Internalization and Subcellular Localization of Transferrin and Transferrin Receptors in HeLa Cells*

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The subcellular location of radiolabeled transferrin (¹²⁵I-Tf), internalized during cellular iron uptake, and the cellular distribution of transferrin (Tf) receptors were studied in cultured HeLa cells. Cells were incubated at 37 °C with ¹²⁵I-Tf(Fe)₂. Forty per cent of the labeled ligand was associated with cell surface receptors. The remaining 60% was internalized as shown by the inability to dissociate ¹²⁵I-Tf from cells by competition with excess Tf(Fe)₂ or treatment of cells with 0.2 M acetic acid containing 0.5 M NaCl. Subcellular fractionation studies using sucrose density gradients indicated that internalized Tf was localized in a membranous vesicle distinct from lysosomes, Golgi apparatus, endoplasmic reticulum, or plasma membranes. The subcellular distribution of Tf receptors was studied using an assay for detergent solubilized receptors. Even without preincubation with ligand, the majority of cellular Tf receptors were localized intracellularly in a vesicle with the same buoyant density as the vesicle containing internalized ¹²⁵I-Tf. Using an assay for occupied receptors, we demonstrated that the same vesicle contained both internalized receptors and internalized ligand. A portion (20%) of the intracellular receptor pool was insensitive to trypsin treatment of whole cells at 37 °C suggesting that during the experimental time period (20-30 min) this portion did not recycle to the cell surface.

We propose that during cellular iron uptake, Tf receptor-ligand complexes are internalized and directed to a nonlysosomal compartment where iron is released, followed by recycling to the cell surface of an intact Tf receptor-apo-Tf complex.

The initial event in the transfer of iron from Tf(Fe)₂ to cells is the binding of Tf(Fe)₂ to specific, high affinity surface receptors. Following the formation of the receptor-ligand complex, cells internalize and accumulate iron. The precise nature of the steps involved in the internalization and accumulation of iron remain to be defined.

Most published data suggest that Tf(Fe)₂ is internalized, the iron is removed, and apo-Tf then is released intact from the cells (1-4). There are conflicting views, however, regarding the precise intracellular location of internalized Tf(Fe)₂ (5-7). Agents which inhibit endocytosis (5) or raise the pH of intracellular compartments prevent cellular iron accumulation (6, 8). The effect of such agents has led to the suggestion that internalized Tf(Fe)₂ is located in either a lysosomal compartment (6) or an acidic nonlysosomal compartment (7).

In this paper, we report that in cultured HeLa cells internalized Tf is localized in a unique intracellular compartment which is distinct from lysosomes, plasma membrane, or Golgi apparatus. The majority of cellular Tf receptors are localized intracellularly and are in the same compartment. Tf may reach this compartment in association with internalized Tf receptors.

EXPERIMENTAL PROCEDURES

Materials

MEM, newborn calf serum, and trypsin (1:250 porcine parvovirus tested) were purchased from Grand Island Biological Co. (Grand Island, NY). Bovine serum albumin (Sigma, fraction V), d-glucose 6-phosphate (disodium salt), p-nitrophenyl-N-acetyl-β-d-glucosamine, l-Leucine-β-naphthylamide-HCl, glucose oxidase, lactoperoxidase, and γ-globulins (human Cohn fraction II) were obtained from Sigma. All radioisotopes, 5'-[3H]AMP, [14C]UDP-Gal, and ¹²⁵I, were obtained from New England Nuclear. Human Tf was purchased from Calbiochem-Behring Corp. (San Diego, California).

Methods

Growth of Cells—HeLa cells were grown on plastic tissue culture dishes (5% CO₂) or in spinner flasks at 37 °C. Monolayers were cultured in MEM containing 10% newborn calf serum, penicillin (200 units/ml), and streptomycin (0.2 mg/ml). Spinner cultures were maintained in MEM (Joklik's modification) containing 10% calf serum and 1 mM L-glutamine.

Ligand Preparation—Tf(Fe)₂ and ¹²⁵I-Tf(Fe)₂ were prepared as described by Ward et al. (9).

Binding Studies—Binding of ligand to whole cells was performed in suspension in 1.5-ml Eppendorf tubes (unless otherwise indicated, all operations were performed at 0-4 °C). Cells were washed three times in PBS (10 mM phosphate, pH 7.2, 3 mM KCl, 140 mM NaCl) and incubated (37 or 0 °C) in 1 ml of MEM containing 4 mg ml⁻¹ of bovine serum albumin and 10 mM ¹²⁵I-Tf(Fe)₂. Nonspecific binding was determined by incubating cells under the same conditions but in the presence of 2-5 μM Tf(Fe)₂. After incubation, cells were washed three times with PBS and solubilized with 0.1% sodium dodecyl sulfate. Both cell protein and ¹²⁵I radioactivity were determined.

