treatment (data not shown). These data demonstrate that only vesicles that contained peroxidase activity exhibited an increase in density. Similar results were obtained using vesicles isolated from peak B. We have observed shifts ranging from 60 to 90% of internalized radioligand. This variability may represent damage to endosomes during preparation.

**Internalization of Different Receptor-Ligand Complexes**

Morphological studies have demonstrated that ligands bound to different receptors enter the endosome very soon after internalization and that different ligand receptor complexes initially utilize the same endocytic pathway (6). In addition, biochemical studies have shown that different ligands exhibit similar subcellular distributions, suggesting that they are localized in the same compartment (13). We used the density shift technique to determine whether different ligands were in the same or different compartments. Cells were incubated at 37°C.
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Percoll gradients and reacted with DAB-H2O2. 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 [125I]-EGF alone or [125I]-Tf plus Tf-HRP. Vesicles isolated from each culture were mixed, reacted with DAB-H2O2 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. 7 a). Cells incubated with [125I]-EGF were mixed and homogenized with cells that had been incubated with [125I]-Tf and Tf-HRP. Peak A was isolated and subjected to the DAB reaction. Again, there was a change in the distribution of [125I]-Tf without a concomitant change in the radioactivity profile of [125I]-EGF (Fig. 7 b). 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 [125I]-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 tri- chloroacetic acid precipitable radioactivity from cells that had been labeled with [35S]methionine. We were able to detect 3-4% of the total acid precipitable 35S in the density-shifted region of the gradient. Most of the 35S-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 125I-Tf to Percoll and boiling using the same conditions used for electrophoresis samples. We next analyzed the ability of detergent to solubilize 125I-Tf from shifted and nonshifted vesicles. Table I illustrates the ability of Triton X-100 to release internalized 125I-Tf into a high speed supernatant after incubation of vesicles with DAB-H2O2. 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 125I-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 125I-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 (7 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 125I-Tf and Tf-HRP for 30 min at 37°C. An equivalent set of cells was incubated with 125I-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 A. Cells from the two cultures were combined and homogenized together. The homogenate was incubated with DAB-H2O2 and applied to 27% Percoll. The figure illustrates the radioactivity profiles from the gradient. (Solid circles) 125I-Tf; (open circles) 125I-EGF.

| Sample                  | Detergent-extractable 125I-Tf (%) |
|-------------------------|-----------------------------------|
| Control (−DAB)          | 70 ± 2                             |
| Control (+DAB)          | 69.8 ± 2                           |
| Tf-HRP (−DAB)           | 68 ± 1                             |
| Tf-HRP (+DAB)           | 14 ± 3                             |
| Control/Tf-HRP mix     | 49 ± 2                             |

Cells were incubated at 37°C with 125I-Tf (1 × 10⁻⁴ M) for 40 min in the presence or absence (control) of Tf-HRP (5 × 10⁻⁴ 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.
Activity Control Control (lysed) Tf-HRP Tf-HRP (lysed)
Tf receptor 95 ± 3 100 51 ± 2 91 ± 5

were lysed with detergent (0.1% Triton X-100) before the reaction (lysed). Addition of detergent did not inhibit the DAB reaction.

Cells were incubated at 37°C in the presence of [125I]-Tf (2 x 10^{-9} M) and Tf-HRP (5 x 10^{-9} M) until steady state binding was reached. Surface-bound ligand was removed at 0°C and cells were harvested and homogenized. The low speed supernatant was applied to a 12% Percoll gradient and centrifuged as described in Materials and Methods. Endosome peaks A and B were isolated from fractionated gradients and incubated with the indicated concentrations of DAB. The DAB-treated material was divided in half and either centrifuged on 27% Percoll gradients or detergent extracted with 1% SDS. The data in the table represent either the percent of total radioactivity shifted to the lower half of the 27% Percoll gradient or the percent of radioactivity that could be pelleted after detergent extraction.

| Concentration DAB (µg/ml final) |
|-------------------------------|
| 450  | 150  | 50  | 17  | 5.5  | 1.9  | 0  |
| Density shifted               |
| 66 ± 2.4                     |
| 65 ± 3                       |
| 60 ± 5                       |
| 51 ± 10                      |
| 30 ± 5                       |
| 16 ± 7                       |
| 12 ± 5                       |
| Insoluble                    |
| 80 ± 2.9                     |
| 80 ± 3                       |
| 81 ± 3                       |
| 78 ± 4                       |
| 70 ± 2                       |
| 37 ± 9                       |
| 13 ± 4                       |

DAB-H_2O_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-β-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, was 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 II. Titrations of DAB**

| Concentration DAB (µg/ml final) | 450 | 150 | 50  | 17  | 5.5  | 1.9  | 0  |
|----------------------------------|-----|-----|-----|-----|------|------|----|
| Density shifted                  | 66 ± 2.4 | 65 ± 3 | 60 ± 5 | 51 ± 10 | 30 ± 5 | 16 ± 7 | 12 ± 5 |
| Insoluble                        | 80 ± 2.9 | 80 ± 3 | 81 ± 3 | 78 ± 4 | 70 ± 2 | 37 ± 9 | 13 ± 4 |

The fact that the cross-linking is confined to vesicles containing peroxidase was shown by mixing vesicles containing [125I]-Tf and Tf-HRP with control vesicles before the addition of DAB-H_2O_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 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_2O_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-β-naphthylamidase, 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 confined to endosomes, the possibility exists that naphthylamidase 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_2O_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-β-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, was 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**

| Activity         | Control     | Control (lysed) | Tf-HRP     | Tf-HRP (lysed) |
|------------------|-------------|-----------------|------------|----------------|
| Tfi receptor     | 95 ± 3      | 100             | 51 ± 2     | 91 ± 5         |
| Hexosaminidase   | 96 ± 4      | 92 ± 1          | 98 ± 1     | 99 ± 1         |
| Naphthylamidase  | 95 ± 5      | 53 ± 5          | 9 ± 3      | 38 ± 3         |

Cells were treated in a manner similar to those in Table I. The enzyme activities of hexosaminidase and leucyl-β-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.
Table IV. Localization of HRP in Lysosomes Affects Lysosomal, but Not Endosomal, Activities

| Activity          | Control | Control (lysed) | HRP     | HRP (lysed) |
|-------------------|---------|----------------|---------|------------|
|                   | %       | %              | %       | %          |
| Hexosaminidase    | 98 ± 3  | 95 ± 1         | 30 ± 3  | 74 ± 5     |
| Tf receptor       | 98 ± 2  | 97 ± 3         | 95 ± 3  | 97 ± 2     |

Cells were incubated at 37°C with HRP (2 mg/ml) for 1-2 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 125I-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 125I 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 125I-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 (11). 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, 125I-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

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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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