Dynamic or Stable Interactions of Influenza Hemagglutinin Mutants with Coated Pits

DEPENDENCE ON THE INTERNALIZATION SIGNAL BUT NOT ON AGGREGATION*

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Ella Fireš, Orit Gutman‡, Michael G. Roth§, and Yoav I. Henis‡†

From the ‡Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel and the §University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

Measurements of the lateral mobility of native and mutated membrane proteins, combined with treatments that alter clathrin lattice structure, are capable of characterizing their interactions with coated pits in live cells (Fire, E., Zwart, D. E., Roth, M. G., and Henis, Y. I. (1991) J. Cell Biol. 115, 1585–1594). To explore the dependence of these interactions on the internalization signal and the aggregation state of the protein, we have extended this approach to investigate the interactions between coated pits and several influenza hemagglutinin (HA) mutants, which differ in the internalization signals in their short cytoplasmic tails. The lack of internalization signals in the trimeric wild-type HA enables a direct comparison between specific internalization signals introduced singly in each mutant. We have selected for these studies HA mutants that showed different internalization rates and varied in their tendency to aggregate into complexes larger than trimers. Our results indicate that the mode of interaction with coated pits (transient association-dissociation versus stable entrapment) depends on the internalization signal and affects the internalization efficiency. Mutants that contain a strong internalization signal and undergo fast endocytosis were entrapped in coated pits for the entire duration of the lateral mobility measurement, suggesting stable association with (slow dissociation from) coated pits. A mutant with a suboptimal internalization signal, which was internalized 10-fold slower, exhibited transient interactions with coated pits. Both types of interactions disappeared or were significantly reduced upon disruption of the clathrin lattices under hyperosmotic conditions, and were modulated following the “freezing” of coated pits by cytosol acidification. Unlike the dependence on the cytoplasmic internalization signal, the interactions with coated pits did not depend on the aggregation state (measured by sucrose gradient centrifugation after solubilization in n-octyl(β-D-glucoside) of the mutants.

Receptor-mediated endocytosis plays important roles in the recycling of membrane proteins, receptor down-regulation, and signal termination (1-4). In many cell types the initial step in entering the endocytic pathway is the clustering of specific membrane proteins in plasma membrane clathrin-coated pits (1, 5–7). A large body of evidence suggests that short cytoplasmic motives on membrane proteins destined for endocytosis are recognized by clathrin-associated proteins (by binding to AP-2, the adaptor protein complex associated with plasma membrane coated pits), and serve as internalization signals (8–13) (reviewed in Refs. 3, 4, and 14). Two distinct classes of such signals were identified (3, 4, 14–16). The first contains at least one aromatic residue, typically tyrosine, in a tight turn conformation (17–21) (reviewed in Refs. 3 and 15). The second is based on leucine (LZ, where Z = L, I, V, M, C, or A), and is thought to be involved mainly in targeting membrane proteins from the trans-Golgi network to endosomes (22, 23) (reviewed in Ref. 16). There are also indications that aggregation of certain receptors may induce their endocytosis, as suggested by the need for dimerization to trigger the internalization of epidermal growth factor (EGF)1, or Neu receptors (2), and by the accumulation of aggregated macrophage Fc receptors in lysosomes (24).

In order to characterize the interactions of membrane proteins with coated pits in intact cells, we have recently developed a method based on comparative studies of the lateral mobilities of native and specifically mutated membrane proteins and demonstrated that it can measure their interactions with coated pits at the surface of living cells (25). In studies conducted on several cell types and several different membrane proteins, we have demonstrated that two modes of interactions with coated pits can be encountered, dynamic interactions (several association/dissociation cycles during the measurement) (25, 26) or stable association with coated pits (27).

In view of the large variety of internalization signals and the possible contribution of additional parameters, such as higher complex formation (aggregation) to the interactions with coated pits, it was desired to explore the relationships between the mode of these interactions (transient association/dissociation versus stable association) and internalization efficiency. To investigate this issue, we applied the lateral mobility-based approach to a series of influenza hemagglutinin (HA) mutants whose cytoplasmic domain contains different internalization signals. The absence of an internalization signal in wild-type HA (HA wt), which is excluded from coated pits (9, 25), enables a direct comparison between specific internalization signals introduced one at a time in each mutant. We have focused on HA mutants that were internalized at different rates (28) and varied in their tendency to form higher complexes (larger than

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† To whom correspondence should be addressed. Tel.: 972-3-6409053; Fax: 972-3-6407643.

