Endocytosis of the ASGP Receptor H1 Is Reduced by Mutation of Tyrosine-5 But Still Occurs via Coated Pits

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Abstract. The clustering of plasma membrane receptors in clathrin-coated pits depends on determinants within their cytoplasmic domains. In several cases, individual tyrosine residues were shown to be necessary for rapid internalization. We have mutated the single tyrosine at position 5 in the cytoplasmic domain of the major subunit H1 of the asialoglycoprotein receptor to alanine. Expressed in fibroblast cells, the mutant protein was accumulated in the plasma membrane, and its rate of internalization was reduced by a factor of four. The residual rate of endocytosis, however, was still significantly higher than that of resident plasma membrane proteins. Upon acidification of the cytoplasm, which specifically inhibits the formation of clathrin-coated vesicles but not uptake of the fluid phase marker Lucifer yellow, residual endocytosis was blocked. By immunoelectron microscopy mutant H1 could be directly demonstrated in coated pits. The fraction of wild-type and mutant H1 present in coated pits as determined by immunogold localization correlated well with the respective rates of internalization. Thus, mutation of tyrosine-5 only partially inactivates recognition of H1 for incorporation into coated pits.

MACROMOLECULES are taken up into eukaryotic cells by endocytosis. The best characterized mechanism for this process is mediated by cell surface receptors that cluster into clathrin-coated domains in the plasma membrane (Goldstein et al., 1985; Hubbard, 1989). Receptors are collected in these regions either constitutively or after binding of ligand, and are internalized by invagination and pinching off of clathrin-coated vesicles (Goldstein et al., 1979; Hopkins and Trowbridge, 1983). In the last years, convincing evidence has been accumulated in support of another, clathrin-independent mechanism for internalization of fluid phase and certain toxins and membrane proteins (reviewed by van Deurs et al., 1989). By biochemical disruption of coated vesicle formation, it was possible to distinguish between clathrin-dependent and -independent pathways.

The determinants for clustering into clathrin-coated pits were shown for several plasma membrane receptors to be contained within their cytoplasmic domain. Deletion of this portion of the receptors for LDL, polymeric immunoglobulins (poly-Ig), epidermal growth factor (EGF),[1] transferrin, and mannose-6-phosphate resulted in a drastic reduction of the internalization rate (Lehrman et al., 1985; Mostov et al., 1986; Prywes et al., 1986; Rothenberger et al., 1987; Lobel et al., 1989). In the case of the macrophage Fc receptor (RII-B2), quantitative immunoelectron microscopy studies have shown that a truncated form lacking the cytoplasmic domain was largely excluded from coated pits (Miettinen et al., 1989). There is increasing evidence that peripheral membrane proteins, so-called assembly or adaptor proteins bind to the cytoplasmic domains of receptors and induce the formation of the clathrin coat (e.g., Moore et al., 1987; Pearse, 1988; Glickman et al., 1989; reviews by Keen, 1990, and by Pearse and Robinson, 1990). As a result, proteins recognized by adaptor proteins are concentrated in coated pits, while other (resident) plasma membrane proteins are passively excluded from these surface patches and internalized only very slowly (Bretscher et al., 1980; Roth et al., 1986).

The cytoplasmic domains of endocytic receptors are very diverse in length, primary structure, and even their orientation with respect to the membrane (some are amino-, others carboxy-terminal). Analysis of natural LDL receptor mutants deficient in LDL uptake revealed that mutation of a single residue, tyrosine-807, strongly affected internalization (Davis et al., 1986). No other amino acid at this position except phenylalanine and, to a lesser degree, tryptophan, mediated efficient endocytosis of LDL (Davis et al., 1987). A general importance of tyrosine residues for internalization was suggested by the finding that insertion of a tyrosine into the short cytoplasmic domain of influenza virus hemagglutinin caused this protein to enter coated pits and to be internalized (Lazarovits and Roth, 1988). The sequence context appeared also to be important, since at only one of three insertion positions tested the tyrosine was functional. Very recently, an extensive mutational analysis of this endocytosis signal has been performed as well as a comparison of sequences flanking tyrosine residues that are either known or presumed to be critical for internalization of endocytosed

1. *Abbreviations used in this paper*: ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; EGF, epidermal growth factor.

proteins (Ktistakis et al., 1990). A degenerate consensus for internalization signals was deduced and was successfully used to predict a position within the cytoplasmic domain of glycophorin A where insertion of a tyrosine directed the mutant protein into the endocytic pathway (Ktistakis et al., 1990). For natural endocytic receptors other than the LDL receptor, reports have been published demonstrating the importance of tyrosine-2360 and/or tyrosine-2362 of the cation-independent mannose-6-phosphate receptor (Lobel et al., 1989), of tyrosine-20 of the transferrin receptor (Jing et al., 1990; Alvarez et al., 1990), and of tyrosine-734 of the polymeric immunoglobulin receptor (Breitfeld et al., 1990). In the cases of the LDL, the transferrin, and the poly-Ig receptor, internalization of mutant receptors lacking the critical tyrosine was significantly reduced in transfected cells, but not entirely eliminated. So far, it has not been addressed by which mechanism this residual endocytosis takes place, i.e., whether the lack of a "tyrosine signal" renders these molecules less efficiently clustered into coated pits or rather diverts them into an alternative endocytic pathway.

In this study we have investigated the characteristics of endocytosis of the wild-type and a mutant subunit H1 of the human asialoglycoprotein (ASGP) receptor. The ASGP receptor is a constituent of the plasma membrane of hepatocytes. It specifically binds galactosyl-terminal oligosaccharides and is responsible for the removal of desialylated glycoproteins from the circulation into the cell for degradation in lysosomes (reviewed by Ashwell and Harford, 1982; Breitfeld et al., 1985). The ASGP receptor is composed of two homologous subunits, H1 and H2 (Spiess and Lodish, 1985), which are assembled in a complex with an H1-H2 ratio of 2-5:1 (reviewed by Spiess, 1990; Henis et al., 1990). Formation of the heterooligomeric complex is necessary for high-affinity ligand binding (Shia and Lodish, 1989). Yet, we have recently shown that the major subunit H1 alone, although unable to bind ligand with high affinity, contains all the signals for constitutive endocytosis and recycling (Geffen et al., 1989).

H1 is a type II membrane protein and contains an amino-terminal cytoplasmic domain of 40 residues with a single tyrosine residue at position 5. The flanking sequences have no striking similarity to those of the tyrosines implicated in endocytosis in other receptors. By site-directed mutagenesis, we have changed this residue to an alanine and characterized endocytosis of the resulting mutant ASGP receptor H1. The rate of internalization was found to be reduced by approximately a factor of four. However, as in other receptor systems, significant residual endocytosis was observed for H1 lacking the cytoplasmic tyrosine. By immunoelectron microscopy and by specific inhibition of clathrin-dependent endocytosis, we could show that the residual internalization still occurs via clathrin-coated pits and vesicles and not by an alternative pathway. Mutation of this tyrosine thus does not entirely inactivate the recognition signal(s) for clustering in coated pits.

Materials and Methods

DNA Constructs

Site-directed mutagenesis was performed using the gapped duplex DNA procedure according to Kramer et al. (1984). The HindIII–BamHI fragment of the cDNA of the ASGP receptor H1 (Spiess et al., 1985), encoding the amino-terminal 60 residues of the protein, was subcloned into M13mp9 and used as the template. Using the synthetic anti-sense oligonucleotide GGTCTTGAGCCTCCTTG (the mismatched nucleotides are underlined), the codon for tyrosine-5 (TAT) was mutated to that for an alanine (GCT). Successfully mutated M13 clones were identified by DNA sequencing and ligated to the 3′ portion of the cDNA. After ligation of a BglII linker into the blunted HindIII site at the 5′ end of the mutant cDNA and of a SalI linker into the blunted EcoRI site at the 3′ end, the full-size cDNA (BglII–SalI) was subcloned into the retroviral shuttle vector pLJ (cut with BamHI and SalI; Korman et al., 1987) to yield the expression plasmid pL1/5A.