Enzyme Assays—Glucose-6-phosphatase, a marker enzyme for smooth endoplasmic reticulum, was assayed by a modification of the method of Nordlie and Arion (10). The final reaction mixture contained 20 mM glucose 6-phosphate, 40 mM Tris-maleate, pH 6.6, and 10 mM MgCl₂ in a final volume of 0.5 ml. The sample was incubated at 37 °C for 30 min. The reaction was terminated by the addition of...
The use of a rubber policeman. The cells were resuspended in 10 mM Tris-washed three times in PBS. Monolayer cells were harvested by the addition of 0.1% Triton X-100. The supernatant (cell extract) was diluted to a protein concentration of 2 mg/ml.

The plasma membrane marker, 5'-nucleotidase, was assayed using a modification of the procedure of Avruch and Wallach (11). Samples were incubated with 45 mM Tris-HCl, pH 9.0, 10 mM MgCl₂, 1.5 mM 5'-AMP, 3.85 mM [3H]AMP (0.035 Ci/mmole, specific activity 15 CI/mmole) in a final reaction mixture of 600 ml. After 36 min at 37°C, 0.2 ml of 0.25 M ZnSO₄ and 0.2 ml of 0.25 M Ba(OH)₂ were added sequentially at room temperature and the samples mixed after the addition of each reagent. The precipitate was removed by centrifugation at 1000 x g for 30 min. An aliquot (0.25 ml) of the supernatant was mixed with 5 ml of scintillation fluid (Aquasol 2, New England Nuclear) and counted in a Beckman LS-233 scintillation counter. A unit of enzyme activity is equivalent to 1 µmol of AMP hydrolyzed per min.

Galactosyl transferase, a marker for the Golgi apparatus, was assayed by a modification of the method of Brew et al. (12) as described by Rome et al. (13). A unit of enzyme activity is equivalent to 1 µmol of galactosyltransferase per min.

The method of Horvat et al. (14) was employed to measure the lysosomal enzyme marker hexoseaminidase. p-Nitrophenyl-N-acetyl-β-D-glucosaminide (3.15 mM) was incubated with 100 µl of a sample containing 0.1% Triton X-100. The final reaction volume was 1.0 ml. After 10 min at 37°C, the reaction was quenched with 1.0 ml of 0.2 M glycine, pH 10.5. The liberated p-nitrophenolate was measured spectrophotometrically at 420 nm. One unit of enzyme activity represents 1 µmol of substrate converted per min.

Leucyl-β-naphthylamide was assayed according to the procedure of Peters et al. (15). One unit of enzyme activity is equivalent to 1 µmol of substrate hydrolyzed per min.

Protein Determination—Protein was measured by the method of Lowry et al. (16) using bovine serum albumin as the standard.

Preparation of Cells for Sucrose Gradient Analysis—HeLa cells, grown either on monolayers or in spinner culture, were harvested and washed three times in PBS. Monolayer cells were harvested by the use of a rubber policeman. The cells were resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.2, containing 250 mM sucrose and homogenized in a tight-fitting Dounce homogenizer. The percentage of disrupted cells (generally greater than 80%) was monitored by phase-contrast microscopy. Intact cells, nuclei, and large organelles (lysosomes, mitochondria, and plasma membranes) were removed by centrifugation (200 X g, 10 min, 0-4°C).

Sucrose Density Gradients—Sucrose density gradients were prepared using a Buckler gradient maker and a peristaltic pump. All sucrose solutions (s/w) were prepared in 10 mM Tris-HCl, and 1 mM EDTA, pH 7.2. One ml of 60% sucrose was placed in the bottom of Beckman 16 × 150 mm sucrose density tubes (5.8 × 4 inches) and a linear (40-0%) sucrose gradient layered over the top. Approximately 1-2 ml of cell homogenate, prepared as described above, were carefully loaded onto the gradients. After centrifuging overnight at 100,000 X g in a Beckman SW 28 rotor at 15°C, the gradients were fractionated manually. The sucrose concentration was measured at 25°C using a Bausch and Lomb refractometer and converted to sucrose density (g/ml).

Soluble Tf Receptor Assay—HeLa cells were washed three times with PBS and then twice with 10 mM Tris-HCl, 150 mM NaCl, pH 8.0, and finally resuspended in the same buffer containing 0.1% Triton X-100. The cells were homogenized using a Brinkman Polytron (Westbury, NY) using 2 30-s bursts at setting 6-7. The supernatant obtained after centrifugation of the homogenate (20,000 X g, 30 min, 4°C) was used as a source of solubilized Tf receptors. The supernatant (cell extract) was diluted to a protein concentration of 2 mg/ml.

When assaying Tf receptor activity across a sucrose density gradient, aliquots were simply made 0.1% in Triton X-100 and mixed thoroughly to solubilize the Tf receptor and disrupt membrane-bound organelles.