1 The abbreviations used are: EGF, epidermal growth factor; HA, influenza hemagglutinin; TMR, tetramethylrhodamine; STI, soybean trypsin inhibitor; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; FPR, fluorescence photobleaching recovery; wt, wild type; KA, potassium-amiloride.
the trimeric HA wt). Our results indicate that, depending on the specific internalization signal, the HA mutants can shift all the way from no interactions to stable entrapment in coated pits, and that a shift from transient to stable entrapment correlates with significantly higher internalization rates. On the other hand, microaggregation (higher complex formation) per se does not have a major effect on the interactions with coated pits.

**EXPERIMENTAL PROCEDURES**

**Materials—** Tetramethylrhodamine (TMR) 5-isothiocyanate was from Molecular Probes, Inc. (Eugene, OR). Restriction enzymes and T4-DNA ligase were from New England Biolabs (Beverly, MA). Trypsin, soybean trypsin inhibitor (STI), N-octylglucoside, amiloride hydrochloride, Hank’s balanced salt solution (HBSS), and bovine serum albumin (BSA) were from Sigma. 35S-labeled methionine and cysteine (Tran 35S-label) was from Molecular Probes, Inc. (Eugene, OR). Restriction enzymes and T4-DNA ligase were from New England Biolabs (Beverly, MA). Trypsin, soybean trypsin inhibitor (STI), N-octylglucoside, amiloride hydrochloride, Hank’s balanced salt solution (HBSS), and bovine serum albumin (BSA) were from Sigma. 35S-labeled methionine and cysteine (Tran 35S-label) was from ICN (Irvine, CA). Polyclonal rabbit antiserum that recognizes all the Jap HA (A/jap/305/57 strain) was employed throughout. Monovalent F(ab’)2 fragments were prepared from the IgGs described earlier (25, 33) and labeled with TMR 5-isothiocyanate using standard procedures (30). The F(ab’)2 preparation was free of contamination by F(ab’), or IgG, as judged by SDS-PAGE under nonreducing conditions.

Recombinant Virus Vectors and Cell Culture—Several mutants were prepared from the cDNA encoding the A/jap/305/57 HA and introduced into the SV40-based vector pSVE (31). The mutants are depicted in Table I. The derivation of the mutants HA + 8 (containing a carboxyl-terminal extension of eight amino acids with four aromatic residues, generating a strong internalization signal), HA + 4 (which is HA + 8 minus the last four residues), and HA + 8-S548, S554 (HA + 8 with the tyrosines at positions 548 and 554 replaced by serines) was described elsewhere (28). HA + 5-S548, 554, which is a point mutant of HA wt (cysteine residues, generating a strong internalization signal), HA + 1 (containing a carboxyl-terminal extension of eight amino acids with four aromatic residues, generating a strong internalization signal), HA + 4 (which is HA + 8 minus the last four residues), and HA + 8-S548, S554 (HA + 8 with the tyrosines at positions 548 and 554 replaced by serines) was described elsewhere (25, 33). Recombinant SV40 virus stocks were prepared in CV-1 monkey fibroblasts (American Type Culture Collection, Rockville, MD) using the appropriate pSVE vector together with the di1055 helper virus as described earlier (32, 33). The CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Biological Industries, Beth Haemek, Israel) or Serum Plus (Hazleton, Lenea, KS), 100 units/ml penicillin, and 100 μg/ml streptomycin. For lateral mobility studies by fluorescence photobleaching recovery (FPR), CV-1 cells were infected with recombinant SV40 virus stocks, and the experiments were performed 36–46 h postinfection (depending on the HA mutant; see below). For experiments on HA complex formation (aggregation), the incubation period post infection was 32 h.

