Relations Between the Intracellular Pathways of the Receptors for Transferrin, Asialoglycoprotein, and Mannose 6-Phosphate in Human Hepatoma Cells

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Abstract. We compared the intracellular pathways of the transferrin receptor (TfR) with those of the asialoglycoprotein receptor (ASGPR) and the cation-independent mannose 6-phosphate receptor (MPR)/insulin-like growth factor II receptor during endocytosis in Hep G2 cells. Cells were allowed to endocytose a conjugate of horseradish peroxidase and transferrin (Tf/HRP) via the TfR system. Postnuclear supernatants of homogenized cells were incubated with 3,3′-diaminobenzidine (DAB) and H_2O_2. Peroxidase-catalyzed oxidation of DAB within Tf/HRP-containing endosomes cross-linked their contents to DAB polymer. The cross-linking efficiency was dependent on the intravesicular Tf/HRP concentration. The loss of detectable receptors from samples of cell homogenates treated with DAB/H_2O_2 was used as a measure of colocalization with Tf/HRP. To compare the distribution of internalized plasma membrane receptors with Tf/HRP, cells were first surface-labeled with ^{125}I at 0°C. After uptake of surface ^{125}I-labeled receptors at 37°C in the presence of Tf/HRP, proteinase K was used at 0°C to remove receptors remaining at the plasma membrane. Endocytosed receptors were isolated by means of immunoprecipitation. ^{125}I-TfR and ^{125}I-ASGPR were not sorted from endocytosed Tf/HRP. ^{125}I-MPR initially also resided in Tf/HRP-containing compartments, however 70% was sorted from the Tf/HRP pathway between 20 and 45 min after uptake. To study the accessibility of total intracellular receptor pools to endocytosed Tf/HRP, nonlabeled cells were used, and the receptors were detected by means of Western blotting. The entire intracellular TfR population, but only 70 and 50% of ASGPR and MPR, respectively, were accessible to endocytosed Tf/HRP. These steady-state levels were reached by 10 min of continuous Tf/HRP uptake at 37°C. We conclude that 30% of the intracellular ASGPR pool is not involved in endocytosis (i.e., is silent). Double-labeling immunoelectron microscopy on DAB-labeled cells showed a considerable pool of ASGPR in secretory albumin-positive, Tf/HRP-negative, *trans*-Golgi reticulum. We suggest that this pool represents the silent ASGPR that has been biochemically determined. A model of receptor transport routes is presented and discussed.

VESICULAR intracellular transport of many macromolecules is mediated by specific membrane-bound receptors. These receptors and their respective ligands traverse various routes, depending on the subcellular source and destination of the ligands. Although much information about the routing of individual receptor species has been obtained, direct comparisons of receptor pathways have seldom been made.

Asialoglycoprotein receptor (ASGPR)[1] is found in hepatic parenchymal cells (Ashwell and Morell, 1974). It mediates the uptake of serum glycoproteins exposing terminal galactose or *N*-acetylgalactosamine residues. The physical characteristics and properties of the ASGPR have recently been reviewed (Schwartz, 1984, 1989; Breitfeld et al., 1985). After ligand binding at the plasma membrane, receptor and ligand are internalized by coated vesicles (Geuze et al., 1983*a*,*b*) with pseudo first-order kinetics (Tolleshaug et al., 1980; Bridges et al., 1982; Schwartz et al., 1982). Dissociation and segregation occurs intracellularly (Bridges et al., 1982; Simmons and Schwartz, 1984) in a prelysosomal tubulovesicular compartment, termed CURL (Compartment of Uncoupling Receptor and Ligand) (Geuze et al., 1983*a*), or endosome. The ASGPR recycles to the plasma membrane, presumably via the tubular CURL elements, whereas the ligand is transported to the lysosomes by means of the vesicular component of CURL (Geuze et al., 1987). The re-

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1. *Abbreviations used in this paper*: ASGP, asialoglycoprotein; ASGPR, asialoglycoprotein receptor; DAB, 3,3′-diaminobenzidine tetrahydrochloride; HRP, horseradish peroxidase; MPR, mannose 6-phosphate receptor; Tf, transferrin; TfR, transferrin receptor; Tf/HRP, transferrin/horseradish peroxidase conjugate; TGR, *trans*-Golgi receptor.

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cycling pathway to the plasma membrane is largely unknown, but may involve the *trans*-Golgi reticulum (TGR) (Geuze et al., 1984*a*, 1987; van den Bosch et al., 1986; Zijderhand-Bleekemolen et al., 1987).

The transferrin receptor (TfR), which mediates the endocytosis of iron-loaded transferrin (Tf), is found in most mammalian cells types. After internalization, Tf releases iron in an acidic endosomal environment and subsequently recycles receptor bound to the plasma membrane where TfR and Tf dissociate (Dautry-Varsat et al., 1983; Klausner et al., 1983). Several studies indicate that at least part of the TfR recycling route coincides with that of the ASGPR (Ciechanover et al., 1983*a*,*b*; Stoorvogel et al., 1987). We have recently shown that the TGR is involved in the recycling of at least part of the TfR (Stoorvogel et al., 1988).

The mannose 6-phosphate receptors (MPRs) function in the transport of newly synthesized acid hydrolases to lysosomes (Sly and Fischer, 1982; Kornfeld, 1986; von Figura and Hasilik, 1986). After synthesis in the endoplasmic reticulum and transport to the Golgi complex, most lysosomal enzymes are phosphorylated in the 6-hydroxy position of one or more mannose residues. They exit the secretory route complexed to either the 270-kD cation-independent or the 46-kD cation-dependent MPR (Hoflack and Kornfeld, 1985; Stein et al., 1987*b*). Both receptors act independently and can partly substitute for one another's function (Stein et al., 1987*a*). We have previously suggested that lysosomal enzymes leave the biosynthetic route by a distinctive class of coated vesicles developing from TGR (Geuze et al., 1988). These vesicles probably mediate enzyme transport to endosomes. After dissociation, MPR escapes from the endosomes via the tubular extensions to TGR (Geuze et al., 1988), whereas the ligand enzymes are delivered to lysosomes (Sahagian and Neufeld, 1983; Willingham et al., 1983; Geuze et al., 1985; Griffiths et al., 1988). However, the possibility of transport of some of the complexes via the plasma membrane cannot be excluded, since ~10% of MPR has been detected on the plasma membrane of Hep G2 (Pfeffer, 1987) and other cells (Braulke et al., 1987; Geuze et al., 1988). This population of receptors appears to be in equilibrium with the total MPR population (von Figura et al., 1984). Recently it has been shown that the 270-kD cation-independent MPR is identical to the insulin-like growth factor II receptor (Kiess et al., 1988).

As outlined above ASGPR, TfR, and MPR mediate transport of ligands from different origins and/or with different destinations. All three receptor species, however, have been localized in TGR, endosomes, and at the plasma membrane. In previous morphological and biochemical studies we addressed questions as to whether and where these routes are interconnected. By using double-labeling immunoelectron microscopy we colocalized ASGPR and MPR in endosomes and TGR of rat liver (Geuze et al., 1984*b*). Recently we used ligands conjugated to horseradish peroxidase (HRP) to label the routes involved in ligand transport with peroxidase activity (Stoorvogel et al., 1987, 1988). It has been shown that 3,3'-diaminobenzidine (DAB) cytochemistry increases the equilibrium density of HRP-containing microsomes in a density gradient (Courtoy et al., 1984; Stoorvogel et al., 1987) and renders their contents detergent insoluble (Ajioka and Kaplan, 1987; Geuze et al., 1988; Stoorvogel et al., 1988). Thus, one can take advantage of both effects to determine the degree of colocalization of proteins with endocytosed HRP. Using this approach, we previously defined the intracellular sorting of asialoglycoprotein (ASGP) and Tf (Stoorvogel et al., 1987), demonstrated a connection between the pathways of recycling Tf and of secretion of secretory proteins in the TGR (Stoorvogel et al., 1988), and defined the accessibility of MPR and a lysosomal membrane glycoprotein to fluid-phase endocytosed HRP (Geuze et al., 1988). In the present study, we have taken advantage of this technique to compare and contrast the intracellular pathways of TfR, ASGPR, and MPR.

Materials and Methods

Materials

The human hepatoma cell line Hep G2, clone A16 (Schwartz and Rup, 1983), was cultured as described earlier (Stoorvogel et al., 1987). Rabbit antisera raised against the human ASGPR and human TfR reacted specifically with the receptors as determined by immunoprecipitation. The antiserum raised against the ASGPR showed only reaction with the H1 polypeptide (Schwartz and Rup, 1983; Bischoff and Lodish, 1987). Rabbit anti-human MPR antiserum was kindly provided by Dr. K. von Figura (Georg-August University, Göttingen, FRG). The IgG fraction of rabbit antiserum against HRP was obtained from Dakopatts (Copenhagen, Denmark). DAB was obtained from BDH Chemicals Ltd. (Poole, England). [^{35}S]Methionine and Na-^{125}I were obtained from Amersham International (Amersham, England).

