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Abstract

Lateral mobility studies comparing native and mutated membrane proteins, combined with treatments that alter clathrin lattice structure, can measure membrane protein-coated pit interactions in intact cells (Fire, E., Zwart, D., Roth, M. G., and Henis, Y. I. (1991) J. Cell Biol. 115, 1585-1594). We applied this approach to study the interactions of the H1 and H2 human asialoglycoprotein receptor subunits with coated pits. The lateral mobilities of singly expressed and coexpressed H1 and H2B (the H2 species that reaches the cell surface) were measured by fluorescence photobleaching recovery. They were compared with mutant proteins, H1(5A) (Tyr-5 replaced by Ala) and H2(5A) (Phe-5 replaced by Ala). While the mobile fractions of H1, H2B, and their mutants were similar, the lateral diffusion rate (measured by D, the lateral diffusion coefficient) was significantly slower for H1, whether expressed alone or with H2B. Coexpression with H1 reduced D of H2B to that of H1. Disruption of the clathrin lattices by hypertonic medium elevated D of H1, H1(5A), H2B, and H2(5A) to the same final level, without affecting their mobile fractions. Cytosol acidification, which retards altered clathrin lattices attached to the membrane and prevents coated vesicle formation, immobilized part of the H1 molecules, reflecting stable entrapment in "frozen" coated pits. H1(5A), H2B, and H2(5A) were not affected; however, coexpression of H2B with H1 conferred the sensitivity to cytosol acidification on H2B. Our results suggest that H1 lateral mobility is inhibited by dynamic interactions with coated pits in which Tyr-5 is involved. H2B resembles H1(5A) rather than H1, and its interactions with coated pits are weaker; efficient interaction of H2B with coated pits depends on complex formation with H1.
Dynamic Interactions of the Asialoglycoprotein Receptor Subunits with Coated Pits

ENHANCED INTERACTIONS OF H2 FOLLOWING ASSOCIATION WITH H1*

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In many cell types, the initial step in receptor-mediated endocytosis is the clustering of specific receptors in plasma mem-
brane clathrin-coated pits (1). The determinants responsible for
the internalization of numerous receptors via coated pits reside in their cytoplasmic domains (2–6). Recent evidence identifies at least one class of a degenerate cytoplasmic peptide “recog-
nition signal” necessary and sufficient for coated pit-mediated internalization (7–10). The common motif in these short se-
quences is at least one aromatic residue, typically a tyrosine, in
a secondary structure that assumes a tight turn conformation
(8, 11–15).

Clathrin-associated assembly proteins (APs),† also called adaptors, were proposed to interact with both clathrin lattices
and membrane proteins destined for endocytosis (7). Two adap-
tor complexes, AP-2 (associated with plasma membrane coated
pits) and AP-1 (associated with trans-Golgi coated pits), were
characterized (16, 17). Recent in vitro studies demonstrated
interactions of AP-2 or their adaptin subunits with cytoplasmic
internalization signals of several receptors (18–22), and the
binding of adaptors to plasma membrane fragments has been
reconstituted in broken cell systems (23–26). Thus far, studies
on the interactions between membrane receptors and coated
pit-associated components have been limited to in vitro assays
employing solubilized or immobilized (on column or by blotting)
proteins (18–22). Clearly, it is desired to characterize these
interactions at the surface of the intact cell.

We have recently demonstrated that comparative studies of the lateral mobility of native and mutated membrane proteins
can measure their interactions with coated pits in intact cells
(27). In these studies, a mutant influenza hemagglutinin (HA)
protein containing a cytoplasmic tyrosine recognition signal
(HAY534S) was shown to interact dynamically with coated
pits, while no interactions were detected for wild type HA,
which lacks a tyrosine signal and is excluded from coated pits
(27). To investigate the nature of such interactions in naturally
occurring membrane receptors, we have now applied this ap-
proach to the human asialoglycoprotein (ASGP) receptor.
The endocytosis and recycling of this hepatocyte receptor, which
internalizes desialylated, galactose-terminal serum glycopro-
teins, have been well characterized (28, 29). It is comprised of
two homologous subunits, H1 and H2 (30, 31), which form a
complex containing H1 and H2 at a (3–4:1 ratio (32). Of the
four alternatively spliced forms of H2, the one that contains the
18-nucleotide insertion (relative to H1) in the cytoplasmic
domain but misses the 5-codon insertion at the exoplasmic end
of the transmembrane domain (termed H2B; Ref. 30) is trans-
ported to the cell surface and participates in the mature recep-
tor complexes (33–36). Although high affinity ligand binding requires co-
expression of H1 and H2 (37, 38), singly expressed H1
undergoes constitutive endocytosis and recycling (39). The ami-