Cell Culture and Transfection

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY) NIH3T3 mouse fibroblasts and ΨAM cells were grown in Dulbecco's modified minimal essential medium (DME) supplemented with 8% newborn calf serum (Inotech, Switzerland), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C with 7.5% CO_2. The cell lines 1-7 (expressing subunit H1) and 1-7-1 (expressing both receptor subunits H1 and H2) were derived from NIH3T3 fibroblasts as described by Shia and Lodish (1989). For expression of tyrosine-mutated H1 in fibroblasts, pL1/5A was first transfected into ΨAM helper cells (Cone and Mulligan, 1984) using polybrene and dimethyl sulfoxide as described by Kawai and Nishizawa (1984). ΨAM cells resistant to 1 mg/ml G418 sulfate (Gibco Laboratories) were grown to confluence. The virus-containing medium was filtered through a 0.45-μm pore-size filter (Millipore Corp., Bedford, MA) and supplemented with 8 μg/ml polybrene. To infect NIH3T3 cells, 2.5 ml were added per 10-cm dish (30% confluent). After 2.5 h at 37°C, 7.5 ml fresh medium was added. After two days, the cells were split into a 15-cm dish with selective medium containing 1 mg/ml G418 sulfate. Resistant colonies were isolated and screened for expression of mutant H1 protein by metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine and immunoprecipitation. Two independent clonal cell lines, Fl(5A)-1 and Fl(5A)-2, were further characterized.

Receptor Distribution and Internalization Assay

The distribution between the cell surface and intracellular membranes and the rate of internalization of wild-type and mutant H1 were determined as described by Geffen et al. (1989). Briefly, H1 distribution was assayed by digesting intact cells with proteinase K (1 mg/ml) at 4°C in PBS with 5 mM EDTA for 30 min. Digestion was stopped by adding 2 mM PMSF. Protease-digested and control cells were lysed in gel sample buffer and subjected to SDS-PAGE and immunoblot analysis, using an affinity-purified rabbit antibody raised against a synthetic peptide to the carboxy-terminal sequence of H1 (residues 277–287). To assay H1 internalization, cells were surface labeled at 4°C using the impermeant reagent [^{125}I]sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate ([^{125}I]sulfo-SHPP; Thompson et al., 1987), incubated at 37°C for up to 45 min, and then digested at 4°C with proteinase K. Protease-resistant labeled receptor was analyzed by immunoprecipitation, SDS-gel electrophoresis, and autoradiography. Autoradiographs were quantitated using a computing densitometer (Model 300A; Molecular Dynamics, Sunnyvale, CA).

Blocking Endocytosis via Clathrin-coated Vesicles

To specifically inhibit receptor internalization by coated pits/vesicles, the method of acidification of the cytoplasm according to Sandvig et al. (1987) was used. Cells grown in six-well clusters were rinsed with DME and equilibrated with DME containing 20 mM Hepes (pH 7.2) and 30 mM ammonium chloride for 30 min at 37°C. The medium was replaced by 1 ml of prewarmed potassium/amiloride buffer (KA buffer; 140 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride (Sigma Chemical Co., St. Louis, MO), 20 mM Hepes, pH 7.2) for 5 min. This treatment lowers the cytoplasmic pH to 5.6–5.8 and blocks the pinching-off of coated vesicles (Sandvig et al., 1987; Heuser, 1989). To maintain the acidic cytoplasmic pH, all subsequent incubations were done in KA buffer.

Ligand Binding and Internalization

Asialoorosomucoid (ASOR) was prepared and ^{125}I-iodinated as described previously (Shia and Lodish, 1989). Ligand binding to 1-7-1 cells (expressing both receptor subunits) was performed for 2 h at 4°C with 1 μg/ml [^{125}I]ASOR in KA buffer containing 0.2 mg/ml cytochrome *c*. Nonspecific binding was determined in the presence of 200-fold excess of unlabeled

ASOR. To monitor ligand internalization, the cells were incubated with [^{125}I]ASOR at 37°C for up to 15 min. Surface-bound ligand was selectively removed by three washes with 5 mM EDTA in PBS at 4°C. Cell-associated ligand was determined by counting the radioactivity, and by gel electrophoresis of the cell lysate and autoradiography.

Endocytosis of Lucifer Yellow

Uptake of Lucifer yellow was measured as described by Swanson et al. (1985). Acidified and control cells were incubated with 1 mg/ml Lucifer yellow (Fluka Chem. Corp., Ronkonkoma, NY) in KA buffer for various times at 37°C. To wash the cells, the whole plate was immersed at 4°C twice in 1 l each of PBS with 1 mg/ml BSA and twice in 1 l each of PBS. The cells were then lysed in 0.05% Triton X-100 in PBS. The lysate was supplemented with 0.1 mg/ml BSA, and Lucifer yellow concentration was determined by fluorescence spectrometry (excitation at 430 nm, emission at 540 nm; background fluorescence of cells not incubated with Lucifer yellow was subtracted).

Immunoelectron Microscopy

Surface distribution of wild-type and mutant H1 was analyzed in living and prefixed cells by immunoelectron microscopy using gold conjugates. For labeling of living cells, colloidal gold was prepared by the method of Slot and Geuze (1985). Affinity-purified rabbit immunoglobulins directed against the carboxy-terminal sequence of H1 were conjugated to 8-nm gold particles essentially as described by Lucocq and Baschong (1986). Subconfluent 1-7, 3T3 and Fl(5A)-1 cells grown in 35-mm dishes were incubated in situ with the immunoglobulin-gold probe in PBS for 1 h at 4°C and then for 10 min at 37°C to allow labeled receptors to distribute in plasma membrane invaginations and to be internalized. After fixation in 1% glutaraldehyde in PBS for 2 h, the cells were rinsed three times with PBS and postfixed in reduced osmium tetroxide for 30 min. Finally, cells were carefully scraped, enclosed in 2% agar and embedded in Epon according to standard procedures. Thin sections were counterstained with 6% aqueous uranyl acetate (30 min) and lead acetate (2 min), and examined in a Phillips EM 300 electron microscope.

For labeling of prefixed cells, monolayer cultures grown in six-well plates were fixed at 4°C for 15 min as described by McLean and Nakane (1974) and then covered with 50 mM NH_4Cl in 100 mM sodium phosphate buffer, pH 7.2, for 20 min to amidinate free aldehyde groups. The cells were washed three times with phosphate buffer for 10 min each and twice for 5 min with TBS (20 mM Tris, pH 8.2, 150 mM NaCl), incubated with 3% BSA in TBS for 40 min and then for 4 h with a rabbit antiserum raised against purified human ASGP receptor (diluted 1:400 in TBS with 1% BSA). After three washes with TBS for 5 min, the cells were incubated for 2 h with sheep anti-rabbit immunoglobulin conjugated to 5-nm gold particles diluted 1:20 in TBS with 0.25% BSA. After three rinses with TBS, the cells were fixed and further processed as described above.