Immuno-fluorescent Labeling—Subconfluent CV-1 cells were infected with recombinant viruses as previously described (25, 33). They were grown postinfection for 36–38 h (for HA wt and HA + 4) or 44–46 h (for HA + 8 and HA + 8-S548, S554). The longer incubations after infection used for lateral mobility studies were required to achieve surface areas closer to that of HA wt, as the percentage of these mutants at the cell surface is significantly lower (28). The cells were washed twice with cold HBSS containing 20 mM HEPES and 2% BSA (HBSS/HEPES/BSA, pH 7.2), and labeled with anti-HA TMR-Fab’ (100 μg/ml, 30 min, 4°C). In cases where the cells were preincubated in specific buffers to alter coated pit structure (see below), the specific buffers employed for each treatment were used throughout all experiments. The experiments were performed in the following steps, including the FPR measurements (see following section). After three washes, the coverslips carrying the live cells were taken for FPR measurements.

Fluorescence Photobleaching Recovery—Lateral diffusion coefficients (D) and mobile fractions (RF) of the HA mutants were measured by FPR (34, 35) using previously described instrumentation (36). After labeling the infected cells as described above, the coverslip was placed (cells facing downward) over a serological slide with a depression filled with HBSS containing 2% BSA (or with the buffers used to alter coated pit structure) and equilibrated at the desired temperature using a thermostated microscope stage. For untreated cells, whose coated pit-mediated internalization was not blocked, samples were replaced within 15 min at the measurement temperature (10 or 22°C) to minimize endocytosis of the mutants that undergo fast internalization (HA + 8 and HA + 8-S548, S554; Table I) which could lead to their immobilization (34). For the same reason, such experiments were not conducted at 37°C. The temperature values of D and RF at the beginning and end of this period demonstrated that the contribution of such effects to the measurements was negligible. The monitoring laser beam (Coherent Innova 70 argon ion laser; 529.5 nm, 1 microwatt) was focused through the microscope (Zeiss Universal) to a gaussian radius of 0.61 ± 0.02 μm, using 100X oil immersion objective. A brief pulse (5 milliwatts, 30–60 ms) bleached 50–70% of the fluorescence in the illuminated region. The time course of fluorescence recovery was followed by the attenuated monitoring beam. D and RF were extracted from the fluorescence recovery curves by nonlinear regression analysis (37). Incomplete fluorescence recovery was interpreted to represent fluorescent-labeled molecules immobile on the experimental time scale (D = 5 × 10–12 cm2/s).

**Treatments Affecting Coated Pit Structure—** The treatments employed were incubation in a hypertonic medium (38–40) or acidification of cytosol (40–42) or acidification of cytosol (40–42). Hypertonic treatment, which blocks endocytosis via coated pits by dispersing the underlying dathrin lattices (39, 40), was performed by 15-min incubation (37°C) in HBSS/HEPES/BSA supplemented with 0.45 M sucrose or 0.225 M NaCl. The cells were kept in hypertonic medium during labeling and FPR measurements. Cytosol acidification, which alters the coated pit structure and eliminates endocytosis by blocking the pinching-off of dathrin-coated vesicles, was performed as described earlier (25, 41). Cells were incubated in HEPES-buffered DMEM (pH 7.2) containing 30 mM NH4Cl (30 min, 37°C), followed by 5 min (37°C) in potassium-amiloride (KA) buffer (0.14 M KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM amiloride HCl, 20 mM HEPES, pH 7.2). This buffer was replaced by cold KA containing 2% BSA, which was also used throughout the TMR-Fab’ labeling and the FPR experiments.

Assay for Complex Formation—Thirty-two h after infection with recombinant SV40 viruses, CV-1 cells were washed with phosphate-buffered saline and incubated for 30 min at 37°C in DMEM lacking methionine and cysteine. A pulse of radiolabeled protein was initiated by subsequent incubation in the presence of Trans–label (15 min, 0.6 μCi/ml) and ended by removal of the labeling medium and addition of complete DMEM. After chasing for 120 min, the cells were lysed in cold buffer containing 20 mM HEPES (pH 7.3), 1% n-octylglucoside, 10 μg/ml STI, and 0.1 unit/ml aprotinin. After removing nuclei and cell debris by centrifugation at 14,000 × g for 12 min, 70% of the lysate was pooled above a 700-μl cushion of 60% sucrose. The gradient was prepared in 20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 μg/ml STI, 0.1 unit/ml aprotinin, and 1% n-octylglucoside. The gradients were centrifuged in a SW41 rotor (Beckman Instruments, Palo Alto, CA) at 40,000 rpm for 17 h at 18°C or 22°C. 700-μl fractions were collected from the bottom of the tube. The amount of HA present in each fraction was analyzed by immunoprecipitation, SDS-PAGE, and densitometry as described elsewhere (43).