Cell Iodination

To deplete cells from exogenous Tf, semiconfluent cell cultures were washed three times with MEMH (Eagle's Minimal Essential Medium supplemented with 20 mM Hepes/NaOH, pH 7.2, lacking bicarbonate) and recultured for 2 h in MEMH at 37°C in a water bath. After 1 h, the medium was replaced with fresh MEMH. The Tf-depleted cells were washed once with MEMH and twice with PBS at 0°C. The plasma membranes were ^{125}I-iodinated, using lactoperoxidase as the oxidizing enzyme, and glucose/glucose oxidase as the H_2O_2 generating system (Hubbard and Cohn, 1972). The cells were incubated for 30 min at 0°C in 1 ml PBS containing 1.7 mM $CaCl_2$, 300 μCi Na-^{125}I, 25 μg glucose oxidase (Cooper Biomedical, Inc., Malvern, PA), 50 μg lactoperoxidase (Sigma Chemical Co., St. Louis, MO), and 0.5 mg glucose on a rocker platform. The reaction was stopped by the addition of 0.5 mM tyrosine and 1 mM sodium metabisulphite. Finally, the cells were washed four times with PBS and once with MEMH.

Endocytosis of Transferrin/Horseradish Peroxidase Conjugate (Tf/HRP)

The Tf/HRP was prepared and analyzed as described (Stoorvogel et al., 1988). In brief, Tf and HRP were linked by a disulphide bond, with ~80% of the conjugation products consisting of one molecule Tf and one molecule HRP. The remaining 20% were larger complexes. The conjugate bound specifically and in a saturable manner to the TfR, and its recycling kinetics were identical to that of nonconjugated Tf.

^{125}I-labeled cells were first incubated for 30 min at 0°C on a rocker platform in MEMH containing 25 μg/ml Tf/HRP (and when indicated also nonconjugated Tf) to bind ligand to plasma membrane TfR. The cold medium was removed and the cells were further incubated in prewarmed medium containing 25 μg/ml Tf/HRP (and when indicated Tf) at 37°C. Under these conditions, recycling TfR was bound by iron-saturated ligand before reentering the cell (Stoorvogel et al., 1988).

If noniodinated cells were used, depletion of serum Tf in the growth medium was as described above. The cells were not preincubated at 0°C with Tf/HRP, but were directly incubated in Tf/HRP-containing medium at 37°C for the indicated periods of time.

Excess ligand was removed by washing the cells four times with MEMH and once with PBS containing 1 mM EDTA at 0°C. Next, cell surface proteins were removed during a 60-min incubation at 0°C on a rocker platform in 1 ml PBS, 1 mM EDTA, 0.5 mg/ml proteinase K (Boehringer-Mannheim GmbH, Mannheim, FRG). The protease activity was neutralized by adding

1 ml homogenization buffer (0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, 10 mM Hepes/NaOH, pH 7.2). The detached cells were collected and washed twice with homogenization buffer by centrifugation at 150 *g* for 5 min at 0°C. Virtually no cells were leaky, as determined by trypan blue exclusion.

DAB Cytochemistry and Receptor Detection

Cells suspended in 0.5 ml homogenization buffer were homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with a tight-fitting pestle (50 strokes). About 80% of the cells were disrupted as determined by trypan blue exclusion. Nuclei and nondisrupted cells were removed by centrifugation for 10 min at 300 *g* at 0°C. The postnuclear supernatant was mixed with an equal volume of homogenization buffer containing 60 μg/ml DAB (unless otherwise stated). The DAB solution was prepared by dilution of a freshly prepared 2 mg/ml stock solution (Stoorvogel et al., 1987). One 400-μl sample of the mixture, and a second one to which H_2O_2 (0.02%) was added were incubated for 60 min at room temperature in the dark. The reaction was stopped by adding 600 μl lysis buffer (PBS, 1% Triton X-100, 1 mM PMSF, 0.02% NaN_3, 1 mM EDTA) for ^{125}I-labeled samples, or 400 μl Laemmli sample buffer lacking reducing agents for nonlabeled samples. No lysis buffer or Laemmli sample buffer was added when Percoll density gradient centrifugation (Stoorvogel et al., 1988) was performed.

^{125}I-TfR, ^{125}I-ASGPR, and ^{125}I-MPR were separately quantitatively immunoprecipitated from 300-μl aliquots of the lysate, and analyzed by SDS-PAGE (Strous and Berger, 1982; Strous et al., 1985). The gels were fluorographed and the fluorograms were scanned with a densitometer for quantitation. The signal was linear within the range of radioactivity measured. The amount of receptor detected from the sample containing H_2O_2 was expressed as a percentage of the amount of receptor extracted from the sample lacking H_2O_2.

For detection of receptors by Western blotting, 20-μl samples were separated by SDS-PAGE. The gels were equilibrated in transfer buffer for 30 min before blotting. ASGPR was transferred to nitrocellulose sheets in a 25 mM Tris/glycine, pH 8.3, buffer containing 20% methanol in 60 min at 30 V in a Genie blotting apparatus (Idea Scientific, Corvallis, OR). Transfer of TfR and MPR to nitrocellulose sheets was attained in 16 h at 30 V in a transblot cell (Bio-Rad Laboratories, Richmond, CA) using 25 mM sodium phosphate, pH 6.5, as transfer buffer. The nitrocellulose sheets were blocked in blotto (5% milk powder [a gift from Elk, DMV Campina BV, Bergeijk, Holland], 50 mM Tris/HCl, pH 7.8, 2 mM $CaCl_2$, 0.01% antifoam [Sigma Chemical Co.], 0.05% Nonidet P-40) for 2 h at room temperature. Antisera were diluted 1:200 in blotto and allowed to react for 90 min. The nitrocellulose sheets were washed intensively with blotto and were finally incubated in blotto containing ^{125}I-protein A (0.1 μg/ml, 2×10^6 cpm/μg) for 1 h. Portions of 200 μg protein A (Sigma Chemical Co.) were iodinated in 500 μl PBS containing 1 mCi ^{125}I (Amersham International) and five iodobeads (Pierce Chemical Co., Rockford, IL) for 30 min at room temperature. Free ^{125}I was removed by chromatography on a Sephadex G-25 column equilibrated in PBS. Quantitative detection of ^{125}I-protein A was performed by scanning a fluorographed film with a densitometer. The percentage of non-cross-linked receptor was calculated as described above. The detection of HRP in cell lysates was also done by means of Western blotting after transfer in 25 mM Tris-glycine, pH 8.3.

In addition to the protease treatment of the intact cells, in some experiments the postnuclear supernatants of cell homogenates were also incubated with proteinase K, to remove receptors from disrupted vesicles. In these experiments the protease incubation of the whole cells was stopped by washing the cells in homogenization buffer lacking PMSF. The DAB cytochemistry was performed as described above, but stopped with 0.02% NaN_3, after which 0.5 mg/ml proteinase K was added. After 60 min on ice, the protease was neutralized by the addition of 1 mM PMSF. The sample was then layered on top of 3 ml 0.35 M sucrose, 1 mM EDTA, 1 mM PMSF, 10 mM Hepes/NaOH, pH 7.2, in a Beckman Instruments, Inc. (Palo Alto, CA) SW-60 tube. The microsomes were centrifuged through the sucrose cushion in 60 min at 200,000 g_{max}. The microsomal pellet was lysed and the receptors were detected either by immunoprecipitation (^{125}I-labeled cells) or Western blotting.

Electron Microscopy

Cells depleted of serum Tf were incubated for 30 min at 37°C in MEMH containing 25 μg/ml Tf/HRP. Cells were fixed and DAB cytochemistry was performed as described previously (Stoorvogel et al., 1988). The cells were scraped and embedded in 10% gelatin as described (Geuze and Slot, 1980). Ultrathin cryosections were immunodouble labeled (Geuze et al., 1981) with 10- and 15-nm protein A/gold particles (Slot and Geuze, 1985). Anti-human albumin was used as first antibody in combination with anti-ASGPR. The sections were stained (Stoorvogel et al., 1988), and embedded in methylcellulose (Slot et al., 1988).