For the quantitation of immunoelectron micrographs, the unlabeled, inaccessible domain of the plasma membrane adjacent to the plastic was ignored. For the accessible surface, the fraction of the plasma membrane occupied by coated pits was determined by counting the relative number of intersections of the lines of a test grid with coated and uncoated membrane, as described by Griffiths et al. (1989). For this a double-lattice test system was used (Weibel, 1979) on a set (n = 28) of randomly selected micrographs with a primary magnification of 10,000 and a final magnification of 40,900. At this magnification the spacing (d) between test lines was 1.47 μm for estimating the total plasma membrane length and 0.12 μm for estimating that of coated pits. The number of gold particles over total plasma membrane and coated pits was determined from the same micrographs.

Results

Mutation of Tyrosine-5 to Alanine

The major subunit H1 of the human ASGP receptor has previously been expressed in NIH3T3 fibroblast cells, and the resulting cell line, called 1-7, has been characterized in detail (Shia and Lodish, 1989; Geffen et al., 1989). In the absence of the second subunit, H1 was unable to bind ASGPs with high affinity, but it was transported to the cell surface and was constitutively internalized and recycled with kinetics very similar to those of the heterooligomeric H1–H2 receptor complex. Subunit H1 thus contains all the signals necessary for rapid internalization and recycling and can serve as a model system to study the requirements for endocytosis.

The cytoplasmic domain of H1 consists of 40 amino acids and contains a single tyrosine residue at position 5:

MTKEYQDLQHLDNEESDHHQLRKGPPPPQPLLQRLCSGPR . . .

There are no phenylalanines and tryptophans, residues which in the LDL receptor could functionally replace tyrosine-807. To study the importance of tyrosine-5 for clustering of H1 into coated pits and endocytosis, we have mutated it to an alanine by site-directed mutagenesis of the cDNA. Using a retroviral expression system (Korman et al., 1987), the mutated cDNA was introduced into mouse NIH3T3 fibroblasts, and stable expressing cell lines were isolated. The amount of mutant H1 expressed in two clones, Fl(5A)-1 and Fl(5A)-2, was similar to the amount of wild-type H1 expressed in 1-7 cells. In all three cell lines, two major forms of H1 with apparent molecular masses of 40 and 46 kD were synthesized (Fig. 1, lanes *2*, *4*, and *6*), which have been previously identified as the high-mannose glycosylated precursor and the complex glycosylated mature form of the protein, respectively (Schwartz and Rup, 1983; Shia and Lodish, 1989). The mature form (which sometimes appears as a doublet, most likely because of glycosylation heterogeneity) migrated with an electrophoretic mobility very similar to that of the ASGP receptor purified from human liver (lane *1*). The ratio of the two forms was almost identical in all three cell lines, suggesting that mutation of tyrosine-5 does not affect maturation of the protein.

Mutant H1 Is Accumulated in the Plasma Membrane

A first indication of whether mutation of tyrosine-5 affects internalization of H1 was obtained by analyzing the distribution of receptor polypeptides between the plasma membrane and intracellular compartments. Proteins defective in endocytosis are expected to accumulate on the cell surface. Fl(5A)-1, Fl(5A)-2, and 1-7 cells were incubated with proteinase K for 30 min at 4°C. At this temperature, membrane traffic is arrested and only proteins located at the cell surface are digested. Protease-resistant receptor was detected by SDS-gel electrophoresis and immunoblot analysis using an H1-specific antiserum (Fig. 1). Upon proteinase K treatment, ~50% of the mature form of wild-type H1 in 1-7 cells was protected from digestion, i.e., intracellular, consistent with previous determinations (Geffen et al., 1989). In contrast, only ~15% of the mature form of mutant H1 in Fl(5A)-1 and Fl(5A)-2 was resistant to exogenous protease. Mutation of tyrosine-5 to alanine thus resulted in a significant accumulation of H1 in the plasma membrane. The intracellular 40-kD precursor remained unaffected by protease treatment in all cell lines, indicating that the cells stayed intact during the experiment.

Internalization Rate of Mutant H1 Is Reduced

We have previously developed a ligand-independent procedure to determine the rate of internalization of subunit H1 (Geffen et al., 1989). The cell surface is first labeled at 4°C by incubation with ^{125}I-iodinated sulfosuccinimidyl-3-(4-

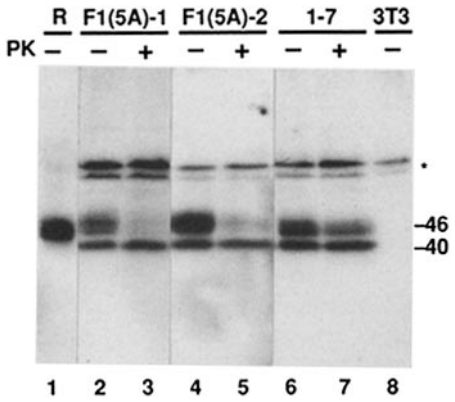

Figure 1. Cellular distribution of wild-type and mutant ASGP receptor H1. The distribution of wild type and mutant H1 between the plasma membrane and intracellular compartments in 1-7 and F1(5A)-1 and F1(5A)-2 cells, respectively, was determined by immunoblot analysis (as described in Materials and Methods). H1 of untreated cells and of cell digested for 30 min at 4°C with proteinase K (*PK*), as indicated, was analyzed. ASGP receptor purified from human liver (lane *1*) and lysate of parental 3T3 cells (lane *8*) were included for comparison. The positions of the 40-kD high-mannose glycosylated precursor form and the 46-kD complex glycosylated mature form are indicated. Nonspecific proteins recognized by the antiserum are marked by an asterisk.

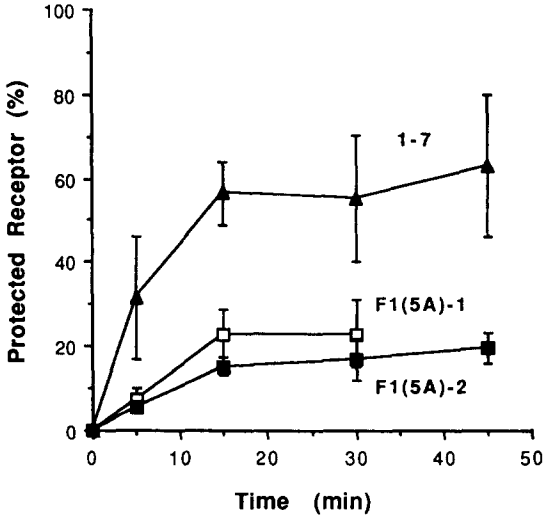

Figure 3. Time course of endocytosis of wild-type and mutant receptor H1. Internalization assays as shown in Fig. 2 were quantitated by densitometric scanning of the autoradiographs. Average values with standard deviations originating from one (F1(5A)-2 cells) or from two (1-7 and F1(5A)-1 cells) experiments, each done in triplicates, are shown.

hydroxyphenyl) propionate (sulfo-SHPP), a water-soluble and thus impermeant, amino group–specific reagent (Thompson et al., 1987). The surface-labeled cells are incubated at 37°C for different times, then chilled on ice and digested with proteinase K at 4°C. Labeled receptors that have been internalized during the 37°C chase and thereby have acquired resistance to added protease are then immunoprecipitated and analyzed by gel electrophoresis and autoradiography.