**RESULTS**

Interactions of membrane proteins with immobile structures are capable of immobilizing them or of retarding their lateral motion (44, 45). The plasma membrane-coated pits are essentially immobile on the FPR experimental time scale (46) and can therefore retard the lateral mobility of membrane proteins destined for endocytosis. This phenomenon can be employed to characterize the interactions between these proteins and coated pits in live cells. Using HA wt (which is excluded from coated pits) as a control, we have used this approach to demonstrate that the internalization-competent HA-Y543 point mutant exhibits dynamic interactions with coated pits (25). The HA system is ideal for the exploration of the dependence of the mode of interactions with coated pits and the internalization rate on the internalization signal of the membrane protein,

| Jap HA mutant in pSVE vector | Cytoplasmic sequence | Endocytosis rate | Reference |
|-----------------------------|----------------------|-----------------|-----------|
| HA wt | NGSLQCRICI | <0.01 | 31 |
| HA-Y543 | · · · · · · · · · | 0.05 | 9 |
| HA + 8 | NGSLQCRICIYDYKSFYN | 0.60 | 28 |
| HA + 5-S548, 554 | · · · · · · · · · · | 0.55 | 28 |
| HA + 4 | · · · · · · · · · · | 0.01 | 28 |
since various internalization signals can be introduced at the HA cytoplasmic tail, keeping the rest of the protein (which is devoid of any other internalization signals) unaltered. We have therefore performed lateral mobility studies on cells expressing HA mutants that differ in their cytoplasmic internalization signal and are internalized at highly different rates (28) (see Table I). To further substantiate the involvement of coated pits in the effects measured, the experimental design also involved treatment of cells to disperse or alter the coated pit structure.

The Mode of Interactions with Coated Pits Depends on the Internalization Signal and Correlates with Internalization Rate—Following the expression of the various HA mutants (see Table I) in CV-1 cells, the HA proteins at the cell surface were labeled in the cold by monoclonal anti-HA TMR-Fab (to eliminate IgG-mediated cross-linking that may affect the mobility). FPR studies were conducted at 10 and 22 °C (see “Experimental Procedures”). Measurements at 37 °C were avoided due to the fast internalization of some of the HA mutants employed. Fig. 1 shows that, while the lateral diffusion coefficients (D) of the three mutants measured (HA +8, HA +8-S548,S554, and HA +4) were similar to each other and to HA wt (representing an HA protein that does not interact with coated pits), the mobile fractions (Rf) of the mutants that undergo fast internalization (HA +8 and HA +8-S548,S554; Table I) were significantly lower than that of HA wt. In contrast, Rf of HA +4 (truncated HA +8, which is internalized very slowly) (28) was similar to the Rf value of HA wt (Fig. 1). The reduction in Rf with no effect on D which was observed for HA +8 and HA +8-S548,S554 indicates that a subpopulation of these mutant molecules is entrapped by stable interactions with immobile structures (presumably coated pits) for the entire duration of the FPR measurement (about 1 min). The remainder of the cell-surface population of these molecules diffuse freely in the plasma membrane and do not show dynamic interactions with the immobile structures, which would reduce the average lateral diffusion rate (25, 44, 45). The reduction in Rf of HA +8 and HA +8-S548,S554 was somewhat more pronounced at 22 °C than at 10 °C, suggesting that the mobility-restricting interactions are elevated at higher temperatures. This is in line with former observations (25) on HA-Y543, which demonstrated temperature-dependent dynamic interactions with coated pits. As for HA +4, whose internalization is extremely slow (28), this mutant did not show detectable reduction in its lateral mobility, and diffused in the membrane in an unrestricted manner, just like HA wt.