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‡ The abbreviations used are: AP, assembly protein; HA, influenza
virus hemagglutinin protein; ASGP, asialoglycoprotein; FPR, fluores-
ence photo bleaching recovery; TMR, tetramethylrhodamine; ASOR,
asialoorosomucoid; DMEM, Dulbecco's modified Eagle's medium;
HBSS, Hank's balanced salt solution; BSA, bovine serum albumin;
RHL-1, rat hepatic lectin 1; GAR, goat anti-rabbit.

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no-terminal cytoplasmic domain of H1 contains a single tyrosine residue (position 5), whose substitution by alanine to produce the tyrosine-deficient mutant H1(5A) reduces the endocytosis rate 4-fold (40). A similar phenomenon occurs upon deletion of residues 4–11 in the H1 cytoplasmic tail, but is not upon replacement of Tyr-5 with Phe (41), suggesting that the aromatic nature of Tyr-5 is essential for efficient endocytosis of H1. The highly homologous H2 subunit contains phenylalanine at position 5 but, in spite of this, is not internalized efficiently (42).

In this study, we investigated the nature of the interactions of H1 and H2B (expressed singly or together) with coated pits in living cells. By combining fluorescence photobleaching recovery (FPR) studies on the lateral mobility of native and mutated ASGP receptor subunits with treatments that alter the clathrin lattice structure, we explored the mode of these interactions (dynamic on/off interactions versus permanent entrapment) and their dependence on Tyr-5 of H1 or Phe-5 of H2. Our results indicate that H1 interacts dynamically with coated pits, and that Tyr-5 is required for these interactions. The interactions of H2 with coated pits are weaker, do not involve Phe-5, and are significantly enhanced upon coexpression with H1.

**EXPERIMENTAL PROCEDURES**

**Materials—**Tetramethylrhodamine (TMR) 5-isothiocyanate was obtained from Molecular Probes (Eugene, OR). Na125I was from Amersham Corp. Amiloride hydrochloride and human mucous were from Sigma. Asialoglycoprotein (ASOR) was prepared by desialylation of human mucus (38, 43). It was iodinated by Na125I using Enzymobeads (Bio-Rad) following the manufacturer’s protocol. Highly specific anti-peptide antibodies raised in rabbits against the COOH-terminal regions of H1 or H2, which did not cross-react with each other, were obtained by us previously (32). Affinity-purified goat IgG directed against rabbit IgG (GAR IgG) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Monovalent Fab’ fragments were prepared from both the rabbit anti-peptide IgG and the GAR IgG as described (44). Both Fab’ preparations were free of contamination by Fab” or IgG, as judged by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. GAR Fab’ tagged with TMR isothiocyanate (TMR-GAR Fab’) was prepared following standard labeling procedures (45). Tissue culture media were from Biological Industries, Beth Haemek, Israel.

**Cell Culture—**The stably transfected cell lines 1-17 (expressing H1), 1-7-1 (expressing H1 and H2B), and 1-7-1H1 (expressing H1) and H2 (41) were derived from NIH 3T3 fibroblasts using the retroviral vector pDO-L (32, 38). The doubly transfected 1-7-1 line expresses H2B together with H1 and is fully active in ligand binding and internalization (38). The F1(5A)-1 line, which expresses the H1(5A) mutant H1 protein (Tyr-5 replaced by Ala), was prepared by transfecting NIH 3T3 cells using the retroviral vector pDO-L5A (41). An NIH 3T3 line expressing H2B(5A) (Phe-5 replaced by alanine) was prepared using the pLJ-derived retroviral vector pBarnHis (42) and is designated F2(5A)-2. The stably expressing NIH 3T3 cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin (Biological Industries).