The soluble transferrin receptor assay was performed by mixing and incubating samples (100 µl) with 70 µl of 20 mg/ml of bovine serum albumin in buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 10 µl of 40 nM 125I-Tf(Fe)₂ (specific activity 200-400 cpm/fmol-1), and 20 µl of PBS. Non-specific binding of 125I-Tf(Fe)₂ was estimated by substituting 20 µl of 10 mg/ml of Tf(Fe)₂ (125 µM) for PBS in the incubation mixture. After incubating receptor and ligand in 1.5-ml Eppendorf tubes at 0°C for 60 min, 100 µl of 2.0 mg/ml of IgG (γ-globulins, human Cohn fraction II) in 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl were added. This was followed immediately by the addition of 245 µl of cold, saturated (NH₄)₂SO₄ (45%). Following centrifugation in a Brinkmann Eppendorf centrifuge (5 min, 4°C) the supernatant was aspirated and the amount of 125I-Tf in the pellet determined.

Iodination of Plasma Membrane—HeLa cells were incubated for 1 h at 37°C in the presence of 1 µM Tf(Fe). After three washes in PBS at 0°C, approximately 23 X 10⁶ cells were iodinated according to a modification of the method of Hubbard and Cohn (17). The final 1.0-ml reaction mixture contained 10 milliliters of glucose oxidase, 20 µmol of glucose, 10 µg of lactoperoxidase, 1 nmol of NaI, and 10 µl of 70% (1-3 MCl). The glucose oxidase was always added last. After 40 min at 0°C, the reaction was quenched with 2 ml of Eagle's MEM. The cells were washed several times with 5.5 mM EDTA and finally with PBS. Iodinated cells were combined with unlabeled control cells which had been incubated with 1 µM Tf(Fe)₂ at 37°C for 1 h, and subjected to Dounce homogenization as described above. Protein-bound 125I was determined by measuring the trichloroacetic acid-precipitable radioactivity in the presence of 50 mM KI.

Removal of Cell Surface 125I-Tf at Low pH—HeLa cells growing on 30-mm diameter tissue culture dishes were incubated with 125I-Tf(Fe)₂ (5 nm) at 37°C for 60 min then shifted to 0°C. After four washes with 2 ml of PBS (4°C), 1.5 ml of acid-stripping solution (0.5 M HCl in 0.2 M acetic acid) was added to each plate. After 8 min at 0-4°C, the acid-stripping solution was removed and placed in counting vials. Each plate was wash added with 1 ml of acid-stripping solution. This wash was added to the initial 1.5 ml and the "acid-strippable" radioactivity in the final 2.5 ml was determined. The cells were then solubilized with 1 ml of 1% sodium dodecyl sulfate and the radioactivity, which represented "acid-resistant" Tf receptors, was determined. During acid-stripping, both cells and solutions were maintained at 4°C.

RESULTS

Evidence for Internalization of 125I-Tf(Fe)₂—Incubation of HeLa cells at 37°C for 60 min resulted in a steady state binding of 125I-Tf(Fe)₂, confirming our earlier observations (9). When high concentrations of unlabeled Tf(Fe)₂ were added to cells at 37°C a time-dependent loss of cell-associated radioactivity occurred (Fig. 1, Group C). Greater than 90% of cell-associated radioactivity was lost after 60 min with appar-
ent first order kinetics, yielding a dissociation rate constant (37 °C) of 9.24 × 10⁻² min⁻¹. When unlabeled Tf(Fe)₂ was added to cells incubated with ¹²⁵I-Tf(Fe)₂ at 0 °C (Fig. 1, Group A), the loss of radioactivity also exhibited first order kinetics. The dissociation rate constant (0 °C) was calculated to be 1.11 × 10⁻² min⁻¹. These values are similar to those we have reported previously (9). However, when cells were incubated at 37 °C with ¹²⁵I-Tf(Fe)₂ and then shifted to 0 °C in the presence of nonradioactive Tf(Fe)₂, only 40% of the cell-associated radioactivity was lost (Fig. 1, Group B). This 40% dissociated with the same kinetics as the radioactivity displaced from cells which had always been maintained at 0 °C. The retained radioactivity remained cell-associated even after 4 h at 0 °C. Upon shifting these cells to 37 °C (Fig. 1, Group B) the remaining cell-associated radioactivity was lost with kinetics which were identical to those calculated for cells maintained continuously at 37 °C. These results suggest that, following incubation of cells with ¹²⁵I-Tf(Fe)₂ at 37 °C, a component of cell-bound Tf is internalized. Shifting cells to 0 °C prevents dissociation of this component.