Results

Selective Elimination of Receptors from the Plasma Membrane

This study focuses on the intracellular distribution of endocytosed TfR, ASGPR, and MPR compared to that of endocytosed Tf/HRP. We therefore had to discriminate between internalized and cell surface-associated receptors. For this purpose, we used proteinase K to digest the plasma membrane-associated proteins (Fig. 1). Digestion of cells which were surface labeled with ^{125}I at 0°C resulted in the total degradation of ^{125}I-TfR, ^{125}I-ASGPR, and ^{125}I-MPR, indicating that all plasma membrane receptors were efficiently removed (Fig. 1, lanes *1* and *2*). Control experiments were performed to check whether the procedure resulted only in the removal of plasma membrane-associated and not intracellular receptors. When both plasma membrane and intracellular receptor populations were metabolically labeled during an overnight incubation with [^{35}S]methionine, only limited amounts of [^{35}S]ASGPR and [^{35}S]TfR were accessible to proteinase K (Fig. 1, lanes *5* and *6*). Almost no [^{35}S]MPR was lost. This is consistent with the finding that only 10% of the receptor is located at the plasma membrane of Hep G2 cells (Pfeffer, 1987). To determine whether the protease was efficiently blocked before cell lysis, half of a ^{125}I-labeled, nonproteinase K-treated cell culture was mixed with an unlabeled, proteinase K-treated cell culture. No loss of signal of either receptor was observed (Fig. 1, lanes *3* and *4*). We conclude that the proteinase K treatment effectively removes plasma membrane receptors, but leaves intracellular receptors unaffected.

Receptor Cross-linking as a Function of the Intravesicular Tf/HRP Concentration

When HRP-containing microsomes are incubated in a DAB- and H_2O_2-containing buffer, intravesicular DAB polymer is formed, resulting in two different effects: (*a*) the equilibrium density of the microsomes in a density gradient is increased (Courtoy et al., 1984; Ajioka and Kaplan, 1987; Stoorvogel et al., 1987, 1988); and (*b*) the intravesicular proteins are entrapped within the DAB polymer, rendering them detergent insoluble (Ajioka and Kaplan, 1986, 1987; Stoorvogel et al., 1988; Geuze et al., 1988). The addition of DAB alone did not influence the amount of ^{125}I-receptor recovered. Only when microsomes were incubated in the presence of both DAB and H_2O_2 were ^{125}I-labeled receptors cross-linked, and consequently lost from detection at the appropriate mobility in acrylamide gels (not shown).

To study the receptor cross-linking as a function of the intravesicular HRP concentration, cells were incubated in media with constant Tf/HRP and increasing Tf concentrations. Since Tf competes with Tf/HRP for TfR binding sites, decreasing amounts of Tf/HRP were expected to be endocytosed at increasing Tf concentrations. The cells were lysed and analyzed for the presence of HRP by Western blotting

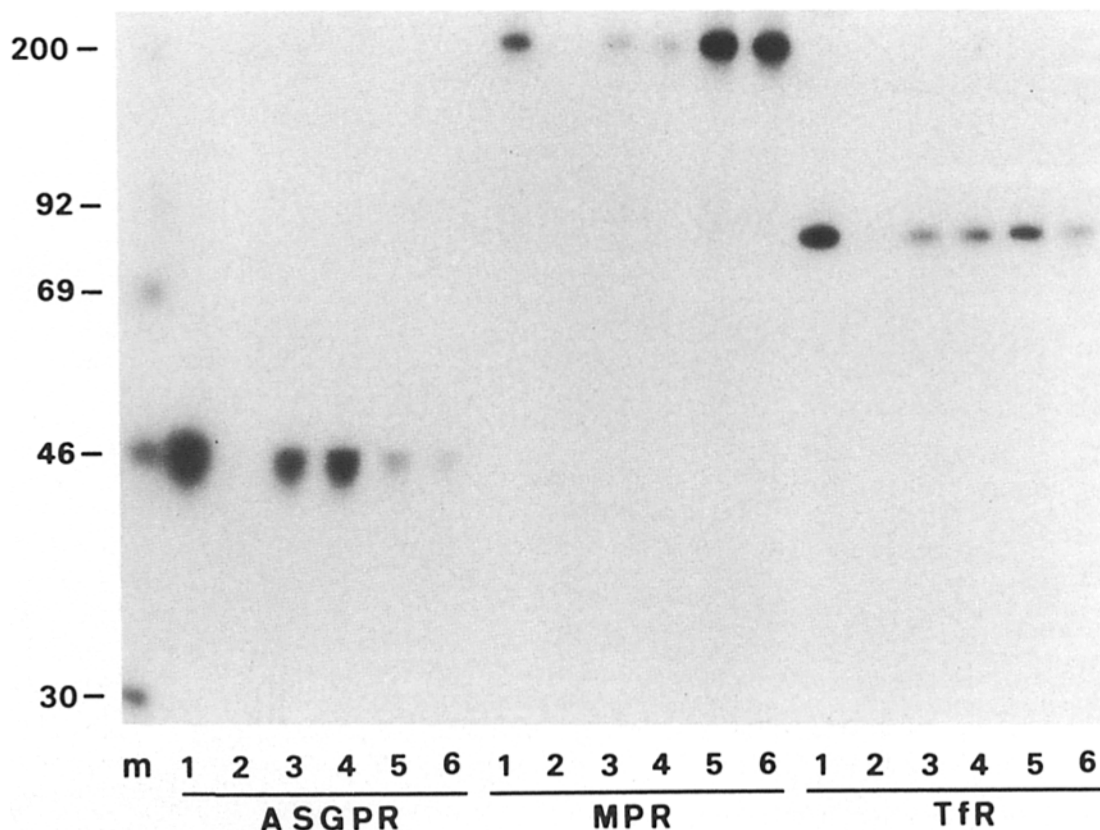


Figure 1. Digestion of receptors by proteinase K. Cells were surface labeled with ^{125}I as described in Materials and Methods (lanes *1* and *2*), or metabolically labeled with [^{35}S]methionine (lanes *5* and *6*) by incubation for 16 h in methionine-free MEM supplemented with dialyzed fetal calf serum and 60 μCi/ml [^{35}S]methionine. After labeling, the cells were suspended by scraping (lanes *1* and *5*) or proteinase K treatment (lanes *2* and *6*). Equal aliquots of a ^{125}I-labeled cell culture were mixed with a nonlabeled cell culture (lanes *3*), or with a nonlabeled, proteinase K–treated and PMSF-treated cell culture (lanes *4*). The cells were solubilized in lysis buffer, from which ASGPR, MPR, and TfR were immunoprecipitated. The immunoprecipitates were analyzed under nonreducing conditions by 10% SDS-PAGE. Molecular mass markers (lane *m*) are shown on the left in kilodaltons.

(Fig. 2). Proteins were separated by SDS-PAGE under reducing conditions. Consequently the disulfide bond of Tf/HRP was reduced, and free HRP could be quantitated. In the presence of constant Tf/HRP but increasing Tf concentrations, Tf/HRP uptake decreased to nondetectable levels. The intravesicular Tf/HRP concentration was decreased at a Tf to Tf/HRP ratio of ~0.2, confirming that Tf/HRP has a fivefold lower affinity for the TfR compared to nonconjugated Tf (Stoorvogel et al., 1988).

In a parallel experiment we used surface ^{125}I-labeled cells under the same conditions (Fig. 2). After endocytosis at 37°C, cell surface receptors were removed by proteinase K treatment leaving only internalized labeled receptors intact. The cells were homogenized and the postnuclear supernatants were subjected to DAB cytochemistry. ^{125}I-labeled TfR, ASGPR, and MPR were immunoprecipitated and quantitated. DAB/H_2O_2 mediated cross-linking of all three receptor species was about equally dependent on the intravesicular HRP concentration (Fig. 2). The addition of low concentration of Tf (3 μg/ml) to the medium, decreased the intravesicular HRP concentration by 35%, but did not affect the extent of cross-linking of the receptor species studied. Receptor cross-linking was not optimally effective if the intravesicular HRP concentration dropped below 25% of its maximal value. If cells were incubated in medium containing 25 μg/ml Tf/HRP and 3 mg/ml Tf, no cross-linking of either receptor type was observed. This demonstrates that Tf/HRP-mediated receptor cross-linking was fully TfR dependent.