Alteration of Coated Pit Structure Changes the Interactions of Internalization-competent HA Mutants with Coated Pits—To validate that the lower Rf values of HA +8 and HA +8-S548,S554 relative to HA wt are due to interactions with coated pits, we have determined the effects of treatments known to alter coated pit structure on the lateral mobility of HA wt and the three HA mutant proteins. The treatments employed were hypertonic medium treatment, which disperses the cisternae of coated pits as well as flat clathrin lattices (39, 40), and cytosol acidification, which alters the morphology of the clathrin lattice structure (40). These treatments were highly effective in CV-1 cells, as they completely inhibited the internalization of HA +8 and the endogenous transferrin receptors of CV-1 cells (25). Analogous results were obtained for HA +8 (28). An added benefit of these treatments was that they enabled FPR measurements on cells expressing the fast-internalized HA mutants also at 37 °C, where the interactions with coated pits may be further enhanced (25), since they blocked the otherwise rapid internalization of these mutants.

The effect of treatments with hypertonic medium (containing sucrose or high NaCl) on the lateral mobilities of the HA proteins is depicted in Fig. 2. The hypertonic conditions induced a marked increase in the Rf values of HA +8 and HA +8-S548,S554, raising them very close to the Rf values observed for HA wt and HA +4, essentially abolishing the differences between the Rf values of the various HA mutants. Thus, this treatment released most of the mutant HAs formerly entrapped in coated pits, in accord with our former observation (25) that the dynamic interactions of HA-Y543 with coated pits are abolished under hypertonic conditions. Interestingly, at 37 °C (where the interactions with coated pits are more pronounced), hypertonic medium lowered the D values for HA +8 and HA +8-S548,S554 relative to HA wt or HA +4 (an effect that was somewhat more pronounced with sucrose, but clearly evident also with NaCl). Since reduced D values are characteristic of transient (dynamic) interactions, this phenomenon could reflect weaker interactions (faster dissociation rates) of these mutant HA proteins with adaptor protein aggregates, which were reported to remain associated with the plasma membrane following dispersal of the clathrin lattices (40).

Fig. 3 shows the effects of acidification of the cytosol by preincubating with NH4Cl on the lateral mobility of the HA mutants. Since this treatment does not disperse the coated pits but “freezes” them at the plasma membrane (40–42), it is not expected to disrupt the interactions with coated pits, but rather to alter them. In the case of HA-Y543, which binds transiently to coated pits in untreated cells, this treatment resulted in stable entrapment of HA-Y543 in the “frozen” coated pits, suggesting tighter interactions (slower dissociation from the coated pit structures) (25). In contrast, acidification of the cytosol of cells expressing HA +8 or HA +8-S548,S554 had little effect on either their Rf or D values at 22 °C (compare Fig. 1 with Fig. 3), with the Rf values remaining below those of HA wt.
wt or HA +4. This is the result expected if HA +8 and HA +8-SS48,SS554 were already stably entrapped in coated pits prior to cytosol acidification, since if the dissociation rate is slow enough such that no exchange occurs on the experimental time scale, a still slower dissociation rate will not alter the observed mode of interaction. Interestingly, the reduction in RF of these two mutants in cytosol-acidified cells was much more pronounced at 37°C (Fig. 3), suggesting stronger interactions with coated pits at this elevated temperature.

Interaction of the HA Mutants with Coated Pits Does Not Depend on Their Aggregation—In several cases, microaggregation (higher complex formation) was shown to be important for receptor-mediated endocytosis or targeting to lysosomes (2, 24). We therefore investigated the correlation between the mode of interactions of HA mutants that differ in their internalization rates and the capacity of the normally trimeric HA proteins to form higher complexes.