When HeLa cells were incubated with ¹²⁵I-Tf(Fe)₂ at 0 °C for 105 min, 92% of the cell-associated radioactivity appeared to be bound at the cell surface, since it could be removed by incubation with 0.2 M acetic acid containing 0.5 M NaCl. If binding was allowed to proceed at 37 °C for 75 min, only 26% of the cell-associated radioactivity could be removed by acid treatment. The remainder of the cell-associated radioactivity (74%) was acid-resistant and presumably located within the cell. Intracellular, cell-associated, ¹²⁵I-Tf determined in these experiments (as a percentage of total cell-associated ¹²⁵I-Tf) is in close agreement with the data obtained by the temperature shift experiments discussed previously and is similar to that reported by Karin and Mintz (5) and Bleil and Bretscher (18).

**Intracellular Localization of ¹²⁵I-Tf**—The experiments described above indicate that in the steady state approximately 60–74% of cell-associated ¹²⁵I-Tf(Fe)₂ is internalized at 37 °C. To determine the subcellular localization of this internalized Tf, we employed linear sucrose density gradients. Cells were incubated with ¹²⁵I-Tf(Fe)₂ at 37 °C for 1 h and then placed at 0 °C in the presence of unlabeled Tf(Fe)₂ (10 μM) for 3–4 h. The cells were washed, homogenized, and the homogenate applied to a sucrose density gradient (15–50%). Subcellular fractions were identified using as enzyme markers glucose-6-phosphatase (endoplasmic reticulum), galactosyl-transferase (Golgi apparatus), hexosaminidase (lysosomes), and 5'-nucleotidase (plasma membranes). Representative sucrose density gradients are shown in Fig. 2. Two peaks of ¹²⁵I radioactivity were always observed. One was a major peak of radiolabeled material which entered the gradient and the other a quantitatively minor component of apparent lower buoyant density. The lower buoyant density peak was precipitable by trichloroacetic acid but was nonsedimentable (100,000 × g, 6 h). ¹²⁵I-Tf(Fe)₂ added to cellular homogenates and then applied to a sucrose gradient exhibited a sedimentation pattern of similar low buoyant density. These observations suggest that the minor component of apparent low buoyant density represents free ¹²⁵I-Tf.

The major peak of ¹²⁵I radioactivity, with a buoyant density of 1.123 g·ml⁻¹, was sedimentable, and the radioactivity in this component could be released by detergent treatment. Thus, the major peak of radioactivity represents membrane-bound ¹²⁵I-Tf. The free ¹²⁵I-Tf was likely generated as a result of mechanical damage to cell membranes during homogenization.

Marker enzyme activities associated with the major radiolabeled peak (presumably ¹²⁵I-Tf in a vesicle) did not reveal any concordance with the peaks of activities of hexosaminidase, 5'-nucleotidase, galactosyl-transferase, or glucose-6-phosphatase. There was, however, a consistent coincidence between the peaks of activity for leucyl-β-naphthylamidase activity and ¹²⁵I-Tf. A, the distribution of hexosaminidase (■–■), leucyl-β-naphthylamidase (△–△), and ¹²⁵I radioactivity (○–○). B, the distribution of galactosyl transferase (▲–▲), 5'-nucleotidase (○–○), and glucose-6-phosphatase (†–†). C, the HeLa cells were incubated as above except that Tf(Fe)₂ was used in both the initial incubation at 37 and at 0 °C. After incubation at 0 °C for 3 h, cells were washed and plasma membrane proteins iodinated at 0 °C using the lactoperoxidase/glucose oxidase procedure (see "Experimental Procedures"). Cellular homogenates were then applied to a linear sucrose density gradient and the distribution of ¹²⁵I radioactivity (■–■) was determined.
cell extract was mixed with either Y-Tf or Tf(Fe)Z as carrier protein. The samples were centrifuged (15,000 g) and 5'-nucleotidase activity was monitored by omitting cell extract. Saturated (NH4)2SO4 (together with 125I-Tf(Fe)2, or both 125I-Tf(Fe)2 and Tf(Fe)) was added to give the concentrations illustrated by omitting cell extract. Saturated (NH4)2SO4 was added (final concentration 45%). The samples were centrifuged and the 125I radioactivity of the precipitate was determined. After 60 min, Tf(Feh (12.5 nM) was added to each sample. Triton X-100 solubilized extracts of HeLa cells were incubated at 0°C with a constant amount of either 125I-Tf(Fe)2, or both 125I-Tf(Fe)2 and Tf(Fe). After 60 min, IgG and saturated (NH4)2SO4 (final concentration 45%) were added. The samples were centrifuged and the 125I radioactivity in the precipitate was determined. Decreasing volumes (0-100 pl) of Triton X-100 extract of HeLa cells were incubated at 0°C with a constant amount of either 125I-Tf(Fe)2, or both 125I-Tf(Fe)2 and Tf(Fe). After 60 min, IgG and saturated (NH4)2SO4 (final concentration 45%) were added. The samples were centrifuged and the 125I radioactivity in the precipitate was determined. Specific binding (total binding minus nonspecific binding) is plotted in all panels.