The human hepatoma cell line HepG2, which naturally expresses both H1 and H2, was grown in minimum essential medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM glutamine, and penicillin-streptomycin as described previously (46). Cells were routinely grown in 75-cm2 flasks, and split upon reaching 80% confluence (with 1:10 dilution) to allow receptor recycling. They were then treated to alter coated pit structure as described above, while untreated control dishes were incubated instead in HBSS/Hepes/cytocrome c buffer. The dishes were incubated with for 2 µg/ml, 2 x 104 cpm/dish, 2 h, 4 °C) in the appropriate buffers of the various treatments, washed with cold buffers, and shifted to 37 °C for various periods to allow internalization. Samples were withdrawn from the warm-up buffer to the amount of 0.1 ml. The amount of 0.1 ml was then recovered at 4 °C (with three washes after each incubation) with the following antibodies: 1) normal goat IgG (50 µg/ml, 30 min), 2) anti-H1 or anti-H2 Fab’ (150 µg/ml, 45 min), and 3) TMR-GAR Fab’ (100 µg/ml, 45 min). All labeling steps were performed in the cold to eliminate internalization and allow only surface labeling by the antibodies. In cases where the cells were preincubated in specific buffers to disperse or alter coated pit structure (see below), the specific buffers employed for each treatment were used throughout all antibody-incubation steps and the lateral mobility measurement.

**Fluorescence Photobleaching Recovery—**Lateral diffusion coefficients (D) and mobile fractions (R) of H1, H1(5A), H2B, or H2(5A) were measured by FPR (47, 48) using previously described instrumentation (49). The bleaching conditions used in the FPR studies were not shown to alter the lateral mobilities measured (50, 51). Following the labeling with fluorescent monovalent Fab’ fragments (see “immunofluorescent labeling and the FPR experiments with the cytosol-acidified cells.”), the cells were extracted in fluorescence noninvasive nonlinear regression analysis (52). Incomplete fluorescence recovery was interpreted to represent fluorophores that are immobile on the FPR experimental time scale (D ≈ 5 x 10-12 cm²/s).

**Treatments Affecting Coated Pit Structure—**The treatments employed to disrupt or alter the clathrin lattice structure were hypotonic medium treatment (53–55) or sodium acidification (55–58). For treatment with hypotonic medium, the cells were incubated (20 min, 37 °C) in HBSS/Hepes/BSA-supplemented with 0.45 mM sucrose. Under these conditions, the clathrin lattices underlying coated pits are disrupted, and coated pit-mediated endocytosis is blocked (54, 55). The cells were kept in the hypertonic buffer during the labeling with Fab’ fragments and the FPR experiments, or during ligand internalization assays; in the latter case, cytochrome c (0.02%) replaced the BSA to eliminate interference by BSA with ASOR binding (56).

**Cytosol acidification was performed as described (27, 56). Cells were incubated in Hepes-buffered DMEM (pH 7.2) containing 30 mM NH4Cl (30 min, 37 °C), and then 5 min at 37 °C in potassium-amiloride (KA) buffer (0.14 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM amiloride hydrochloride, 20 mM Hepes, pH 7.2). The buffer was replaced by cold KA supplemented with 2% BSA (KA/BSA), which was employed for the Fab’ labeling and the FPR experiments with the cytosol-acidified cells. Similarly, in the case of cytchrome c (0.02%), the cell were replaced by the KHCl buffer (55). In this case, the Fab’ labeled the cells, and the FPR experiments were performed as above. The above treatment results in cytoplasmic pH values of 5.6–5.8 and blocks coated-pit-mediated endocytosis by altering and “freezing” the coated pits, thus eliminating the pinching-off of coated vesicles (55, 56, 58).