Thirty-two hours postinfection, cells expressing one of the HA proteins (HA wt, HA-Y543, HA +8, HA +8-SS48,SS554, or HA +4) were subjected to a pulse-chase protocol with Tran35S-label (see “Experimental Procedures”). The cells were lysed in a buffer containing 1% n-octylglucoside and analyzed by velocity sedimentation on sucrose gradients made in 1% of the same detergent, chosen since it was shown to maintain protein-protein interactions in many cases. This choice is justified by the failure to detect higher complex formation (described below) when n-octylglucoside was replaced by the more disruptive detergents Triton X-100 or Nonidet P-40 at the lysis step (followed by analysis on sucrose gradients containing Triton X-100). A typical experiment comparing HA +8 and HA-Y543 is shown in Fig. 4. While HA-Y543 was detected almost entirely in fractions 10–11, the position normal for HA trimers under the conditions employed, most of the HA +8 migrated lower in the gradient, corresponding to the position of larger, faster sedimenting species. The long chase time in these experiments (120 min) causes both HA-Y543 and HA +8 (and all the other HA mutants employed) to exit the exocytic pathway and become distributed both at the cell surface and in internal endocytic organelles (28). To determine whether the aggregation occurred to different levels at the cell surface and after internalization, trypsin was added to the extracellular medium at 4°C at the end of the chase, to distinguish between HA molecules present at the cell surface (which are cleaved by trypsin to disulfide-bonded HA1 and HA2) and those localized intracellularly (which are inaccessible to trypsin and remain in the HA0 form). Fig. 4 demonstrates that the ratio of (HA1 + HA2)/HA0 (cleaved/ uncleaved) was the same in gradient fractions containing larger complexes and in those containing trimeric HA +8. This indicates that the higher HA +8 complexes contained both proteins that were present at the cell surface at the end of the chase, and proteins that had entered internal compartments. In addition, there was no loss of HA +8 after trypsin treatment, indicating that the HA +8 in the aggregated form contained well folded HA +8 trimers.

The average results of several such experiments (with five samples for each HA protein), performed in two separate sets, are depicted in Fig. 5 and Table II. Each set (A and B) included HA +8 and a trimeric HA protein (HA wt or HA-Y543), to enable direct comparison and exemplify the consistency of the data. It is apparent that HA-Y543 and HA +8-SS48,SS554 sediment almost exclusively (85% or higher) as trimers, similar to HA wt, with very low percentages migrating below the trimer peak. In contrast, only 20 and 40% of HA +8 and HA +4, respectively, sedimented as trimers, and the two mutants exhibited a strong tendency to associate into faster sedimenting complexes. These results demonstrate that the ability of an HA mutant protein to form higher complexes does not correlate with the mode of its interactions with coated pits. Thus, HA +8
characteristic lateral diffusion time) reduces ("permanent entrapment" for times long relative to the character of the interaction with coated pits (25). Finally, HA +4, which contains a truncated form of the HA internalization signal (YK), is hardly internalized (Table I) and has no such effect was found for HA +4, whose lateral mobility was similar to that of HA wt. These results are compatible with the notion that HA +8 and HA +8-S548,S554 become stably associated with immobile structures, most likely coated pits, for the entire duration of the measurement. The identification of these structures as coated pits is supported by the correlation between the reduction in Rf (Fig. 1) and the internalization rates of the HA mutants (Table I). In addition, two independent treatments that alter the structure of coated pits, hypotonic treatment and cytosol acidification (discussed below), also affect the lateral mobility of those mutants that undergo internalization (Figs. 2 and 3). The mode of the interaction with coated pits (stable versus transient) appears to depend on the internalization signal and to affect the internalization efficiency. Thus, HA +8 and HA +8-S548,S554, which contain a strong internalization motif (YKSF) closely resembling that of the transferrin receptor (Table I), show stable interactions with coated pits and fast internalization. HA-Y543, which has an internalization motif (YKSF) closely resembling that of the transferrin receptor (Table I), shows stable interactions with coated pits and fast internalization. HA-Y543, which has an internalization motif (YKSF) closely resembling that of the transferrin receptor (Table I), shows stable interactions with coated pits and fast internalization. HA-Y543, which has a suboptimal internalization signal (33), is internalized 10-fold slower than the former mutants (Table I), and was shown to interact transiently (without permanent entrapment) with coated pits (25). Finally, HA +4, which contains a truncated form of the HA +8 internalization signal (YK), is hardly internalized (Table I) and has no detectable interactions with coated pits in our experiments.