The finding that only 70% of the ^{125}I-TfR could be cross-linked was surprising, since all endocytosed TfRs were expected to be associated with ligand. We next investigated whether noncross-linked ^{125}I-labeled receptors could have orginated from disrupted microsomes, with the leakage of Tf/HRP as a consequence of cell homogenization. To test this, postnuclear supernatants were incubated for an additional period with proteinase K after DAB cytochemistry. If the receptors were localized in sealed vesicular structures, it was expected that only their cytoplasmic tails would be digested. In contrast, receptors originating from leaky microsomes would be expected to be totally digested. The TfR and the H1 species of the ASGPR have NH_2-terminal cytoplasmic domains of ~7 (McClelland et al., 1984; Schneider et al., 1984) and 6 kD (Spiess et al., 1985), respectively. MPR has a 15-kD proteinase K cleavable COOH-terminal cytoplasmic extension (Von Figura et al., 1985). Proteinase K treatment resulted in slightly decreased molecular masses of all three receptor species (Fig. 3). A fraction of the total ^{125}I-label, which was located at the luminal side of sealed

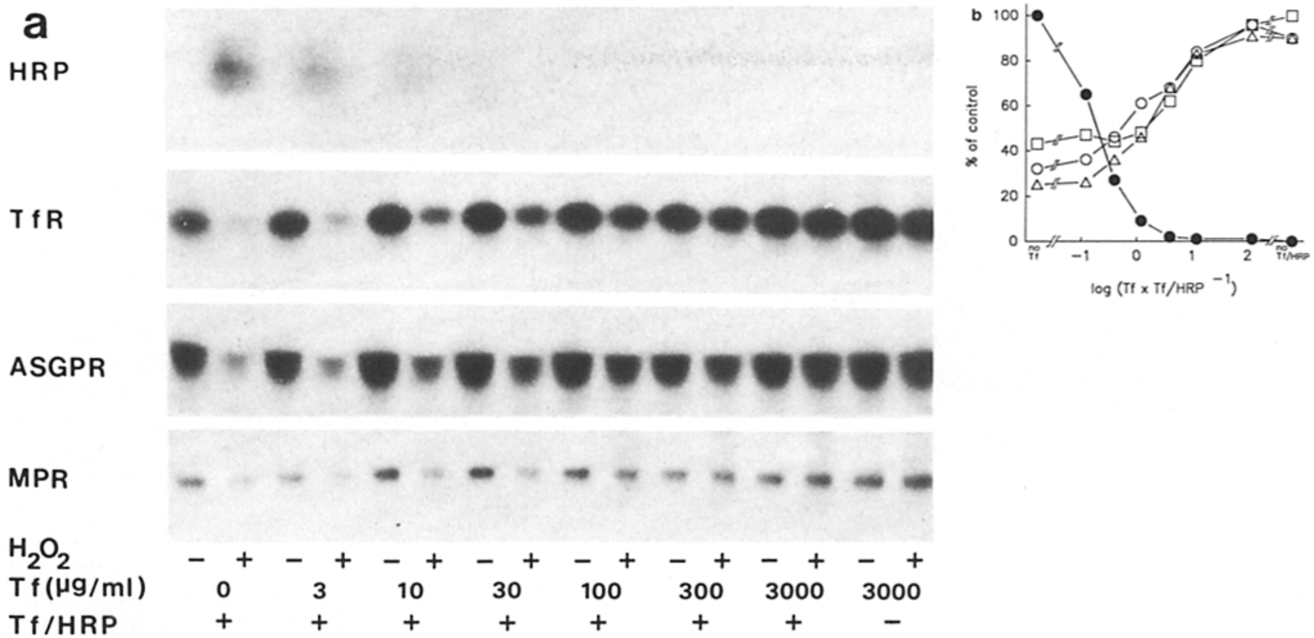


Figure 2. Receptor cross-linking as a function of the intravesicular Tf/HRP concentration. Tissue cultures were depleted of exogenous Tf and incubated for 10 min in medium containing 25 μg/ml Tf/HRP and varying Tf concentrations. One culture dish contained only 25 μg/ml Tf/HRP (no Tf) and another one only 3 mg/ml Tf (no Tf/HRP). The cells were treated with proteinase K at 0°C and lysed. HRP in the cell lysates was detected by immunoblotting (*a*), quantitated, and expressed as a percentage of its maximum value (*b*, ●). A parallel series of dishes was identically treated except that they were labeled with ^{125}I before they were incubated with ligand. After proteinase K treatment of cells and DAB cytochemistry on postnuclear supernatants, the ^{125}I-labeled receptors were immunoprecipitated and analyzed by SDS-PAGE. (○) ^{125}I-ASGPR; (△) ^{125}I-TfR; (□) ^{125}I-MPR. The amount of ^{125}I-ASGPR recovered from each sample containing H_2O_2 is expressed as a percentage of ^{125}I-ASGPR recovered from samples lacking H_2O_2.

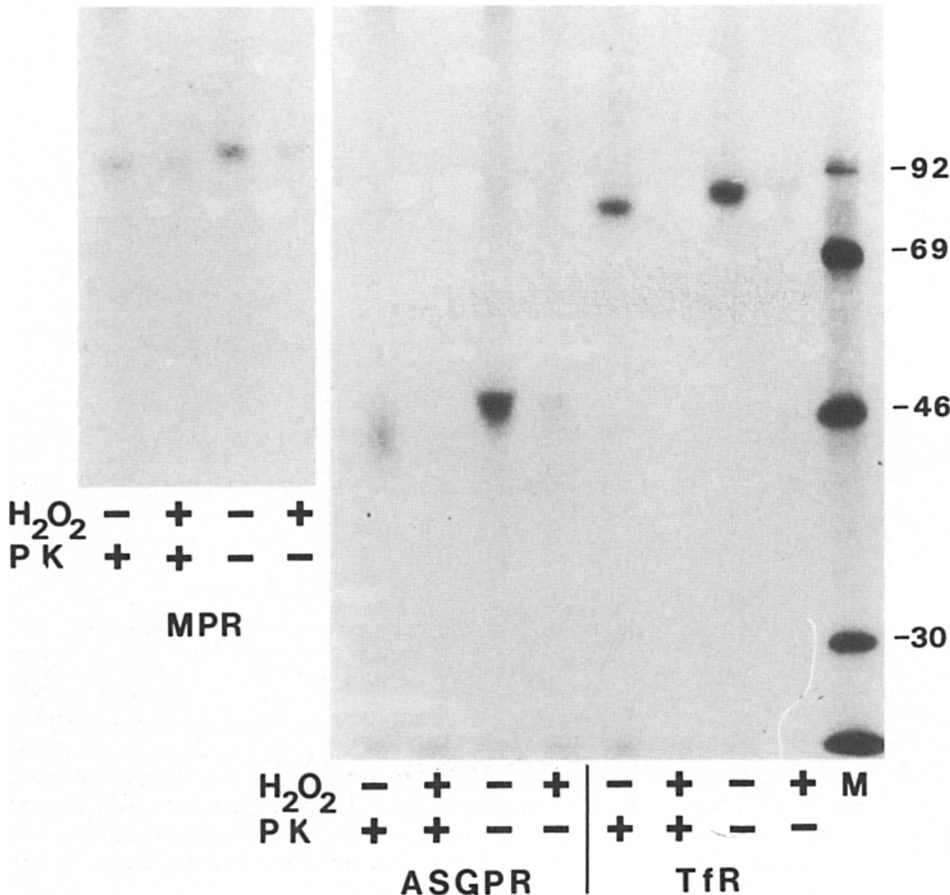


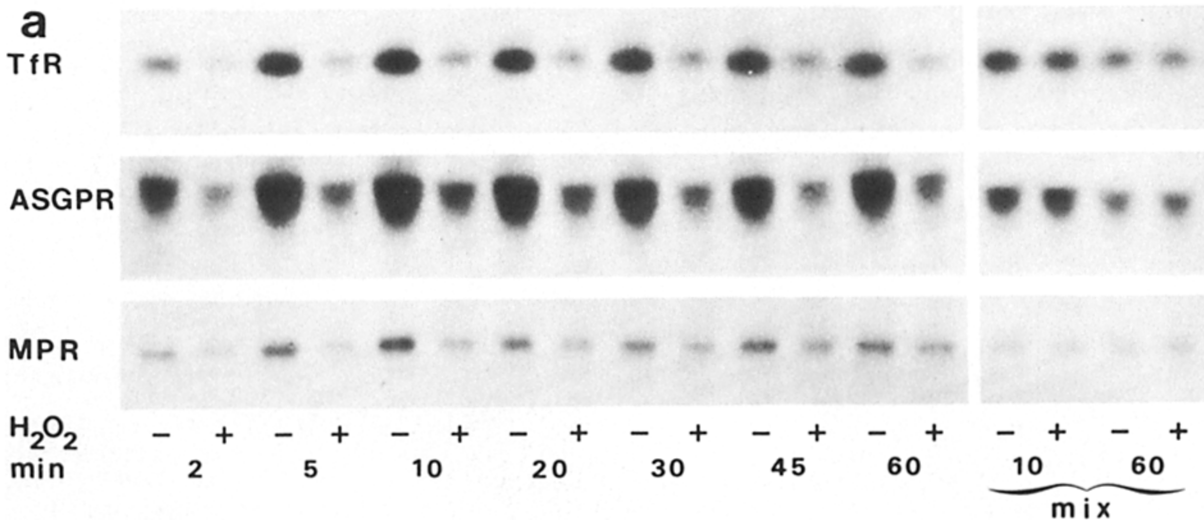
Figure 3. Proteinase K digestion of microsomes. ^{125}I-labeled cells were incubated for 30 min in Tf/HRP-containing medium. After proteinase K digestion, half of the cells were washed in the presence and half in the absence of PMSF. After DAB cytochemistry, the samples lacking PMSF were additionally incubated with proteinase K (*PK+*) which was blocked by the addition of PMSF, and the membranes were centrifuged through a sucrose cushion. The samples were analyzed for labeled receptor by immunoprecipitation and SDS-PAGE (10% polyacrylamide for ^{125}I-ASGPR and ^{125}I-TfR; 5.5% polyacrylamide for ^{125}I-MPR). Molecular mass markers (*M*) for the 10% PAGE are shown on the right in kilodaltons.