**Internalization Assay—**Internalization of 125I-ASOR was measured essentially as described (60, 61). Cells grown in 35-mm dishes to 80–90% confluence were incubated (30 min, 37 °C) in serum-deficient Hepes-buffered medium to allow receptor recycling. They were then treated to alter coated pit structure as described above, while untreated control dishes were incubated instead in HBSS/Hepes/cytocrome c buffer. The dishes were incubated with 125I-ASOR (2 µg/ml, 2 x 105 cpm/dish, 2 h, 4 °C) in the appropriate buffers of the various treatments, washed with cold buffers, and shifted to 37 °C for various periods to allow internalization. Samples were withdrawn from the warm-up buffer to the amount of 0.1 ml. The amount of 0.1 ml was then recovered at 4 °C (with three washes after each incubation) with the following antibodies: 1) normal goat IgG (50 µg/ml, 30 min), 2) anti-H1 or anti-H2 Fab’ (150 µg/ml, 45 min), and 3) TMR-GAR Fab’ (100 µg/ml, 45 min). All labeling
RESULTS

The approach employed in the present study is based on the theoretical and experimental demonstration that interactions of membrane proteins with either membranous or submembranous immobile structures are capable of retarding their lateral motion or fully immobilizing them (32, 62, 63). Plasma membrane coated pits can induce such effects, since they are essentially immobile in lateral mobility measurements by FPR (64), and have been shown to retard the lateral motion of a mutant influenza HA protein containing a tyrosine internalization signal (27). Therefore, if H1 and/or H2 interact with coated pits, their lateral mobility should be restricted relative to mutant proteins that carry a defective internalization signal (e.g. H1(5A)). Furthermore, this restriction should depend on the integrity and state of the coated pits underlying the plasma membrane. Thus, the experimental design involved treatment of cells under conditions that disperse or alter coated pit structure, followed by measurements of their effects on the lateral mobilities of native or mutant ASGP receptor proteins.

Ligand Endocytosis by ASGP Receptors is Blocked by Treatments That Alter Coated Pit Structure—Since alterations in the lateral mobility of ASGP receptor subunits following specific treatments to disrupt or change the structure of clathrin-coated pits (hypertonic medium treatment and cytosol acidification) are an important feature of the current studies, it was necessary to demonstrate the effectiveness of these treatments. To that end, their effects on the endocytosis of ligand (125I-ASOR) by the ASGP receptors were measured in the cell types employed (NIH 3T3 and HepG2 cells). The data (Fig. 1) demonstrate that both treatments completely blocked 125I-ASOR endocytosis in the two cell types (1-7-1 and HepG2). Thus, either disassembly of the clathrin lattices by the hypertonic medium (53–55) or acidification of the cytosol to eliminate the pinching-off of coated pits (55, 56, 58) effectively block ligand internalization via the ASGP receptors, in accord with the notion that it occurs via clathrin-coated pits (29, 65). This is in agreement with the finding (39, 40) that under identical conditions, cytosol acidification also blocked the constitutive endocytosis (in the absence of ligand) of singly expressed H1 (in 1-7 cells) or H1(5A) (in F1(5A)-1 cells).