Fig. 2 shows the result of a typical endocytosis assay performed with 1-7 and F1(5A)-2 cells. The fraction of surface-labeled H1 that acquired protease resistance is significantly smaller for mutant H1 in F1(5A)-2 cells than for wild-type H1 in 1-7 cells (compare lanes *2–5* with lane *1*, and lanes *7–10* with lane *6*). Fig. 3 shows the quantitation of experiments performed with 1-7 cells and the two cell lines expressing

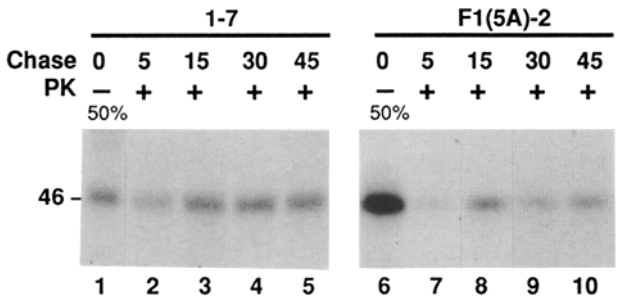

Figure 2. Endocytosis of surface-labeled H1 in 1-7 and F1(5A)-2 cells. Intact cells were labeled at 4°C with ^{125}I-sulfo-SHPP and incubated for increasing times at 37°C (as indicated in minutes). After digestion of the cell surface by proteinase K at 4°C, protease-resistant labeled receptor was analyzed by immunoprecipitation, SDS-gel electrophoresis, and autoradiography (lanes *2–5* and *7–10*). In lanes *1* and *6*, 50% aliquots of the respective labeled cells not digested with protease were loaded.

mutant H1. Within 15 min of incubation at 37°C, surface-labeled and unlabeled receptor proteins had mixed, and an equilibrium distribution was reached: ~50% of wild-type H1 and 15–20% of mutant H1 were intracellular. This is in agreement with the results obtained by the immunoblot analysis in Fig. 1. Judged from the values determined after 5 min of chase, the rate of internalization of mutant H1 was reduced by a factor of three to four. This change of the internalization rate alone can explain the change in receptor distribution from an intracellular-to-surface ratio of 1:1 for the wild-type to ~1:4 for the mutant. It is therefore likely that the recycling rate has not been significantly affected by mutation of tyrosine-5 to alanine. The half-life of surface-labeled mutant H1 was determined to be 6–7 h (data not shown) and was indistinguishable from that of wild-type H1 in 1-7 cells (Geffen et al., 1989). Turnover of receptor protein was thus negligible within the time of the experiments.

Residual Endocytosis of Mutant H1 Is Blocked by Acidification of the Cytoplasm

Endocytosis of mutant H1 is clearly reduced, yet internalization still occurs at a low but significant rate which is higher than expected for a bona fide resident plasma membrane protein. Similar levels of residual endocytosis have been reported for the tyrosine mutants of the LDL, the poly-Ig, and the transferrin receptor (Davis et al., 1986; Breitfeld et al., 1990; Jing et al., 1990). Interestingly, mutation of tyrosine-807 of the LDL receptor of cysteine reduced LDL internalization to ~25% of wild-type levels in transfected IdlA-7 hamster fibroblasts, whereas the same mutant in the fibroblasts of the hypercholesterolemia patient from which it was isolated took up LDL at only 5% of the rate of the wild-type receptor (Davis et al., 1986). Based on this observation, it was suggested that the mutant receptor, depending on the cell type it is expressed in, might be endocytosed by a pathway not involving clathrin-coated pits. Similarly, it has been pro-

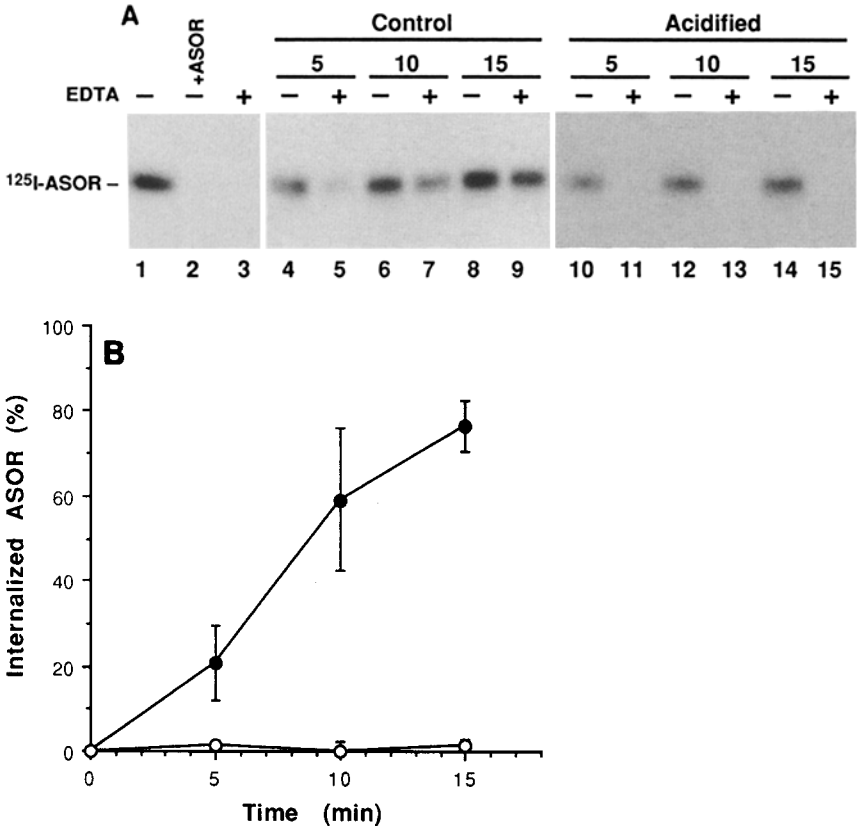

Figure 4. Internalization of [^{125}I]ASOR in 1-7-1 cells under conditions of cytosolic acidification. (*A*) ^{125}I-ASOR was bound to 1-7-1 cells for 2 h at 4°C without (lane *1*) or with (lane *2*) a 200-fold excess of unlabeled ASOR. The cells were lysed and the radioactive ligand was analyzed by SDS-gel electrophoresis and autoradiography. Cells incubated with ^{125}I-ASOR at 4°C and then washed with EDTA were analyzed in lane *3*. Untreated (lanes *4–9*) and acidified cells (lanes *10–15*) were incubated with ^{125}I-ASOR at 37°C for 5, 10, and 15 min, and then washed at 4°C without or with EDTA as indicated to determine total cell-associated and internalized ligand. (*B*) Internalization assays including those shown in *A* were quantitated by densitometric scanning of the autoradiographs. The average percentage of EDTA-resistant ^{125}I-ASOR of the total cell-associated ligand was calculated from triplicate samples for control (*filled circles*) and acidified cells (*open circles*).

posed that ligand-independent endocytosis of the EGF receptor occurs through noncoated pits (Lund et al., 1990).

To identify by which pathway mutant ASGP receptor H1 is taken up into the cell, we examined internalization of wild-type and mutant H1 under conditions that selectively block endocytosis of clathrin-coated vesicles. This was achieved by acidification of the cytoplasm using the procedure developed by Sandvig et al. (1987). The cells are initially equilibrated with up to 30 mM ammonium chloride and then washed with medium free of ammonium chloride. As ammonia (but not ammonium ions) diffuse out of the cell, the cytoplasmic pH is lowered to <6, depending on the initial ammonium chloride concentration used. If no sodium ions are present in the medium and amiloride is added to inhibit the Na^+/H^+ exchanger of the plasma membrane, the cytoplasm can be maintained acidified for >1 h. These conditions have been shown to block the pinching off of coated pits (Heuser, 1989) and to eliminate endocytosis of EGF and transferrin, whereas internalization of ricin and of the fluid-phase marker Lucifer yellow was only partially reduced (Sandvig et al., 1987).