vesicles, was lost. These receptors were fully accessible to proteinase K, and were thus localized in disrupted vesicles. If DAB cytochemistry was performed before the protease treatment, neither ^{125}I-TfR and ^{125}I-ASGPR could be recovered. This demonstrates that the remaining 30% of ^{125}I-TfR and ^{125}I-ASGPR after DAB cytochemistry (Fig. 2) originated from disrupted vesicles. Thus, all intracellular ^{125}I-TfR and ^{125}I-ASGPR were located in Tf/HRP-containing structures before cell homogenization. In contrast, some ^{125}I-MPR was not affected by Tf/HRP, and was protected against proteinase K, consistent with it having been segregated from the Tf/HRP pathway (see also next section).

The Pathways of Endocytosed TfR, ASGPR, and MPR Compared to Tf/HRP

The DAB cross-linking technique allowed us to study whether, during intracellular transport, ^{125}I-TfR, ^{125}I-ASGPR, and cation-independent ^{125}I-MPR remained in the endocytic pathway of Tf/HRP (Fig. 4). First, we saturated ^{125}I-labeled cell surface TfR with Tf/HRP at 0°C. The cells were further incubated at 37°C in Tf/HRP-containing medium under conditions in which recycling TfR binds Tf/HRP before reentering the cell (Stoorvogel et al., 1988). During 60 min of endocytosis, a constant amount of 70% of the surface-labeled TfR was quenched; no sorting of Tf/HRP from its receptor could be detected. Similar results were obtained for ^{125}I-ASGPR, indicating that this receptor is also not sorted from Tf/HRP during endocytosis. However, different results were obtained for ^{125}I-MPR. During the first 20 min, similar amounts of ^{125}I-MPR, comparable to ^{125}I-TfR and ^{125}I-ASGPR, could be cross-linked. Between 20 and 45 min however, ^{125}I-MPR was gradually sorted from Tf/HRP-containing compartments. At 45 min, 80% escaped Tf/HRP-mediated cross-linking. Taking into account that 30% originated from disrupted vesicles, we calculate that ~70% (80–30/100–30) was sorted from the Tf/HRP pathway. To ascertain whether ^{125}I-labeled receptors were cross-linked if they were localized in vesicles lacking Tf/HRP, we performed control experiments, in which cell surface ^{125}I-iodination and Tf/HRP (60 min) labeling was performed in two different cell cultures. After endocytosis for 10 or 60 min and proteinase K treatment, the suspended cells were combined, homogenized, and incubated with DAB/H_2O_2. In this experiment ^{125}I-labeled receptors and Tf/HRP were located in different vesicles. As expected, DAB cytochemistry had no effect on the amount of ^{125}I-receptors detected (Fig. 4, *solid symbols*).

Under the conditions for our system, sorting of endocytosed receptors from Tf/HRP-containing compartments was only detectable if the intravesicular HRP concentration fell below 75% of its maximal value. Therefore, we may have not detected sorting of a limited amount of Tf/HRP from endocytosed ^{125}I-TfR or ^{125}I-ASGPR, if the HRP concentration in receptor-containing microsomes did not fall below the critical concentration for optimal cross-linking. We therefore repeated the experiment at a suboptimal intravesicular Tf/HRP concentration (25 μg/ml Tf/HRP and 30 μg/ml Tf in the



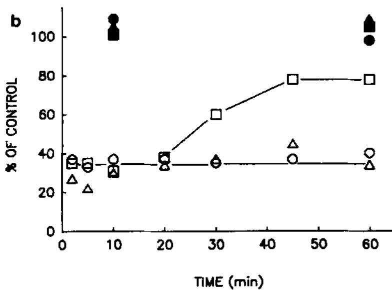


Figure 4. Distribution of endocytosed ^{125}I-TfR, ^{125}I-ASGPR, and ^{125}I-MPR compared to Tf/HRP. Cell cultures were labeled with ^{125}I and incubated at 37°C for different periods of time in Tf/HRP-containing medium. After the proteinase K treatment of the cells and incubation of the postnuclear supernatant with DAB, the three receptor species indicated were immunoprecipitated and analyzed by SDS-PAGE (*a*). (*b*) The fluorograms were quantitated and the percentages of noneffected ^{125}I-TfR (△), ^{125}I-ASGPR (○), and ^{125}I-MPR (□) were calculated as in Fig. 2. The mean values of two independent experiments are shown. Mixing experiments (see text) at 10 and 60 min (*mix*) were plotted as solid symbols: (▲) ^{125}I-TfR; (●) ^{125}I-ASGPR; (■) ^{125}I-MPR.

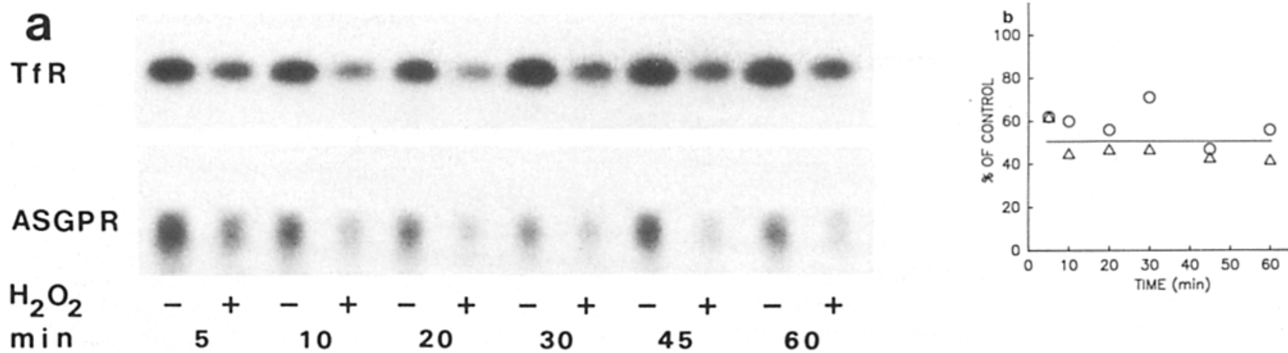


Figure 5. Receptor cross-linking in the presence of a suboptimal Tf/HRP concentration. (*a*) The experiment in Fig. 4 was repeated, except that in addition to 25 μg/ml Tf/HRP, 30 μg/ml Tf was added to the incubation medium. (*b*) (△) ^{125}I-TfR; (○) ^{125}I-ASGPR.

medium). No sorting was observed up to 60 min (Fig. 5). This demonstrates that the Tf/HRP concentration in ^{125}I-TfR- and ^{125}I-ASGPR-containing compartments remained constant during receptor recycling. Thus, we conclude that both the TfR and endocytosis-involved ASGPR follow the same endocytic pathway(s).

The Accessibility of Intracellular TfR, ASGPR, and MPR for Endocytosed Tf/HRP

To study the accessibility of the total intracellular receptor populations for endocytosed Tf/HRP, Western blotting was used in combination with the Tf/HRP-mediated receptor cross-linking technique for receptor detection (Fig. 6). Cross-linked receptors are unable to enter the acrylamide gel, and are consequently not detected on Western blots. Serum Tf-depleted cell cultures were incubated for various periods of time at 37°C in medium containing an amount of Tf/HRP sufficient to saturate TfR. Next, the cells were incubated with proteinase K at 0°C, and DAB cytochemistry was performed on postnuclear supernatants. If the cells were not incubated in Tf/HRP (0 min), DAB/H_2O_2 treatment had no effect on the amount of receptor detected. After 10 min of Tf/HRP uptake, a maximum of 70% of the intracellular TfR pool could be cross-linked. The remaining 30% originated from disrupted microsomes (see below), and was therefore not affected by Tf/HRP. These results show that the full complement of the intracellular TfR population was accessible to Tf/HRP by 10 min of continuous ligand uptake at 37°C.