H1 Mobility Is Restricted Relative to H1(5A) in Untreated Cells but Not following Hypertonic Medium Treatment—In order to measure the lateral mobilities of H1 and H1(5A), these receptors were labeled in the cold at the cell surface by Fab' fragments of highly specific anti-peptide antibodies directed against the COOH-terminal region of H1, followed by TMR-GAR Fab'; we have shown earlier (32) that under the conditions employed this labeling method by monoclonal Fab' fragments results in lateral mobilities identical to those obtained using TMG-tagged anti-H1 Fab' but yields a significantly improved signal to noise ratio. The FPR studies were conducted at 22 °C. This temperature was chosen in order to minimize internalization of the labeled receptors during the FPR measurements; lower temperatures were avoided since earlier studies (27) showed that, while the interactions of the endocytosis-competent HA(Y543) mutant with coated pits were rather similar at 37 and 22 °C, they became significantly weaker at 12 °C. Typical FPR curves obtained for the lateral mobility of H1 on the surface of 1-7 cells under normal and hypertonic medium conditions are depicted in Fig. 2. The average results of many analogous measurements performed on several cell types, expressing H1 (singly or together with H2B) or H1(5A), are shown in Fig. 3. Several observations can be made on the basis of these data. First, it is apparent that the hypertonic medium treatment, which disperses the clathrin lattices underlying coated pits (54) as well as flat clathrin lattices (55), induced a significant increase in the lateral diffusion rate (reflected in the D values) of H1, while the mobile fractions (Rv values) were not affected. This is exactly the effect expected if H1 molecules at the cell surface interact dynamically with coated pits, i.e. undergo several association-dissociation cycles with coated pits during the FPR measurement. Such dynamic interactions would reduce the apparent diffusion rate of H1, since each H1 molecule will spend a fraction of the time bound to coated pits but will be free to diffuse in the membrane during the dissociation cycle (27, 32, 62, 63; see "Discussion"). The notion that the lateral diffusion rate of H1 is slowed down by interactions with coated pits gains further support by comparing the lateral diffusion of singly expressed H1 (in 1-7 cells) with that of singly expressed H1(5A) (in F1(5A)-1 cells); while the H1(5A) mutant protein exhibits a higher D value in untreated cells, disruption of the coated pit structure by hypertonic medium elevates the D values of H1 and H1(5A) to the same level (Fig. 3). This phenomenon suggests that the slower lateral mobility of H1 relative to H1(5A) is due to stronger interactions of H1 with coated pits, in accord with the finding that the internalization of the tyrosine-deficient H1(5A) is slower than that of H1 but still occurs through the clathrin-coated pits pathway (40). Finally, it
FIG. 2. Typical FPR curves of the lateral mobility of H1 in the plasma membrane of 1-7 cells. Cells were grown on glass coverslips and labeled with anti-H1 Fab' followed by TMRGAR Fab' as described under "Experimental Procedures." The FPR measurements were performed in HBSS/Hepe/BSA at 22°C. The dots represent the experimentally determined fluorescence intensity; the solid lines are the best fit to the lateral diffusion equation using nonlinear regression (52). A, a representative curve depicting H1 lateral diffusion at the surface of untreated 1-7 cells (NIH 3T3 line singly transfected with H1). The curve shown yielded $D = 1.6 \times 10^{-9} \text{ cm}^2/\text{s}$, and $R_p = 0.52$. B, a representative curve on the same cells following hypertonic medium treatment. This experiment yielded $D = 4.8 \times 10^{-10} \text{ cm}^2/\text{s}$, and $R_p = 0.48$.

is interesting to note that the rise in the $D$ value of H1 following hypertonic medium treatment is detected in all of the cell types examined, whether they express H1 alone or together with H2. Furthermore, examination of the $D$ and $R_p$ values for H1 expressed singly or together with H2B in the same cell type (NIH 3T3; 1-7 and 1-7-1 cells, respectively) reveals that they are essentially identical (Fig. 3). Since H1 and H2 in these cells reside within mutual complexes (32, 66), this suggests that the interactions of H2 with coated pits provide at most a minor contribution relative to those of H1 (see "Discussion"). This notion is supported by the studies on H2 mobility (Figs. 5 and 6) and is in line with the report that H2 is endocytosed at a rate similar to that of H1(5A) rather than H1 (42). As for the somewhat different lateral mobility parameters measured on HepG2 cells, they most likely reflect cell type differences (different densities of coated pits, different levels of other transmembrane proteins competing for binding to coated pits, and other factors such as differences in cytoskeletal organization and/or in the extracellular matrix).

H1 but Not H1(5A) Is Permanently Entrapped in Coated Pits following Cytosol Acidification—Acidification of the cytosol was shown to block receptor-mediated endocytosis via clathrin-coated pits, but by a mechanism different from that of the hypertonic medium treatment; it alters the morphology of the clathrin lattices and leaves the coated pits associated with the plasma membrane (56, 58). Thus, this treatment is not expected to eliminate the interactions of a given membrane protein with coated pits, but rather to alter them. We have demonstrated (27) that cytosol acidification shifts the dynamic interactions of HA(Y543), an endocytosis-competent HA mutant protein, into "permanent" entrapment in coated pits (for the entire duration of the measurement). Thus, acidification of the cytosol provides an alternative test to validate that the mobility restricting interactions experienced by H1 (and to a lesser degree by H1(5A)) are indeed with coated pits. The ef-

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Fig. 3. Effect of hypertonic medium on the lateral mobility of H1 and H1(5A). Cells (HepG2 and 1-7-1, coexpressing H1 and H2; 1-7, expressing H1; F1(5A)-1, expressing H1(5A)) were either not treated (control, blank bars) or subjected to treatment with sucrose-containing hypertonic media (cross-hatched bars) as described under "Experimental Procedures." The FPR experiments were conducted at 22°C, as in Fig. 2. Each bar is the mean ± S.E. of 30–60 measurements. A, $D$ values; B, $R_p$ values.
**Effects of Cytosol Acidification on the Lateral Mobility of H1 and H1(5A)**