The effect of dispersing the clathrin lattices by hypertonic medium on the lateral mobilities of the HA mutants supports the role of coated pits in retarding the mobility of the internal...
The studies with cytosol-acidified cells provide further support for the involvement of coated pits in the mobility-restricting interactions and for their dependence on temperature. Cytosolic acidification alters the structure of coated pits, “freezing” them at the cell surface (40, 42). We have formerly shown that this causes membrane proteins that interact transiently with coated pits (HA-Y543, and the H1 subunit of the human asialoglycoprotein receptor) to shift from transient to stable entrapment (25, 26). Such a shift is not expected for HA wt and HA-8-S548,554 could provide an alternative explanation to the reduced D values of these mutants after clathrin lattice dispersal, assuming that the fraction of aggregated proteins is preferentially localized to coated pits prior to the disruption of the clathrin lattices. However, this possibility can be ruled out, since these two mutants only HA-8 forms higher complexes (Fig. 5). Furthermore, a significant reduction in D would require formation of large aggregates, due to the very weak dependence of the lateral diffusion of membrane proteins on molecular size (47). If this were the case, HA-4 + (which shows aggregation on sucrose gradients; Fig. 5) would be expected to diffuse at a slower rate than the nonaggregating HA wt; however, the two proteins exhibit similar D and Rv values (Fig. 1).

The studies with cytosol-acidified cells provide further support for the involvement of coated pits in the mobility-restricting interactions and for their dependence on temperature. Cytosolic acidification alters the structure of coated pits, “freezing” them at the cell surface (40, 42). We have formerly shown that this causes membrane proteins that interact transiently with coated pits (HA-Y543, and the H1 subunit of the human asialoglycoprotein receptor) to shift from transient to stable entrapment (25, 26). Such a shift is not expected for HA wt and HA-8-S548,554, since they are stably entrapped in coated pits even prior to the acidification treatment. Under such conditions, one expects that the Rv values of these two mutants will remain lower than those of HA wt and HA-4, and this expectation is met (Fig. 3). These experiments also provide additional evidence for the enhancement of the interactions with coated pits at 37 °C. While the nature of the effect mediated by interactions with immobile structures (transient or stable) is determined by the binding kinetics (mainly the dissociation rate relative to the lateral diffusion rate), the extent of the effect is determined by the thermodynamic binding parameters (25, 44). The reduction in Rv of HA B and HA-8-S548,554 is significantly more pronounced at 37 °C (Fig. 3), indicating that their affinity to coated pits increases at this temperature. It is also possible that the number of binding sites for the HA mutants increases under these conditions.

Apart from the internalization signal, higher complex formation (aggregation) may also play a role in the interactions of membrane proteins with coated pits. The mutants employed in the current study enable us to explore this possibility, since the various HA mutants investigated all fold properly into protease-resistant trimers (28) and differ only in specific sequences in the cytoplasmic domain. These sequences are therefore likely to be responsible for aggregate formation in the case of HA-8 and HA-4. This aggregation is not likely to stem from instability of these mutants in detergent, because higher complexes were observed only when cells were lysed with the mild detergent n-octylglucoside but not with Triton X-100 or Nonidet P-40. Furthermore, no disulfide bonds are involved, as one might expect for aggregates formed after lysis, as is evident by the dissociation of the aggregates in Triton X-100. At this stage, we do not know whether the aggregates are comprised exclusively of HA trimers, or contain additional proteins. At any rate, our results (Fig. 5, Table II) clearly demonstrate that aggregate formation per se is neither sufficient nor required for interactions with coated pits and the ensuing internalization. However, due to the solubilization with n-octylglucoside in these studies, the possibility that some complexes (e.g. HA-8-S548,554) are disrupted by the detergent cannot be ruled out. Finally, our results do not exclude the possibility that in some cases, such as for the EGF and Neu receptors, dimerization can be a prerequisite for endocytosis (reviewed in Ref. 2). However, unlike constitutively endocytosed membrane proteins, EGF or Neu receptor dimerization is also intimately involved in their activation, manifested by enhanced tyrosine kinase activity and autophosphorylation (2); these could well trigger their internalization, rather than the dimerization itself (27, 48).

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