Of the intracellular ASGPR pool, a maximum of only 50% was accessible to Tf/HRP. The kinetics of this process were similar to that seen with TfR. Assuming that, after cell homogenization, ~30% of the ASGPR originated from disrupted vesicles, it follows that 70% (50/70 × 100%) of intracellular ASGPR resided in Tf/HRP-containing vesicles. In contrast 100% of the endocytosed ^{125}I-ASGPR was detected in Tf/HRP-containing compartments (Fig. 4). The unaffected ASGPR (30%) cannot be explained by release from DAB polymer due to the boiling in Laemmli sample buffer; the distribution in a Percoll gradient of noncross-linked ASGPR from the DAB/H_2O_2-treated sample was similar to that of the total ASGPR (not shown), and thus did not originate from Tf/HRP, DAB polymer–containing vesicles. To assure that the unaffected ASGPR did not originate from dis-

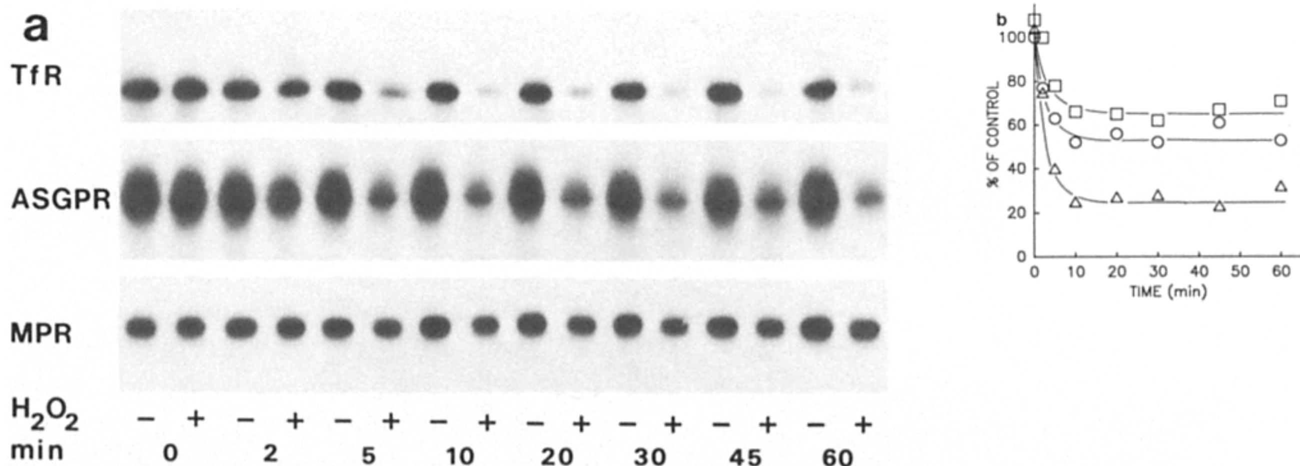


Figure 6. The accessibility of intracellular receptors for endocytosed Tf/HRP. Nonradiolabeled cell cultures were incubated for the indicated period of time in Tf/HRP-containing medium. After proteinase K treatment of whole cells, and DAB cytochemistry of the postnuclear supernatant of the homogenized cells, the membranes were lysed. Noncross-linked receptors were detected by a Western blot (*a*). For ASGPR, TfR, and MPR, 10, 5.5, and 5.5% polyacrylamide gels were used, respectively. (*b*) The fluorograms were quantitated and the percentage of unaffected TfR (△), ASGPR (○), and MPR (□) was calculated as in Fig. 2.

rupted vesicles, postnuclear supernatants were digested with proteinase K after the DAB/H_2O_2 incubation (Fig. 7). Receptors originating from disrupted vesicles were totally digested, whereas those in sealed vesicles were just trimmed of their cytoplasmic tail. No TfR was detected after DAB/H_2O_2 and proteinase K treatment of postnuclear supernatants. This confirmed that all sealed TfR-carrying vesicles also contained Tf/HRP. Significant amounts of both the ASGPR and MPR were found associated with vesicles that were neither disrupted nor contained HRP. Loss of the cytoplasmic tail of receptors might cause an altered blotting efficiency or remove epitopes that otherwise would contribute to the signal on a Western blot. The signals obtained from the trimmed receptors could therefore not be compared to those which were not trimmed. Since ASGPR labeled at the plasma membrane did not sort from Tf/HRP during endocytosis, and only 70% of the intracellular ASGPR were accessible to endocytosed Tf/HRP, we conclude that 30% of the intracellular ASGPR pool is not involved in endocytosis. We next examined whether this "silent" ASGPR pool could be accessed by Tf/HRP after incubation in medium containing a high ligand concentration. We repeated the experiment in Fig. 6, except that the cells were incubated with 10 mM *N*-acetylgalactosamine starting 1 h before Tf/HRP addition. Incubation with *N*-acetylgalactosamine, a ligand for the ASGPR, had no effect on the accessibility of ASGPRs to endocytosed Tf/HRP (not shown).

Maximally, 35% of the intracellular MPRs were affected by Tf/HRP (Fig. 6). If disrupted vesicles were taken into account, we calculate that 50% (35/70 × 100%) of the total intracellular MPR was accessible to endocytosed Tf/HRP. Therefore, we conclude that there is a partial but significant overlap between the intracellular pathways of TfR and MPR.

When the incubation of cells with Tf/HRP was performed in the presence of excess nonconjugated Tf (3 mg/ml), no receptor loss was detected (recovery, 100 ± 0, 100 ± 6, and 96 ± 2% of TfR, ASGPR, and MPR, respectively; mean values ± SD of two independent experiments). Thus, the accessibility of the different receptor species to Tf/HRP is completely TfR dependent.

Morphological Localization of Tf/HRP, ASGPR, and MPR

These studies have demonstrated biochemical evidence for the concomitant intravesicular presence of endocytosed Tf/HRP with ASGPR and MPR. Next, we combined DAB cytochemistry with immunogold cytochemistry on ultrathin cryosections, to compare the subcellular distribution of endocytosed Tf/HRP with that of ASGPR in situ. In previous reports, we described the localization of ASGPR in Hep G2 cells (Zijderhand-Bleekemolen et al., 1987). ASGPR is present at the plasma membrane, in coated pits and vesicles, in endosomal (CURL) vesicles and tubules, and in TGR. We also demonstrated that the detection of secretory albumin can serve to distinguish endosomes (lacking albumins) from TGR (containing albumin).

Hep G2 cells were preincubated in serum-free medium to deplete the cells of serum albumin and Tf. The cells were then incubated for 30 min with Tf/HRP, fixed, and incubated with DAB. Cryosections were immunogold double labeled for the demonstration of ASGPR and albumin. ASGPR was detected in albumin-containing TGR and Tf/HRP-containing endosomes (Fig. 8). Since DAB polymer considerably hampers the detection of ASGPR, only a weak immunogold reaction was found in endosomes. Many gold particles were located just at the cytoplasmic side of the DAB-positive organelles. This diminished immunoreactivity may have resulted in underestimates of Tf/HRP-containing TGR. Only a small amount of albumin-positive TGR was labeled for both Tf/HRP and ASGPR. Most ASGPR in TGR did not colocalize with DAB reaction product. These ASGPRs probably represent the "silent" ASGPR pool which is inaccessible to Tf/HRP, as defined above.