Cells expressing H1 and H2 together (HepG2 and 1-7-1), H1 (1-7 cells), or H1(5A) (F1(5A)-1 cells) were grown on glass coverslips (precoated with fibronectin in the case of the NIH 3T3-derived lines) and subjected to the cytosol acidification treatment (see "Experimental Procedures"). The FPR experiments were conducted at 22 °C on untreated cells (blank bars) or on cytosol-acidified cells (cross-hatched bars). The bars represent the mean ± S.E. of 30-60 measurements in each case. A, D values; B, R values.

The effects of cytosol acidification on the lateral mobilities of H1 and H1(5A) in several cell lines are shown in Fig. 4. For these experiments, the NIH 3T3 cell lines (1-7-1, 1-7 and F1(5A)-1) were grown on coverslips precoated with fibronectin (100 μg/ml, 30 min); this was done in order to prevent the rounding up and detachment of these cells during the cytosol acidification treatment, which resulted in cell motion that interfered with the FPR experiments. These conditions induced a minor increase in the D values of H1 and H1(5A), for which reason separate control measurements on untreated cells growing on fibronectin coats were performed (Fig. 4). The fibronectin coating was not required for HepG2 cells, whose attachment and morphology were not significantly affected by the cytosol acidification treatment.

The effects of cytosol acidification on the lateral mobilities of H1 and H1(5A) clearly differed from those of the hypertonc medium treatment. For all cells expressing H1, the R value dropped significantly, while the D values were elevated close to the levels observed under hypertonic conditions. Such a phenomenon is suggestive of the formation of two populations of H1 molecules, which differ in their dynamic properties and which do not exchange on the FPR experimental time scale (about 1 min); while one population becomes essentially immobile for the entire duration of the FPR measurement (presumably in coated pits, and resulting in a decrease in the R value), the other is free to diffuse in the plasma membrane without being slowed down by transient exchange into coated pits. Interestingly, while the effect of cytosol acidification on singly expressed H1 (1-7 cells) was identical to that observed for H1 coexpressed with H2 (1-7-1 cells), the lateral mobility of the H1(5A) mutant was essentially unaffected. This suggests that unlike H1, the interactions of H1(5A) with coated pits are not altered significantly by the low pH-mediated change in the latter structures, most likely due to the lack of Tyr-5 in the mutant H1 molecule.

**Effects of Hypertonic Medium on the Lateral Mobility of H2B and H2(5A)**

Cells coexpressing H1 and H2B (HepG2 and 1-7-1), singly expressing H2B or H2(5A) (3-8 and F2(5A) cells, respectively) were either not treated (control, blank bars) or subjected to hypertonic medium treatment (cross-hatched bars) as described under "Experimental Procedures." The FPR experiments were conducted at 22 °C, as in Fig. 2. Each bar is the mean ± S.E. of 30-60 measurements. A, D values; B, R values.

H2 and H2(5A) Resemble H1(5A) and Show Only Weak Restriction of Their Lateral Mobility

H2B has a Phe instead of Tyr at position 5 in the cytoplasmic domain. Since Phe can replace Tyr in the internalization signals of several receptors, including H1 (41), it was of interest to investigate whether the lateral mobility of H2B is also retarded by interactions with coated pits and whether Phe-5 plays a role in such an effect. Fig. 5 depicts the results of measurements of the lateral mobility of H2B (expressed singly or together with H1) and H2(5A), along with the effects of dispersing the clathrin lattices by the hypertonc medium. Unlike the more pronounced lateral mobility inhibition of H1 relative to H1(5A) (Fig. 3), singly expressing H2B (3-8 cells) and its Phe-deficient mutant H2(5A) (F2(5A)-2 cells) exhibited identical D and R values (Fig. 5), suggesting that Phe-5 in H2B does not contribute significantly to the interactions with coated pits. Furthermore, the mobility-restricting interactions experienced by H2B or H2(5A) are significantly weaker than those experienced by H1, as indicated by their higher D values in untreated cells and by the smaller rise in D following the hypertonic medium treatment (Fig. 5). In this case as well, the elevation in D was similar to that measured for H1(5A) on F1(5A)-1 cells, demonstrating that H2B and H2(5A) resemble H1(5A) rather than H1 in the retardation of their lateral mobility. This resemblance holds also for the cytosol acidification treatment (Fig. 6), which had no effect on either R or D of singly expressed H2B and H2(5A). Thus, H2B and H2(5A) also resemble H1(5A) in their lack of sensitivity to the low pH-mediated alteration in the coated pit structure. The weaker interactions of H2B and H2(5A) with coated...
immobile structure, where the complex lifetime is long relative to the immobile structure for the entire duration of the molecule will be bound to the immobile structure for the entire duration of the membrane protein will be bound to the immobile structure for the entire duration of the protein between free and bound states relative to its lateral diffusion rate) determine whether their D or R values; A, D values; B, R values.