Identification of "Internal" Tf Receptors—Karin and Mintz (5) and Ward et al. (9) have suggested the existence of a pool of intracellular Tf receptors. We developed an assay for Triton X-100 solubilized Tf receptors in order to investigate the relationship between surface Tf receptors and internal receptors, and to determine whether Tf receptors were present in the vesicle in which we detected internalized 125I-Tf. The assay capitalized on the observation that receptor-bound 125I-Tf could be specifically precipitated with (NH4)2SO4. The data in Fig. 3 and Fig. 4 define some of the properties of the assay. In the presence of a Triton X-100 extract of cells, 125I-Tf could be precipitated at a concentration of 45% (NH4)2SO4, while the precipitation of 125I-Tf(Fe)2 alone required a higher concentration (Fig. 3). This differential precipitation of 125I-Tf(Fe)2 could be completely abrogated by the inclusion of a high concentration of nonradioactive Tf(Fe). At a ligand concentration of 2 nM, the assay was linear with respect to added extract and at least 60% of the added 125I-Tf(Fe)2 was specifically precipitated (Fig. 4b). The data in Fig. 4b demonstrate that the formation of a precipitable ligand-receptor complex was time dependent, saturable, and reversible. Scatchard analysis (20) of binding of 125I-Tf(Fe)2 to Triton X-100 solubilized receptors at 0°C revealed a straight line (Fig. 4c), indicating a homogeneous population of noninteracting receptors. A Kd of 18.8 nM was determined by linear regression analysis according to the method of Scatchard (20). This value is in good agreement with that obtained for cell-associated Tf receptors (9). Thus, Triton X-100 solubilized Tf receptors appear to possess binding characteristics similar to those of cell-associated receptors described previously (9).

In order to verify the specificity of the assay for soluble receptors, we examined a Triton X-100 extract of mature red blood cells. These cells have no Tf receptors (21). No Tf binding activity could be demonstrated in the Triton X-100 extract employed. Increasing volumes (0-100 µl) of a Triton X-100 extract of HeLa cells were incubated at 0°C with a constant amount of either 125I-Tf(Fe)2, or both 125I-Tf(Fe)2 and Tf(Fe). After 60 min, IgG and saturated (NH4)2SO4 (final concentration 45%) were added. The samples were centrifuged and the 125I radioactivity in the precipitate was determined. Specific binding (total binding minus nonspecific binding) is plotted in all panels. A, time course for association and dissociation of 125I-Tf and Triton X-100 solubilized Tf receptors. 125I-Tf(Fe)2 (2 nM) was incubated (0°C) with a Triton X-100 extract of HeLa cells. At specified times, saturated (NH4)2SO4 was added to the samples to yield a concentration of 45%. The samples were centrifuged and the 125I radioactivity of the precipitate determined. After 90 min, Tf(Fe)2 (12.5 µM) was added to each sample. B, Scatchard analysis of the binding of 125I-Tf(Fe)2 to solubilized Tf receptors. Triton X-100 solubilized Tf receptors (100 µg of protein) were incubated with 125I-Tf(Fe)2 (5-50 nM) in the presence and absence of Tf(Fe)2 (26 µM). After 60 min at 0°C, (NH4)2SO4 was added (final concentration 40%) and the 125I radioactivity in the precipitate was determined. The data are plotted according to the method of Scatchard (20). The Kd, calculated by linear regression analysis, was 18.8 nM and the maximal binding capacity was 13.4 fmol of 125I-Tf µg-1 of protein. E/F, bound/free.
extract (data not shown).

Identification of a Nonrecycling Internal Pool of Receptors—Trypsinization of HeLa cells at 37 °C for 20–30 min leads to almost complete loss of Tf receptor binding activity (5% of control value) (9). However, when Triton X-100 extracts of trypsinized cells were assayed for solubilized receptor binding activity, 20–40% of total control activity was detected (Fig. 5). When Triton X-100 extracts from control and trypsinized cells were mixed, the total Tf binding activity of the mixture equaled the sum of the components. This finding demonstrates that trypsinization of whole cells has no inhibitory effect on the soluble receptor assay. These results suggest that some portion of the Triton X-100 soluble Tf receptor activity was refractory to the effects of trypsinization of whole cells.

Subcellular Localization of Tf Receptors—In order to determine the location of intracellular Tf receptors, cells were incubated in Tf-free media for 60 min at 37 °C, homogenized, applied to a sucrose gradient, and each fraction assayed for soluble Tf receptor activity. As demonstrated in Fig. 6, the peak of detectable receptor activity was coincident with leucyl-β-naphthylamidase activity with a broad shoulder in more dense fractions. When Triton X-100 was omitted from the assay mixture, only 15% of the Tf receptor binding activity could be detected (Fig. 6). This observation indicates that a large proportion of cellular Tf receptors are membrane bound and are located in a nonlysosomal, intracellular vesicle. Some of the receptors assayed probably included those on plasma membranes. To discriminate between surface receptors and internal receptors, cells were trypsinized at 0 °C, homogenized, and the homogenate applied to a sucrose gradient. Gradient fractions were assayed for soluble Tf receptor activity. Eighty-five per cent of control activity was detected, the bulk of which was coincident with leucyl-β-naphthylamidase activity (Fig. 7). A broad shoulder of Tf receptor activity was detected in higher density fractions.