The use of 30 mM ammonium chloride in the acidification procedure was found to be sufficient in our fibroblast cell lines to completely inhibit endocytosis via clathrin-coated vesicles without blocking fluid-phase uptake, as is illustrated in Figs. 4 and 5. ASOR is a ligand of the ASGP receptor that has previously been shown by electron microscopy to be internalized specifically via coated pits (Wall et al., 1980). In Fig. 4, the binding and uptake of [^{125}I]iodinated ASOR by 1-7-1 cells is shown. 1-7-1 cells are derived from 1-7 cells and express both subunits of the ASGP receptor (Shia and Lodish, 1989). [^{125}I]ASOR was specifically bound to 1-7-1 cells, and its binding could be competed with a 200-fold excess of cold ASOR (Fig. 4 *A*, lanes *1* and *2*). After binding of [^{125}I]ASOR to the cells at 4°C, the bound ligand could be completely released by washing the cells with EDTA, which removes calcium ions necessary for ligand binding (lane *3*).

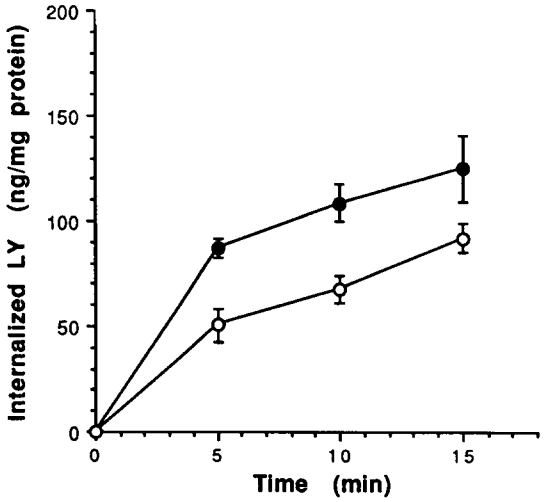

Figure 5. Fluid-phase endocytosis of Lucifer yellow in 1-7 cells under conditions of cytosolic acidification. Acidified and control cells were incubated for the indicated times at 37°C with 1 mg/ml Lucifer yellow. After extensive washing at 4°C, internalized Lucifer yellow was determined by fluorescence spectrophotometry. Average values and standard deviations of two experiments done in triplicates are shown for acidified (*open circles*) and for control cells (*closed circles*).

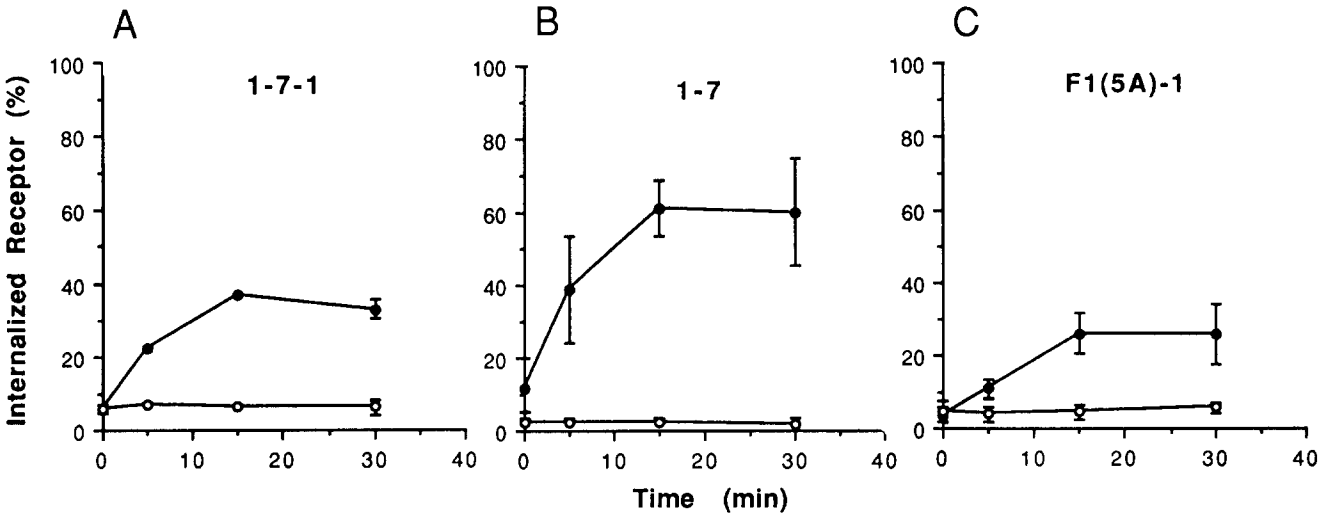

Figure 6. Time-course of constitutive endocytosis of ASGP receptor protein in 1-7-1, 1-7 and Fl(5A)-1 cells under conditions of cytosolic acidification. Internalization assays were performed and quantitated as described for Figs. 2 and 3. The average values of triplicate samples with standard deviations are shown for acidified (*open circles*) and for control cells (*closed circles*).

To monitor ligand internalization, 1-7-1 cells were incubated with [^{125}I]ASOR at 37°C for increasing times, and total cell-associated ligand and EDTA-resistant, intracellular ligand was determined. In untreated cells, an increasing fraction of bound ligand had acquired resistance to EDTA stripping (lanes *4–9*; *filled circles* in *B*). After acidification, however, all cell-associated ligand remained on the surface and could be released with EDTA (lanes *10–15*; *open circles* in *B*). Under the same conditions, uptake of Lucifer yellow by 1-7-1, 1-7, and Fl(5A)-1 cells, as determined after incubation for 10 min at 37°C, was never reduced >40% by acidification. In Fig. 5, a time course of Lucifer yellow uptake by 1-7 cells is shown. The difference in Lucifer yellow accumulation between control (*filled circles*) and acidified cells (*open circles*) most likely reflects the fraction of fluid phase internalization occurring via coated vesicles.

To analyze constitutive endocytosis of wild-type and mutant receptor, the same acidification conditions were applied to 1-7-1, 1-7, and Fl(5A)-1 cells immediately followed by the ligand-independent internalization assay used in Figs. 2 and 3. The results are shown in Figs. 6 and 7. Constitutive endocytosis of the heterooligomeric ASGP receptor in 1-7-1 cells (Fig. 6 *A*) and of H1 expressed alone in 1-7 cells (*B*) was completely blocked after acidification (*open circles*), indicating that internalization occurs exclusively via clathrin-coated pits. Likewise, the residual endocytosis of mutant H1 was entirely inhibited by acidification of the cytoplasm (Fig. 7 and Fig. 6 *C*), suggesting that upon mutation of tyrosine-5 to alanine the recognition signal for association with clathrin-coated pits was not completely inactivated and that mutant H1 was still recognized by coat-associated components, although with reduced affinity. The acid-resistant pathway for fluid-phase uptake is likely to internalize also considerable amounts of membrane, suggesting that a sorting mechanism exists that excludes many membrane proteins, among them the ASGP receptor proteins.

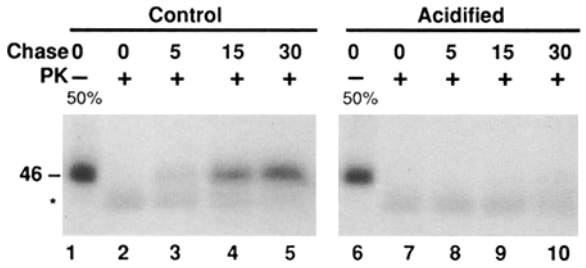

Figure 7. Endocytosis of mutant H1 in Fl(5A)-1 cells under conditions of cytosolic acidification. The internalization assay as described in the legend to Fig. 2 was performed on untreated and acidified Fl(5A)-1 cells. The asterisk indicates a proteolytic fragment of the receptor.