Fig. 6. Effect of cytosol acidification on the lateral mobility of H2 and H2(5A). The cell lines are those described in Fig. 5. The cells were grown on glass coverslips precoated with fibronectin (except for HepG2 cells). They were either left untreated (control, blank bars) or subjected to cytosol acidification as described under "Experimental Procedures" (cross-hatched bars). The FPR experiments were carried out at 22°C. The bars represent the mean ± S.E. of 30–60 measurements in each case. A, D values; B, R values.

and their similarity to H1(5A) are in agreement with the slower (relative to H1) and similar internalization rates measured for these proteins (42). Interestingly, coexpression of H2B together with H1 altered the lateral mobility of H2B and conferred upon it the characteristics of H1 mobility under all the conditions employed (compare H2B on 1-7-1 and HepG2 cells in Figs. 5 and 6 to H1 on the same cells in Figs. 3 and 4). Thus, coexpression with H1 reduces D of H2B to the same level measured for H1 (Fig. 5) and results in a reduced Rp and elevated D for H2B following cytosol acidification (Fig. 6). These results are in accord with the coexpression experiment in which H1 and H2B reside in mutual heterooligomers (32, 33). Furthermore, they suggest that H2B is capable only of weak interactions with the plasma membrane coated pits, and experiences stronger interactions following association with H1.

DISCUSSION

The binding of membrane proteins to structures that are immobile on the time scale of their lateral mobility may affect either their D or Rp, depending on the lifetimes of the complexes formed relative to the membrane protein diffusion rate (32, 62, 63). The binding kinetics (i.e. the rate of the association dissociation cycles of the protein between free and bound states relative to its lateral diffusion rate) determine whether D or Rp will be affected, while the thermodynamic parameters (i.e. the affinity) and the surface densities of the immobile structures and the proteins interacting with them determine the extent of the mobility-restricting effect (27, 62). Formation of stable complexes between the membrane protein and an immobile structure, where the complex lifetime is long relative to the characteristic lateral diffusion time, reduces Rp; this occurs since a given molecule of the membrane protein will be bound to the immobile structure for the entire duration of the mobility measurement. If a protein molecule undergoes several association-dissociation cycles with the immobile entity during the lateral mobility measurement (i.e. dynamic or transient interactions), a reduction in the diffusion rate (measured by D) is expected, since the protein molecule is bound for a fraction of the measurement time and free to diffuse during the dissociation cycle (27, 62).

The data in Fig. 3 demonstrate that the lateral diffusion rate of H1 is significantly lower than that of the tyrosine-deficient H1(5A) mutant, but its Rp value is not; in fact, Rp of H1 singly expressed in the 1-7 cell line is somewhat higher than that of H1(5A) on F1(5A)-1 cells or H1 on 1-7-1 cells, probably reflecting minor individual differences between the cell lines. The reduction in the lateral diffusion rate (but not Rp) of H1 relative to H1(5A) can be explained by mobility-restricting dynamic interactions with immobile structures, which are weaker for H1(5A) due to the absence of Tyr-5. These structures are most likely coated pits, as suggested by the effects of two independent treatments that disrupt or alter the structure of coated pits, and which blocked effectively endocytosis via the ASGP receptors in the current studies (Fig. 1). Thus, treatment with hypertonic medium to disperse the clathrin lattices (54, 55) elevated D of both H1 and H1(5A) to the same final level (Fig. 3); the rise in D of H1 was higher, as expected if the initial restriction of its mobility was stronger. The notion that the immobile structures involved in restricting H1 mobility are coated pits gains further support by the effect of the alternative treatment (acidification of the cytosol), which leaves the clathrin lattices associated with the plasma membrane but unable to detach as coated vesicles (55, 56, 58). This treatment caused a marked reduction in Rp of H1 (Fig. 4), accompanied by a rise in the D values close to the levels measured following disruption of the clathrin lattices by the hypertonic treatment (compare Figs. 3 and 4). This suggests that cytosol acidification, which freezes the membrane-associated coated pits (55, 56), enhanced the mobility-restricting interactions experienced by H1 and shifted them from transient to permanent entrapment (i.e. stable association for the entire duration of the lateral mobility measurement). A similar shift (from labile to stable interactions) following cytosol acidification was encountered earlier for the internalization-competent HA mutant protein HA(Y543D) (27).