To define the location of trypsin-resistant receptors, cells were trypsinized at 37 °C for 20 min, homogenized, and then applied to a sucrose gradient. Under these conditions, only 30% of control Tf receptor activity was detected using the soluble Tf receptor assay. The bulk of receptor activity was coincident with leucyl-β-naphthylamidase activity (Fig. 7). Again, a broad shoulder of Tf receptor activity was detected in higher density fractions. Thus, the trypsin-resistant recep-

**Fig. 5.** Binding of 125I-Tf(Fe)3 to Triton X-100 extracts of control cells and cells trypsinized at 37 °C. HeLa cells were incubated with trypsin (0.25%) in PBS for 20 min at 37 °C. The cells were washed with media containing 10% fetal calf serum to inactivate trypsin activity. Triton X-100 extracts of trypsinized cells and control cells were prepared. 125I-Tf(Fe)3 was added to the Triton X-100 cell extracts (0–200 μl) and binding was determined. Specific binding to extracts of control (Ο—Ο) and trypsinized cells (Ο—Ο).

**Fig. 6.** Distribution of Tf receptors on linear sucrose density gradients assayed in the presence and absence of Triton X-100. HeLa cells were homogenized and an aliquot of the homogenate applied to a linear sucrose density gradient. The gradient was fractionated and specific transferrin receptor activity was assayed in each fraction in the presence (Ο—Ο) or absence (Ο—Ο) of Triton X-100. The peak fractions of activity of hexosaminidase (*) and leucyl-β-naphthylamidase (**) are indicated.

**Fig. 7.** Distribution of Tf receptors from control cells and cells trypsinized at 37 °C and 0 °C. HeLa cells were incubated for 20 min (37 or 0 °C) with either PBS or PBS containing trypsin (0.25%). Cells were then washed with media containing 10% fetal calf serum to inactivate trypsin activity. The cells were homogenized and samples of the homogenate applied to a linear sucrose density gradient. After fractionation of the gradient, Triton X-100-solubilized Tf receptors, hexosaminidase, and leucyl-β-naphthylamidase were assayed. The peak fractions of activity of hexosaminidase (*) and leucyl-β-naphthylamidase (**) are shown. Specific receptor binding in control cells (Ο—Ο), cells trypsinized at 37 °C (Δ—Δ), or cells trypsinized at 0 °C (Ο—Ο) are illustrated.
In another set of experiments, cells were incubated at 37 °C for 1 h in the absence of any Tf. The cells were then incubated at 0 °C for 4 h in the presence of unlabeled Tf(Fe)₃ (6 μM). The leucyl-β-naphthylamidase-associated vesicle was isolated as above, and solubilized with Triton X-100. [125I]-Tf(Fe)₃ was added to the solubilized extract. (NH₄)₂SO₄ was added either immediately or after a 60-min incubation at 0 °C and the precipitable radioactivity determined. When (NH₄)₂SO₄ was added immediately, either in the presence or absence of unlabeled Tf(Fe)₃, precipitable radioactivity did not differ from background levels. When (NH₄)₂SO₄ was added after a 60-min incubation at 0 °C in the presence of added unlabeled Tf(Fe)₃, precipitable radioactivity did not differ from background levels. When (NH₄)₂SO₄ was added after a 60-min incubation at 0 °C in the absence of added unlabeled Tf(Fe)₃, a significant amount of radioactivity was precipitable. The amount of [125I]-Tf(Fe)₃ added (Fig. 8B) to the solubilized vesicular extract was identical (cpm) to the amount present in the assay mixture when vesicles from whole cells (preincubated with [125I]-Tf(Fe)₃) were used (Fig. 8A). The amount precipitated by (NH₄)₂SO₄, however, was significantly lower (compare Fig. 8, A and B). This difference likely reflects a higher concentration of both the ligand and the receptor within the vesicle recovered from cells preincubated with [125I]-Tf(Fe)₃. These results indicate that the immediate addition of (NH₄)₂SO₄ to Triton X-100 solubilized vesicles, "traps" the ligand-receptor complexes.

Examination of subcellular gradient fractions (Fig. 9) using the above assay, revealed that ligand-receptor complexes had the same buoyant density as either intracellular Tf receptors or intracellular [125I]-Tf. These results indicate that internalized Tf and cellular receptors were present in the same vesicle.