Localization of Mutant H1 in Coated Pits

Using an independent approach to directly detect the mutant protein in clathrin-coated pits, the distribution of mutant and wild-type H1 in the plasma membrane of 1-7 and Fl(5A)-1 cells was analyzed by immunoelectron microscopy. Living cells were incubated for 1 h at 4°C with an H1-specific, affinity-purified antibody conjugated to 8-nm gold particles. Incubation was continued for another 10 min at 37°C to allow the receptor protein to be internalized and to potentially enter also noncoated invaginations, which might not be easily accessible for gold particles directly. Like wild-type H1 in 1-7 cells (Fig. 8, *A-C*), mutant H1 could be found along the plasma membrane and in endosomes, as well as in coated pits (*D–F*). No label was found in typical flask-shaped, noncoated invaginations. To exclude the possibility that the immunoglobulin-gold complexes caused receptor cross-linking on the surface of unfixed cells, receptor distribution was also analyzed in prefixed cells. After in situ fixation, the cells were incubated with a polyclonal receptor-specific antiserum followed by sheep anti-rabbit immunoglobulin coupled to 5-nm gold. Also by this procedure mutant H1 could be detected in coated pits (Fig. 9), confirming the biochemical data.

Quantitation of gold particles on the total plasma membrane and in coated pits of in situ-fixed 1-7 and Fl(5A)-1 cells

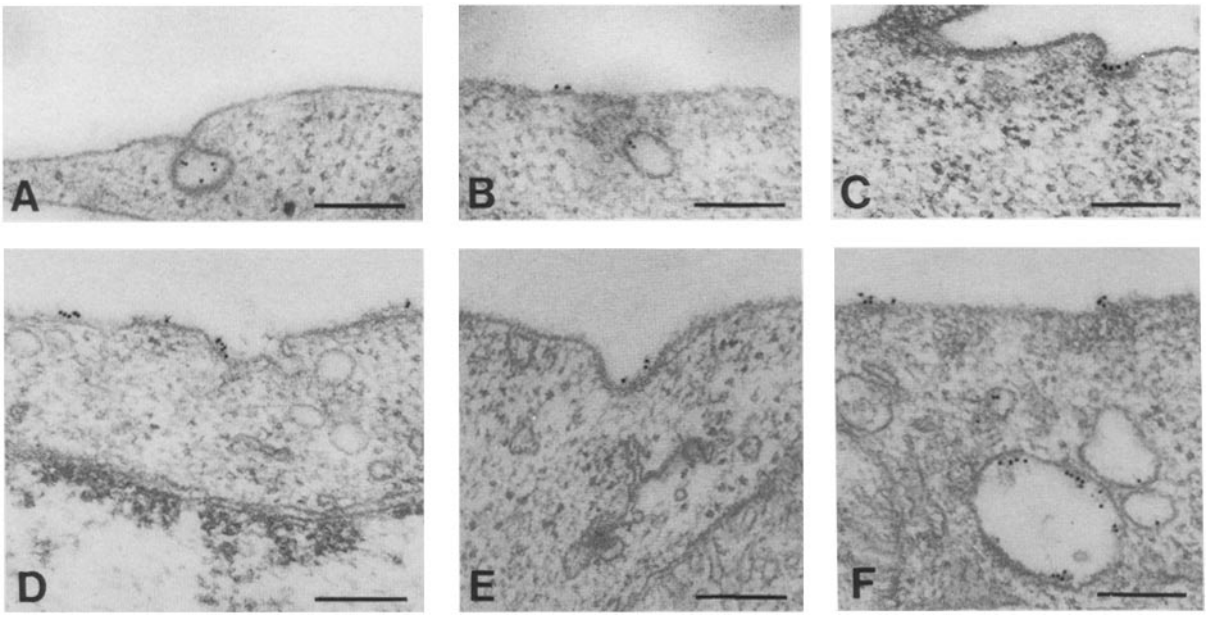

Figure 8. Immunolocalization of H1 protein on the plasma membrane and in endocytic compartments of 1-7 and Fl(5A)-1 cells. Living cells were first incubated for 1 h at 4°C with affinity-purified H1-specific antibodies coupled to 8 nm gold. Cells were then shifted to 37°C for 10 min. In 1-7 cells, gold label was found on the plasma membrane as well as in coated pits and endosomal compartments (*A–C*). Similarly, mutant H1 could also be localized in coated pits of Fl(5A)-1 cells (*D–F*) but not in smooth membrane invaginations. In untransfected 3T3 cells, no plasma membrane label was detectable (not shown). Bars, 200 nm.

is summarized in Table I. Coated pits were found to account for a similar fraction, ~2%, of the plasma membrane in both cell lines. Based on the number of gold particles, ~6% of wild-type H1, but only 1.7% of mutant H1 were localized in coated pits. These numbers are in excellent agreement with the internalization rates determined biochemically. Comparison of the gold densities in coated pits and in the total plasma membrane shows that wild-type H1 was clearly concentrated in coated pits by a factor of 2.3. The density of the mutant protein in coated areas, however, was very similar to that in the uncoated plasma membrane and even somewhat lower. Yet, mutant H1 was not dramatically excluded from coated pits as has been shown for resident proteins (Bretscher et al., 1980; Miettinen et al., 1989; Ktistakis et al., 1990; see Discussion). These findings confirm that the ASGP receptor subunit H1 retains a reduced affinity to the clathrin-coated plasma membrane domains independently of tyrosine residues.

Discussion

The large diversity of cytoplasmic sequences of endocytic receptors suggests that the recognition signal for clustering in coated pits is degenerate, perhaps comparable to the signals that target proteins to the ER or to mitochondria (Verner and Schatz, 1988). This notion is confirmed by the finding that replacement of the cytoplasmic portion of the chicken liver glycoprotein receptor with unrelated sequences did not entirely eliminate internalization and coated pit localization (Verrey et al., 1990). A naturally occurring mutant of the LDL receptor indicated the involvement of a tyrosine (Davis et al., 1986), a residue that is also present in the cytoplasmic domains of most other endocytic receptors. Studies by Roth and co-workers (Lazarovits and Roth, 1988; Ktistakis et al., 1990) showed that mutation of a single cytoplasmic residue to a tyrosine can be sufficient to direct the resident plasma membrane proteins hemagglutinin and glycophorin A to the

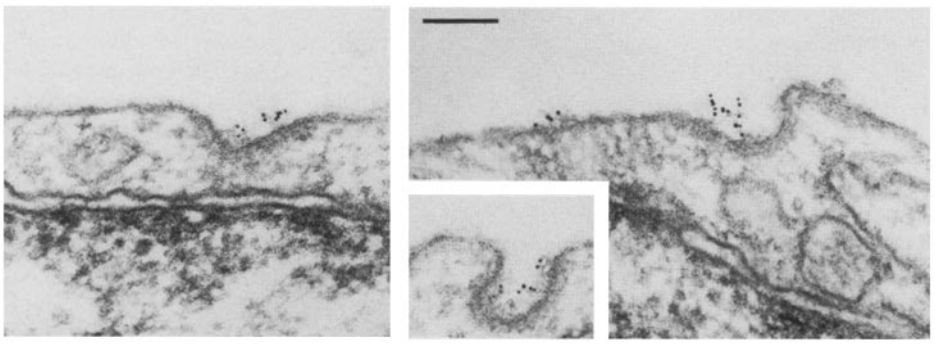

Figure 9. Immunolocalization of mutant H1 on the plasma membrane of prefixed Fl(5A)-1 cells. Cells fixed in situ were incubated for 4 h at 4°C with a polyclonal antiserum raised against ASGP receptor purified from human liver, and immunoreactive products were detected by incubation for 2 h at 4°C with secondary sheep anti-rabbit immunoglobulins coupled to 5-nm gold particles. Gold particles are found on the plasma membrane as well as in coated pits. Bar, 100 nm.