The current findings, which suggest that H1 interacts dynamically with plasma membrane coated pits and that these interactions involve Tyr-5, are in excellent agreement with the reports (40, 41) that mutation of Tyr-5 to Ala or deletion of the surrounding residues 4–11 reduced H1 internalization rate 4-fold, whereas deletion of residues 12–33 affected endocytosis only slightly. Furthermore, the YQDL sequence (residues 5–8 of H1) was shown to be a functional internalization signal when transplanted into the cytoplasmic domain of the transferrin receptor (67), and the inhibition of AP-2 binding to plasma membrane fragments by a peptide corresponding to the cytoplasmic tail of rat hepatic lectin 1 (RHL-1; the equivalent of H1 in the rat) depended on the cytoplasmic tyrosine (position 4 in RHL-1) (26). Interestingly, H1(5A) is still internalized via coated pits, albeit at a slower rate (40); this is in accord with our finding (Fig. 3) that although the lateral diffusion rate of H1(5A) is higher than that of H1, it is still inhibited to some degree, and this inhibition is abolished by the hypertonic treatment. This indicates either that H1(5A) contains a second endocytosis signal or that the mutation of Tyr-5 to Ala reduces the efficiency of the internalization signal but does not fully destroy it. At any rate, the insensitivity of the lateral mobility of H1(5A) to cytosol acidification (unlike H1) suggests that the weak internalization signal retained in H1(5A) is incapable of sensing the low pH-mediated alteration in the coated pit structure.
Thus, Tyr-5 must play a role in the enhanced interactions of H1 with coated pits under these conditions.

H1 and H2B form stable heterooligomers in the cell membrane (32, 33). Together with the high degree of sequence similarity between H1 and H2, this raises the question whether H2B contains an independent internalization signal, which may contribute to the endocytosis of the ASGP receptor complex. Measurement of the lateral mobility of H2B and H2(5A) yielded very different results than the equivalent studies on H1 and H1(5A). Singly expressed H2B and H2(5A) displayed very similar lateral mobility parameters (D and Rσ) (Fig. 5), resembling those measured for H1(5A) (Fig. 3). The analogy to H1(5A), which is not endocytosed efficiently and interacts weakly with coated pits, persisted for the hypertonic and cytosol acidification treatments (compare Figs. 5 and 6 with 3 and 4). This behavior is in accord with the slow internalization rate of H2B, resembling that of H1(5A) (42). These findings suggest that H2B experiences only weak interactions with coated pits, as does H1(5A), and that Phe-5 in H2 is not part of an internalization signal. The fact that H2B and H2(5A) still interact weakly with coated pits, as indicated by their slower but distinct internalization and by the residual inhibition of their lateral mobility, supports the notion that there is another (weak) endocytosis signal. The fact that these findings resemble those measured for a receptor that participates in the growth of the lattice structure during coated pit assembly (70-73). Rather, the ASGP receptor appears to belong to the same class of membrane proteins as HA(Y543), which interact transiently with coated pits and are therefore likely to be internalized via interaction with preformed coated pits. The nucleation of coated pits could involve other membrane receptors that exhibit higher affinity to and stable association with coated pits; alternatively, aggregates of AP-2 adaptors interacting with putative adaptor localization protein(s) distinct from receptor tails may serve as nucleation centers for the formation of plasma membrane coated pits (24-26, 74).

REFERENCES


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