Discussion

In the present study we used DAB cytochemistry to compare the intracellular pathways of TfR, ASGPR, and MPR with those of endocytosed Tf/HRP, a ligand of the TfR. For this purpose we first optimized the conditions for DAB cyto-

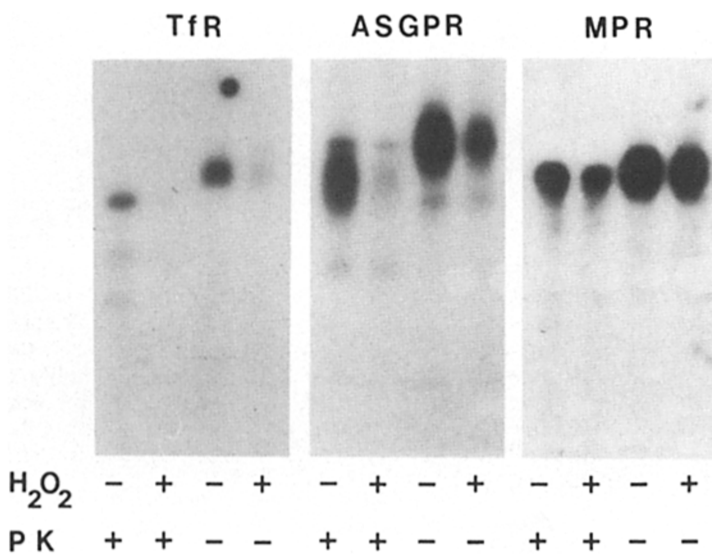
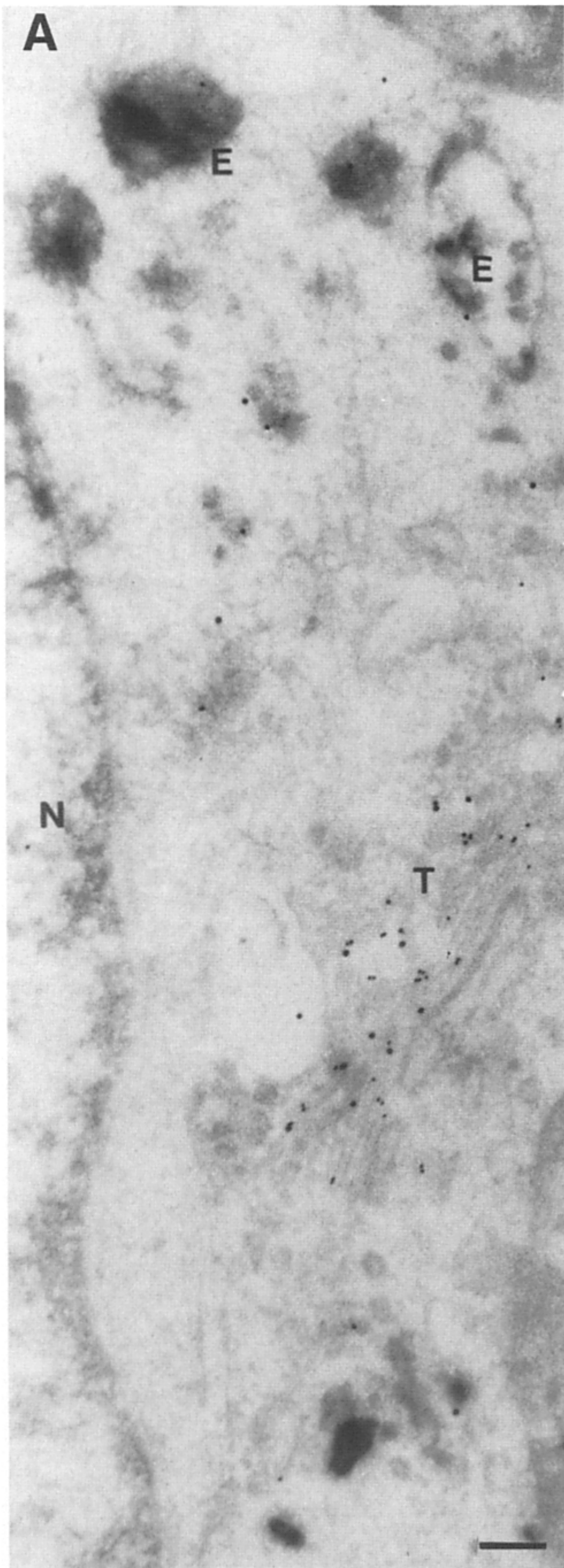


Figure 7. Proteinase K digestion of microsomes. Unlabeled cells were incubated for 30 min in Tf/HRP-containing medium. After proteinase K treatment, half of the suspended cells were washed in the presence, and half in the absence, of PMSF. After DAB cytochemistry, the samples lacking PMSF were additionally incubated with proteinase K (*PK+*), which was inhibited by the addition of PMSF, and centrifuged through a sucrose cushion. The samples were then analyzed for receptor by Western blotting.



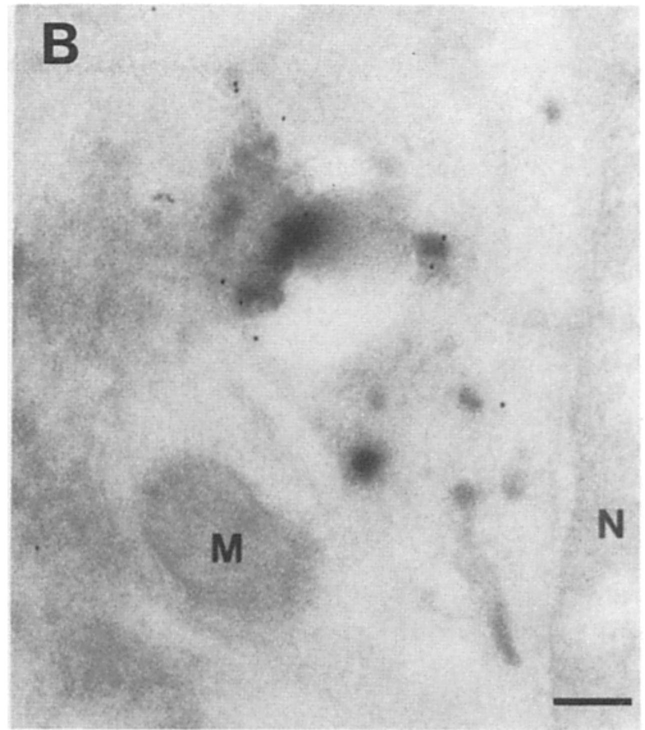


Figure 8. Localization of Tf/HRP and ASGPR. Cells were incubated for 30 min with Tf/HRP, fixed, and processed for DAB cytochemistry. (*A*) The ultrathin cryosection was double immunolabeled for the demonstration of albumin (10-nm gold) and ASGPR (15-nm gold). ASGPR is present in TGR (*T*) together with albumin, and colocalizes with Tf/HRP in endosomes (*E*). (*B*) As in the previous figure, but single immunolabeled for ASGPR with 10-nm gold. The receptor colocalizes with Tf/HRP in an endosome. Note the presence of gold particles just at the cytoplasmic side of the Tf/HRP-positive structures. *M*, mitochondrion; *N*, nucleus. Bars: (*A*) 0.24 μm; (*B*) 0.17 μm.

chemistry. We (Stoorvogel et al., 1988; Geuze et al., 1988) and others (Ajioka and Kaplan, 1986, 1987) previously reported the protein cross-linking effect of DAB cytochemistry. We detected maximal cross-linking of proteins in Tf/HRP-containing vesicles at low DAB concentrations (30 μg/ml). At this concentration, a less pronounced density shift of Tf/HRP-containing vesicles was obtained in a Percoll density gradient, compared to that found at 1 mg/ml DAB (not shown). This indicates the formation of little intravesicular DAB polymer. It is therefore most likely that the protein immobilization is due to a covalent bond between protein and DAB polymer rather than to protein encapsulation.

Several studies have provided evidence that the endocytic pathway(s) of the TfR and the ASGPR coincide at least partly. Both ASGPR and TfR enter the cell by means of coated vesicles (Geuze et al., 1983*a*,*b*; Willingham et al., 1984) with a half-time of 2–3 min (Schwartz et al., 1982, Ciechanover et al., 1983*a*,*c*). We have previously reported that ASGP and Tf are sorted immediately after receptor-mediated uptake (Stoorvogel et al., 1987). Watts (1984) also showed the concomitant intravesicular presence of TfR and ASGP. They used lactoperoxidase conjugated to an ASGP, after its endocytosis by the ASGPR, to iodinate intracellular TfR. Regoeczi and co-workers (1982, 1985) showed that asialotransferrin, which was endocytosed via the ASGPR, could recycle TfR bound to the plasma membrane. They concluded that the ligand switched receptor within an acidic compartment. The present study is consistent with all of these observations, as the entire endocytic pathways of the ASGPR and the TfR coincide. At 37°C, receptor-bound Tf is released as apo-Tf into the medium, implying a half-time of 5 min for TfR recycling in Hep G2 cells (Ciechanover et al., 1983*a*,*c*). Similar recycling kinetics have been reported for the ASGPR (Schwartz et al., 1982; Ciechanover et al., 1983*a*). Virtually all ASGPRs participating in endocytosis were encountered by endocytosed Tf/HRP within 10 min of continuous uptake (Fig. 6). A pulse of endocytosed ^{125}I-Tf was encountered only to a limited extent by subsequently endocytosed Tf/HRP (Ajioka and Kaplan, 1986; our results not shown). Therefore, virtually all ASGPRs involved in endocytosis must pass the plasma membrane within 10 min. Thus, after 60 min (the maximum incubation time in our present study), multiple rounds of endocytosis have been completed. Under these conditions, ^{125}I-labeled plasma membrane ASGPR has had opportunity to completely mix with unlabeled recycling ASGPR populations. Thus, if any portion of the ASGPR recycling route did not coincide with that of the TfR, an equivalent portion of ^{125}I-ASGPR would have have been expected to escape cross-linking during 60 min at 37°C. We therefore conclude that the recycling pathways of ASGPR and TfR are identical.