Table I. Surface Distribution of Wild-Type and Mutant H1 Determined by Immunogold Electron Microscopy

Cell line	Fraction of PM occupied by CP	Fraction of gold particles in CP	Gold particles/μm PM	Gold particles/μm CP	Ratio of densities CP/PM
	%				
1-7	2.60 ± 0.41	5.97 ± 1.05	2.93 ± 0.33	6.73 ± 1.41	2.30 ± 0.55
F1(5A)-1	1.97 ± 0.35	1.72 ± 0.45	3.77 ± 0.38	3.21 ± 0.96	0.87 ± 0.28

Cells fixed in situ with 4% paraformaldehyde were incubated for 1 h at room temperature with a polyclonal antiserum against ASGP receptor. Immunoreactive products were detected by incubation for 1 h with protein A coupled to 9-nm gold particles. Samples were processed for electron microscopy and a set of 28 randomly selected micrographs were quantitated as described in Materials and Methods. The means with standard errors are listed. *CP*, coated pits; *PM*, total plasma membrane.

endocytic pathway. However, there are also proteins lacking cytoplasmic tyrosines or aromatic residues in general, which are nevertheless internalized via clathrin-coated vesicles. An example is the gC glycoprotein of Herpes simplex virus (Roth et al., 1986). It is thus important to experimentally demonstrate the involvement of tyrosines in clustering in coated pits for each endocytic protein. This has been published for the LDL, the mannose-6-phosphate, the transferrin, and the poly-Ig receptor (Davis et al., 1986, 1987; Lobel et al., 1989; Jing et al., 1990; Alvarez et al., 1990; Breitfeld et al., 1990). In the present study, we have shown for the major subunit H1 of the human ASGP receptor that tyrosine-5 is important for efficient internalization. Mutation of this residue to an alanine reduced the rate of constitutive endocytosis by a factor of approximately four. Consistent with this reduction of the internalization rate, the fraction of H1 present on the cell surface at steady-state was increased from 50% for the wild-type protein to 80–85% for mutant H1.

However, when the critical tyrosine of the LDL (Davis et al., 1986), the poly-Ig (Breitfeld et al., 1990), and the transferrin receptor (Jing et al., 1990; Alvarez et al., 1990) was mutated, the mutant receptors were still more efficiently internalized than the respective tail-less proteins. Furthermore, for these receptors and also for the ASGP receptor H1, mutation of the critical tyrosine to a nonaromatic amino acid did not reduce the internalization rate to the level of bona fide resident plasma membrane proteins. While wild-type H1 was taken up at a rate of ~6% of the surface population per minute, mutant H1 was still internalized at 1.5%/min. In contrast, hemagglutinin internalization was not detectable within 10 min and was <10% in 2 h (Lazarovits and Roth, 1988; Ktistakis et al., 1990). Internalization of the mutant hemagglutinin HA-Y543, which occurred at a rate of 4.5%/min (Lazarovits and Roth, 1988), is therefore solely due to mutation of cysteine-543 to tyrosine. The same situation pertains to the mutation of serine-106 to tyrosine in glycophorin A (Ktistakis et al., 1990).

In the four natural endocytic receptors mentioned above, the tyrosine residue is required only for rapid internalization, but significant endocytosis occurs even in its absence. Two mechanisms could explain this behavior. The mutant receptors might enter the cell through a clathrin-independent pathway, as has been previously suggested (Davis et al., 1986). Alternatively, the signal for sorting into coated pits might be (at least partially) redundant, resulting in incomplete inactivation by mutation of any single amino acid. To distinguish these possibilities, we have analyzed internalization of wild-type and mutant ASGP receptor under conditions that selectively block the formation of clathrin-coated vesicles but still allow endocytosis of fluid phase and of certain surface markers (e.g., surface-bound ricin; Sandvig et al., 1987). For this purpose, the cytoplasm of the cells was acidified using the procedure by Sandvig et al. (1987). Under conditions where uptake of the fluid phase marker Lucifer yellow is reduced by less than 40%, ligand internalization by the functional, heterooligomeric ASGP receptor in 1-7-1 cells was entirely blocked. Ligand-independent, constitutive endocytosis of the heterooligomeric H1-H2 complex in 1-7-1 cells and of the wild-type subunit H1 alone in 1-7 cells, as well as the residual endocytosis of mutant H1 in F1(5A)-1 cells was also eliminated upon acidification. These results strongly suggest that ASGP receptor proteins are internalized exclusively via clathrin-coated pits.

This conclusion was confirmed by direct localization of wild-type and mutant H1 in coated pits by immunogold electron microscopy. The fractions of wild-type and mutant H1 detected in coated pits, 5.9 and 1.7%, respectively, correlate well with the relative internalization rates of the two proteins. The value for the mutant receptor is significantly higher than the coated pit fractions determined for the resident plasma membrane protein glycophorin A, 0.4% (Ktistakis et al., 1990), and the tail-less Fc receptor, 0.55% (Miettinen et al., 1989). Mutant H1 is neither concentrated in coated pits like wild-type H1 nor is it significantly excluded. From our data it cannot be formally ruled out that some proteins might neither bind to nor be excluded from coated pits resulting in a distribution similar to that of the tyrosine mutant of H1. However, our data are consistent with the model that membrane proteins are sorted into coated pits by a cytoplasmic signal interacting with specific components of the coat (the assembly or adaptor proteins; Pearse and Robinson, 1990; Keen, 1990). The extent to which a protein is concentrated in coated pits depends on the affinity by which its cytoplasmic domain interacts with the coat. As endocytic proteins are accumulated, other proteins are passively excluded from the coated area. The fact that the mutant H1 density in coated pits was only slightly reduced in comparison to the density in the uncoated membrane therefore indicates that it still retained some affinity for assembly proteins. In addition, proteins may be prevented from entering coated pits by interaction with cytoskeletal elements. This has been suggested for the B1 isoform of the FcRII receptor, since it was found to be almost completely absent from coated pits (only 0.12% were localized in coated pits; Miettinen et al., 1989).

These findings thus show that mutation of tyrosine-5 only partially inactivates recognition of H1 for clustering in coated pits. Whether natural receptors contain more than one independent recognition signal with different affinities to clath-

rin-coated pits or a single signal with a tyrosine residue essential only for high affinity recognition remains to be investigated.

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References

Alvarez, E., N. Girones, and R. J. Davis. 1990. A point mutation in the cytoplasmic domain of the transferrin receptor inhibits endocytosis. *Biochem. J.* 267:31-35.

Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* 51:531-554.

Breitfeld, P. P., C. F. Simmons, G. J. A. M. Strous, H. J. Geuze, and A. L. Schwartz. 1985. Cell biology of the asialoglycoprotein receptor system: a model of receptor-mediated endocytosis. *Int. Rev. Cytol.* 97:47-95.

Breitfeld, P. P., J. E. Casanova, W. C. Mckinnon, and K. E. Mostov. 1990. Deletions in the cytoplasmic domain of the polymeric immunoglobulin receptor differentially affect endocytotic rate and postendocytotic traffic. *J. Biol. Chem.* 265:13750-13757.