**Evidence That Internal Tf Receptors and Internalized Tf Are in the Same Vesicle**—To determine whether Tf receptors and internalized [125I]-Tf were present in the same vesicle, we designed an assay for occupied internal receptors. Cells were incubated at 37 °C with [125I]-Tf for 60 min and then shifted to 0 °C for 4 h in the presence of excess unlabeled Tf(Fe)₃. The cells were then harvested and homogenized. The homogenate was applied to a sucrose gradient. Each gradient fraction was assayed for soluble Tf and cellular receptors were present in the same vesicle. The peak fraction of internalized [125I]-Tf co-sedimenting with leucyl-β-naphthylamidase activity was collected. Samples of this fraction were mixed with either Triton X-100 (control, open bar) or Triton X-100 and excess Tf(Fe)₃ (background, hatched bar). [125I]-Tf-occupied receptors were precipitated either immediately (0 min) or after a 60-min incubation at 0 °C. The quantity of [125I]-Tf-occupied receptors precipitated is shown in both A and B. B, HeLa cells were incubated as in A but in the absence of [125I]-Tf(Fe)₃. The peak fractions of leucyl-β-naphthylamidase activity were collected. Samples of this fraction were mixed with either Triton X-100 and [125I]-Tf(Fe)₃ (control, open bar) or Triton X-100, [125I]-Tf(Fe)₃, and unlabeled Tf(Fe)₃ (background, hatched bar). In the assay mixture, the identical amount of [125I]-Tf(Fe)₃ was added as was present in the samples used in A. [125I]-Tf-occupied receptors were precipitated either immediately (0 min) or after a 60-min incubation at 0 °C.

FIG. 8. Ammonium sulfate precipitation of [125I]-Tf-occupied receptors from endocytic vesicles. A, HeLa cells were incubated in the presence of [125I]-Tf(Fe)₃ for 1 h at 37 °C and then shifted to 0 °C for 4 h in the presence of excess Tf(Fe)₃. Cells were homogenized and aliquots of the homogenate applied to linear sucrose density gradients. The peak fraction of internalized [125I]-Tf-co-sedimenting with leucyl-β-naphthylamidase activity was collected. Samples of this fraction were mixed with either Triton X-100 (control, open bar) or Triton X-100 and excess Tf(Fe)₃ (background, hatched bar). [125I]-Tf-occupied receptors were precipitated either immediately (0 min) or after a 60-min incubation at 0 °C. The quantity of [125I]-Tf-occupied receptors precipitated is shown in both A and B. B, HeLa cells were incubated as in A but in the absence of [125I]-Tf(Fe)₃. The peak fractions of leucyl-β-naphthylamidase activity were collected. Samples of this fraction were mixed with either Triton X-100 and [125I]-Tf(Fe)₃ (control, open bar) or Triton X-100, [125I]-Tf(Fe)₃, and unlabeled Tf(Fe)₃ (background, hatched bar). In the assay mixture, the identical amount of [125I]-Tf(Fe)₃ was added as was present in the samples used in A. [125I]-Tf-occupied receptors were precipitated either immediately (0 min) or after a 60-min incubation at 0 °C.

The above experiment was repeated but Triton X-100 treated vesicles were incubated at 0 °C for 60 min prior to the addition of (NH₄)₂SO₄. Under these conditions, there was an approximate 50% reduction in precipitable radioactivity (Fig. 8A). This reduction probably represents fewer occupied receptors owing to a dilution of the ligand and receptor following disruption of the vesicle. The addition of excess unlabeled Tf(Fe)₃ to the solubilized vesicle, followed by incubation at 0 °C for 60 min reduced the radioactivity detected in the (NH₄)₂SO₄ precipitate to background levels. This reduction represents the dissociation of internalized [125I]-Tf(Fe)₃ from internal receptors and subsequent binding of the excess unlabeled ligand.
Cellular Localization of Transferrin and Transferrin Receptors

DISCUSSION

The studies presented here support the concept that Tf is internalized in the process of cellular iron uptake. Internalization of Tf has been reported by others using morphologic techniques, subcellular fractionation studies, and temperature shift experiments. The evidence for internalization of Tf in our studies was derived from both temperature shift experiments (Fig. 1) and subcellular fractionation studies (Fig. 2).

Morgan (8) and Octave et al. (6) suggested that internalized Tf was localized within lysosomes. Their conclusions were based largely on the finding that agents which increase intracellular pH cause a decrease in cellular iron uptake. This finding is compatible with the fact that the release of iron from Tf is facilitated by an acidic pH (25).