ASGP transport however is not an unidirectional process. In both rat hepatocytes (Weigel and Oka, 1984) and Hep G2 cells (Simmons and Schwartz, 1984) a portion of endocytosed ASGP returns to the cell surface bound to ASGPR. In Hep G2 cells, single cycle kinetics showed that 50% of the ligand returns to the plasma membrane with a half-time of 24 min. To relate these observations with the present results (rapid ASGPR recycling, see above), we postulate a "slipping–coupling" mechanism, in which all ASGPs dissociate from their receptor after uptake. In this model, a portion of the dissociated ligand escapes delivery to lysosomes by means of vesicles containing free recycling ASGPR. Rebinding could occur before fusion with the plasma membrane. Indeed, endocytosed ASGPs have been shown to reside temporarily in ASGPR-containing vacuoles after dissociation and before degradation (Wolkoff et al., 1984; Mueller and Hubbard, 1986). In the present model, rapid recycling ASGPRs can mediate slow receptor-bound ligand recycling.

We have previously shown that in Hep G2 cells, a portion of endocytosed receptor bound Tf/HRP enters the TGR, where it mixes with newly synthesized secretory protein (Stoorvogel et al., 1988). This process occurs in <10 min, which is consistent with rapid accessibility of TfR to Tf/HRP (Fig. 6). Our finding that Tf/HRP is not sorted from endocytosed ASGPR would imply that very little TfR or ASGPR is also transported to the TGR. In a previous study, we detected a substantial cycloheximide-resistant ASGPR population (35% of the intracellular pool) in the TGR of Hep G2 cells (Zijderhand-Bleekemolen et al., 1987). Furthermore, the finding that some of the receptor-bound recycling ASGPs become resialylated also supports the involvement of the TGR in ASGPR recycling (Schwartz, 1989). However, most ASGPR in TGR did not colocalize with Tf/HRP (Fig. 8). This suggests that most of these receptors were not involved in endocytosis. Indeed, the intracellular ASGPR pool was not entirely accessible to endocytosed Tf/HRP (Fig. 6), whereas ASGPRs taken up from the cell surface were not sorted from the Tf/HRP recycling pathway (Fig. 4). Taken together these observations suggest that ~30% of the intracellular ASGPR do not participate in endocytosis. The existence of such silent ASGPR pool in Hep G2 cells has recently been suggested by others (Fallon et al., 1988). The detection of ASGPR in DAB polymer–negative TGR (Fig. 8), suggests that at least a portion of the silent ASGPR pool resides in TGR, which is consistent with our earlier findings that 35% of the intracellular ASGPR is localized in the TGR (Zijderhand-Bleekemolen et al., 1987). However, a quantitative evaluation of ASGPR in TGR is not possible at present, since immunogold labeling of DAB polymer–containing organelles is quenched to a variable degree.

We found that up to 70% of endocytosed ^{125}I-MPR was sorted from the TfR/ASGPR route within 45 min (Fig. 4). These results might seem incompatible with a mean TfR recycling time of 8 min. However, two factors probably contribute to this relatively late escape of ^{125}I-MPR from Tf/HRP mediated cross-linking: (*a*) sorting was only detected if the intravesicular Tf/HRP concentration dropped below 25% of its maximal value (Fig. 2); and (*b*) as the sorting of ^{125}I-MPR from the Tf/HRP route was measured during continuous Tf/HRP uptake, endocytosed ^{125}I-MPR may have contacted subsequent endocytosed Tf/HRP via a mechanism analogous to the slipping–coupling mechanism discussed above. ^{125}I-MPR, which originated from the cell surface, escaped cross-linking after 20–45 min of uptake (Fig. 4), by sorting from the Tf/HRP pathway rather than sorting from concomitant endocytosed Tf/HRP. Only 50% of intracellular MPR was accessible to Tf/HRP, whereas 70% of surface labeled ^{125}I-MPR was sorted from the Tf/HRP pathway. Thus, we conclude that ^{125}I-MPR was not completely mixed with the total MPR pool after 60 min of uptake.

In Fig. 9 we propose a model based on the results obtained in this study, in which the pathways of the TfR, the ASGPR, and the MPR are shown. The three receptor species are in-

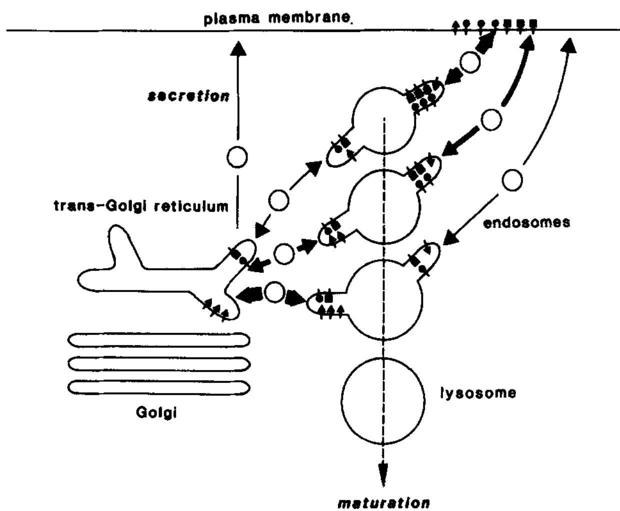


Figure 9. Receptor transport routes. Receptor-containing vesicles budding from the plasma membrane fuse with all, but preferably with early, endosomes. Vesicles budding from the tubular extensions of early endosomes preferably fuse with the plasma membrane. Endosomes mature during this fusion and fission process. During this process they lose their competence for interaction with the plasma membrane, while interaction with the TGR increases. TfR and ASGPR preferentially exit directly after arrival from endosomes and consequently shuttle mainly between the plasma membrane and endosomes. In contrast, MPRs preferentially exit late endosomes and multivesicular bodies, and are thus mainly shuttling between these organelles and the TGR. (⧫) TfR; (⧫) ASGPR; (▲) MPR.

ternalized together during endocytosis (Fig. 4). After uptake via coated vesicles, receptors are localized in larger structures; i.e., endosomes (Fig. 8). Therefore, membrane fusion between endocytic vesicles has occurred. Thus, one prediction of the model is that two subsequent pulses of endocytosed TfR-ligand complexes would interact within a common compartment if the compartment was static. However, we (not shown) and others (Ajioka and Kaplan, 1986), have found that this only occurs to a limited degree. The intracellular mixing of two subsequent endocytosed pulses of ligand is dependent on the time interval between the pulses. Therefore, TfRs must pass through this endosomal compartment at a high rate. Thus, intracellular TfR is expected to be mainly found in transient vesicles shuttling between endosomes and the plasma membrane. In this model, endocytosed MPR and ASGP mainly enter early endosomes and remain there while they maturate into late endosomes, during which recycling Tf/HRP and ASGPR continue to transit this compartment (slipping-coupling, discussed above). Our finding that a maximal amount of intracellular MPR was accessible to endocytosed Tf/HRP within 10 min (Fig. 6), is comparable with the kinetics of accessibility of MPR to fluid-phase endocytosed HRP (Geuze et al., 1988). Both results are fully consistent with the proposed model, as it implies that endosomal MPR resides in compartments which all fuse directly with plasma membrane derived vesicles. MPRs are sorted from the TfR route when endosomes have matured, and vesicular transport to the TGR is increased. In a previous study we showed that at least a portion of endocytosed Tf/HRP entered the TGR as well (Stoorvogel et al., 1988). According to the present model, TfR routing through the TGR is a minor one. TfR and MPR in TGR are transported to the plasma membrane and endosomes, respectively. We have previously shown that MPR is sorted from and shuttles between TGR and late endosomes (Geuze et al., 1988). Therefore, sorting between MPR and Tf/HRP is likely to occur both in endosomes and TGR. In our model, early endosomes communicate mainly with the plasma membrane, and late endosomes with TGR. TfR and ASGPR tend to exit early, and MPR exit late from the endocytic pathway, thereby determining their destination.

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