Bretscher, M. S., J. N. Thomson, and B. M. F. Pearse. 1980. Coated pits act as molecular filters. *Proc. Natl. Acad. Sci. USA.* 77:4156-4159.

Cone, R. D., and R. C. Mulligan. 1984. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc. Natl. Acad. Sci. USA.* 81:6349-6353.

Davis, C. G., M. A. Lehrman, D. W. Russell, R. G. W. Anderson, M. S. Brown, and J. L. Goldstein. 1986. The J. D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. *Cell.* 45:15-24.

Davis, C. G., I. R. van Driel, D. W. Russell, M. S. Brown, and J. L. Goldstein. 1987. The low density lipoprotein receptor. Identification of amino acids in cytoplasmic domain required for rapid endocytosis. *J. Biol. Chem.* 262: 4075-4082.

Geffen, I., H. P. Wessels, J. Roth, M. A. Shia, and M. Spiess. 1989. Endocytosis and recycling of subunit H1 of the asialoglycoprotein receptor is independent of oligomerization with H2. *EMBO (Eur. Mol. Biol. Organ.) J.* 8: 2855-2862.

Glickman, J. N., E. Conibear, and B. M. F. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor-II receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1041-1047.

Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature (Lond.).* 279: 679-685.

Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* 1:1-39.

Griffiths, G., R. Back, and M. Marsh. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. *J. Cell Biol.* 109:2703-2720.

Henis, Y. I., Z. Katzir, M. A. Shia, and H. F. Lodish. 1990. Oligomeric structure of the human asialoglycoprotein receptor: nature and stoichiometry of mutual complexes containing H1 and H2 polypeptides assessed by fluorescence photobleaching recovery. *J. Cell Biol.* 111:1409-1418.

Heuser, J. 1989. Effects of cytoplasmic acidification on clathrin lattice morphology. *J. Cell Biol.* 108:401-411.

Hopkins, C. R., and I. S. Trowbridge. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97:508-521.

Hubbard, A. L. 1989. Endocytosis. *Curr. Op. Cell Biol.* 1:675-683.

Jing, S. Q., T. Spencer, K. Miller, C. Hopkins, and I. S. Trowbridge. 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. *J. Cell Biol.* 110:283-294.

Kawai, S., and M. Nishizawa. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.* 4:1172-1174.

Keen, J. H. 1990. Clathrin and associated assembly and disassembly proteins. *Annu. Rev. Biochem.* 59:415-438.

Korman, A. J., J. D. Frantz, J. L. Strominger, and R. C. Mulligan. 1987. Expression of human class II major histocompatibility complex antigens using retrovirus vectors. *Proc. Natl. Acad. Sci. USA.* 84:2150-2154.

Kramer, W., V. Drutsa, H. W. Jansen, B. Kramer, M. Pflugfelder, and H. J. Fritz. 1984. The gapped duplex DNA approach to oligonucleotide-directed mutation construction. *Nucleic Acids Res.* 12:9441-9456.

Ktistakis, N. T., D. Thomas, and M. G. Roth. 1990. Characterization of the tyrosine recognition signal for internalization of transmembrane surface glycoproteins. *J. Cell Biol.* 111:1393-1407.

Lazarovits, J., and M. Roth. 1988. A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. *Cell.* 53:743-752.

Lehrman, M. A., J. L. Goldstein, M. S. Brown, D. W. Russell, and W. J. Schneider. 1985. Internalization-defective LDL receptors produced by genes with nonsense and frameshift mutations that truncate the cytoplasmic domain. *Cell.* 41:735-743.

Lobel, P., K. Fujimoto, R. D. Ye, G. Griffiths, and S. Kornfeld. 1989. Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. *Cell.* 57: 787-796.

Lucocq, J. M., and W. Baschong. 1986. Preparation of protein colloidal gold complexes in the presence of commonly used buffers. *Eur. J. Cell Biol.* 42:332-337.

Lund, K. A., L. K. Opresko, C. Starbuck, B. J. Walsh, and H. S. Wiley. 1990. Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization. *J. Biol. Chem.* 265:15713-15723.

McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077-1083.

Miettinen, H. M., J. K. Rose, and I. Mellman. 1989. Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic domain heterogeneity. *Cell.* 58:317-327.

Moore, M. S., D. T. Mahaffey, F. M. Brodsky, and R. G. W. Anderson. 1987. Assembly of clathrin-coated pits onto purified plasma membranes. *Science (Wash. DC).* 236:558-563.

Mostov, K. E., A. de Bruyn Kops, and D. L. Deitcher. 1986. Deletion of the cytoplasmic domain of the polymeric immunoglobulin receptor prevents basolateral localization and endocytosis. *Cell.* 47:359-364.

Pearse, B. M. F. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3331-3336.

Pearse, B. M. F., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151-171.

Prywes, R., E. Livneh, A. Ullrich, and J. Schlessinger. 1986. Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2179-2190.

Roth, M. G., C. Doyle, J. Sambrook, and M. J. Gething. 1986. Heterologous transmembrane and cytoplasmic domains direct functional chimeric influenza virus hemagglutinins into the endocytic pathway. *J. Cell Biol.* 102: 1271-1283.

Rothenberger, S., B. J. Iacopetta, and L. C. Kühn. 1987. Endocytosis of the transferrin receptor requires the cytoplasmic domain but not its phosphorylation site. *Cell.* 49:423-431.

Sandvig, K., S. Olsnes, O. W. Peterson, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* 105:679-689.

Schwartz, A. L., and D. Rup. 1983. Biosynthesis of the human asialoglycoprotein receptor. *J. Biol. Chem.* 258:11249-11255.

Shia, M. A., and H. F. Lodish. 1989. The two subunits of the human asialoglycoprotein receptor have different fates when expressed alone in fibroblasts. *Proc. Natl. Acad. Sci. USA.* 86:1158-1162.

Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 38:87-93.

Spiess, M. 1990. The asialoglycoprotein receptor: a model for endocytic transport receptors. *Biochemistry.* 29:1009-1018.

Spiess, M., and H. F. Lodish. 1985. Sequence of a second human asialoglycoprotein receptor: conservation of two receptor genes during evolution. *Proc. Natl. Acad. Sci. USA.* 82:6465-6469.

Spiess, M., A. L. Schwartz, and H. F. Lodish. 1985. Sequence of human asialoglycoprotein receptor cDNA. An internal signal sequence for membrane insertion. *J. Biol. Chem.* 260:1979-1982.

Swanson, J. A., B. D. Yirinec, and S. C. Silverstein. 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *J. Cell Biol.* 100:851-859.

Thompson, J. A., A. L. Lau, and D. D. Cunningham. 1987. Selective radiolabeling of cell surface proteins to a high specific activity. *Biochemistry.* 26:743-750.

van Deurs, B., O. W. Petersen, S. Olsnes, and K. Sandvig. 1989. The ways of endocytosis. *Int. Rev. Cytol.* 117:131-177.

Verner, K. and G. Schatz. 1988. Protein translocation across membranes. *Science (Wash. DC).* 241:1307-1313.

Verrey, F., T. Gilbert, T. Mellow, G. Proulx, and K. Drickamer. 1990. Endocytosis via coated pits mediated by glycoprotein receptor in which the cytoplasmic tail is replaced by unrelated sequences. *Cell Reg.* 1:471-486.

Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. *Cell.* 21:79-93.

Weibel, E. R. 1979. Stereological Methods. I. Practical Methods for Biological Morphometry. Academic Press, Inc., New York.