We found no association between the buoyant density of internalized 125I-Tf and hexosaminidase activity, a lysosomal marker enzyme associated with all types of lysosomes; nor did we find any association between internalized 125I-Tf and marker enzymes for the Golgi apparatus (galactosyltransferase), smooth endoplasmic reticulum (glucose-6-phosphatase), or plasma membrane (5'-nucleotidase). We did, however, find coincident buoyant densities for leucyl-β-naphthylamidase activity and intracellular 125I-Tf. It was initially suggested that leucyl-β-naphthylamidase activity was a marker for plasma membranes (19). However, we observed a clear separation between the buoyant density of leucyl-β-naphthylamidase activity and that of 5'-nucleotidase activity (Fig. 2). When HeLa cell plasma membranes were iodinated at 0 °C and fractionated on sucrose gradients, the buoyant density of the 125I-labeled membrane fraction was the same as that of 5'-nucleotidase activity. There was no coincidence between the peak fractions of 125I radioactivity and leucyl-β-naphthylamidase activity. The precise role of leucyl-β-naphthylamidase is not known but its activity was useful as it was always coincident with intracellular vesicles containing 125I-Tf.

From our studies, we concluded that internalized 125I-Tf was localized in a nonlysosomal compartment. Van Renswoude et al. (7), using erythroleukemia cells, fractionated on colloidal silica gradients, also concluded that internalized Tf was localized in a nonlysosomal compartment. We cannot exclude the possibility that internalized 125I-Tf was localized in a minor lysosomal species.

The results of our studies with an assay for solubilized Tf receptors demonstrate that 70-80% of cellular receptors are intracellular. This result is independent of preincubation of cells with ligand. This figure corresponds with that reported by Bleil and Bretscher (18), who localized HeLa cell Tf receptors using an antibody technique. Intracellular pools of receptors for insulin, mannose receptors, mannosyl terminal glycopolypeptides (27), α1-macroglobulin-protease complexes (28), and mannose phosphate terminal glycopolypeptides (28) have been demonstrated by others. A large proportion of the intracellular receptor pool is sensitive to trypsinization at 37 °C (Fig.5) suggesting that this population of receptors communicates with the cell surface through the endocytic pathway. There was, however, a pool of trypsin-insensitive receptors representing about 30% of the total soluble cellular receptor population. We detected no major differences in the buoyant densities of the trypsin-sensitive and trypsin-resistant receptor pools. Analysis of the equilibrium binding behavior of the total soluble cellular Tf receptor population also did not reveal any evidence for receptor heterogeneity (Fig. 4c). Deutsch et al. (26) have reported similar findings for the insulin receptors of 3T3L1 cells. They noted that 70% of cellular insulin receptors were intracellular and a significant proportion of these were protease resistant. Deutsch et al. (26) demonstrated that protease-resistant receptors under certain circumstances could repopulate the cell surface. Studies are in progress in our laboratory to extend this observation to the protease-resistant pool of Tf receptors.

Intracellular Tf receptors appeared to be located in the same vesicular compartment as internalized 125I-Tf (Fig. 8) suggesting that Tf receptor-125I-Tf(Fe3) complexes were internalized as an intact unit. As discussed previously, this vesicular compartment was distinct from lysosomes. At 37 °C, a large proportion of intracellular Tf receptors can be hydrolyzed by extracellular proteases. This demonstrates that intracellular Tf receptors are susceptible to proteolysis. Since one of the hallmarks of lysosomes is high concentrations of proteases and other hydrolyases, the above observations further indicates that intracellular Tf receptors are not localized in lysosomes.

Studies in a variety of systems have suggested that internalized ligands pass through a nonlysosomal compartment prior to localization in lysosomes (29-33). Aside from a low luminal pH (7, 34), few properties of this nonlysosomal compartment are known. We suggest that in most systems dissociation of ligand-receptor complexes occurs in this compartment. This would allow for separate fates for the ligand and the receptor. The ligand being localized in the fluid phase might be directed to lysosomes while membrane-bound receptors might recycle to the plasma membrane. In the case of Tf(Fe3)-Tf receptor complexes, the acidic pH of the nonlysosomal vesicle would promote dissociation of iron from 125I-Tf(Fe3) (25) and actually enhance binding of apo-Tf to the Tf receptor, thus preventing dissociation of the complex (7, 21, 35). This would permit both "unloading" of iron and recycling of an intact apo-Tf-Tf receptor complex.

Raising the pH of acidic intracellular compartments prevents the release of iron from Tf (8) but does not appear to prevent the recycling of Tf (8) or of the Tf receptor. However, for those ligands whose ultimate fate is the lysosome, increases in the luminal pH traps both unoccupied receptors and receptor-ligand complexes (28, 32, 36-38). These differences in response to increased vesicular pH suggest either that the Tf receptor is directed to a different acidic compartment from other receptors or that different receptors within the same compartment respond differently.

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Note Added in Proof—Subsequent to the submission of this manuscript, two recent publications (Dautry-Varsat, A., Ciechanover, A., and Lodish, H. F.; Klausner, R. D., Ashwell, G., Vanrenswoude, J., Harford, J. D., and Bridges, K. R., Proc. Natl. Acad. Sci. U. S. A. (1983) 80, 2258-2262; 2263-2266) demonstrate that following the internalization of TF, apo-TF is recycled to the cell surface via the TF receptor.

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