Evidence from Lateral Mobility Studies for Dynamic Interactions of a Mutant Influenza Hemagglutinin with Coated Pits

Ella Fire,* David E. Zwart,‡ Michael G. Roth,‡ and Yoav I. Henis*

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

Abstract. Replacement of cysteine at position 543 by tyrosine in the influenza virus hemagglutinin (HA) protein enables the endocytosis of the mutant protein (Tyr 543) through coated pits (Lazarovits, J., and M. G. Roth. 1988. *Cell.* 53:743–752). To investigate the interactions between Tyr 543 and the clathrin coats in the plasma membrane of live cells, we performed fluorescence photobleaching recovery measurements comparing the lateral mobilities of Tyr 543 (which enters coated pits) and wild-type HA (HA wt, which is excluded from coated pits), following their expression in CV-1 cells by SV-40 vectors. While both proteins exhibited the same high mobile fractions, the lateral diffusion rate of Tyr 543 was significantly slower than that of HA wt. Incubation of the cells in a sucrose-containing hypertonic medium, a treatment that disperses the membrane-associated coated pits, resulted in similar lateral mobilities for Tyr 543 and HA wt. These findings indicate that the lateral motion of Tyr 543 (but not of HA wt) is inhibited by transient interactions with coated pits (which are essentially immobile on the time scale of the lateral mobility measurements). Acidification of the cytoplasm by prepulsing the cells with NH_4Cl (a treatment that arrests the pinching-off of coated vesicles from the plasma membrane and alters the clathrin lattice morphology) led to immobilization of a significant part of the Tyr 543 molecules, presumably due to their entrapment in coated pits for the entire duration of the lateral mobility measurement. Furthermore, in both untreated and cytosol-acidified cells, the restrictions on Tyr 543 mobility were less pronounced in the cold, suggesting that the mobility-restricting interactions are temperature dependent and become weaker at low temperatures. From these studies we conclude the following. (*a*) Lateral mobility measurements are capable of detecting interactions of transmembrane proteins with coated pits in intact cells. (*b*) The interactions of Tyr 543 with coated pits are dynamic, involving multiple entries of Tyr 543 molecules into and out of coated pits. (*c*) Alterations in the clathrin lattice structure can modulate the above interactions.

COMPLEXES of cell-surface receptors and their ligands are commonly internalized by receptor-mediated endocytosis via coated pits. This process plays a major role in the action mechanism and in the down-regulation of a variety of membrane receptors (2, 19, 28, 61; reviewed in 21, 29). During the initial stage of receptor-mediated endocytosis, specific cell-surface receptors such as those for low density lipoprotein (LDL), EGF, transferrin, and others collect in coated pits (21, 29). Other transmembrane proteins, such as the Thy-1 antigen and the influenza virus hemagglutinin (HA)[1], are excluded from coated pits and are internalized very slowly (7, 40, 52). Recent evidence indicates that the cytoplasmic domains of transmembrane proteins destined for endocytosis contain structural determinants responsible for their rapid internalization, as deduced from studies on mutants of the receptors for LDL (39), EGF (9, 51), poly-Ig (47), transferrin (30, 46, 53), mannose-6-phosphate (42), and Fc (45). Several lines of evidence suggest that a correctly positioned tyrosine residue in the cytoplasmic region generates a "recognition feature" that enables interaction with coated pits and endocytosis. In the LDL receptor, Tyr 807 was shown to play a crucial role in its location in coated pits and endocytosis (8, 11). Tyrosine residues in the cytoplasmic domains in both of the mannose-6-phosphate receptors (33, 42) and in the human transferrin receptor (1, 10, 32, 44) were demonstrated to be important for the internalization of these proteins. A positive demonstration of the ability of a "cytoplasmic tyrosine" motif to serve as an internalization signal was obtained with the influenza HA protein: while wild-type HA (HA wt) was excluded from coated pits, a mutant HA containing a tyrosine in place of cysteine at position 543 in the short cytoplasmic tail (Tyr 543) assembled into coated pits and underwent endocytosis and recycling (37, 38).

1. *Abbreviations used in this paper*: *D*, lateral diffusion coefficient; FPR, fluorescence photobleaching recovery; HA, influenza virus hemagglutinin protein; R_f, mobile fraction.

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The above findings raise several questions on the mechanistic basis for the sorting of cell-surface proteins into the endocytic pathway. What is the nature of the interactions between transmembrane proteins destined for endocytosis and coated pits? Do these proteins get permanently trapped in coated pits, or do they associate with them transiently? How are these interactions affected by alterations in coated pit structure? We investigated these questions by fluorescence photobleaching recovery (FPR) studies on the lateral mobility of influenza HA proteins expressed in the plasma membrane of CV-1 cells. The parameters whose effects on the lateral mobility of the HA proteins were examined include the presence or absence of the "cytoplasmic tyrosine" signal (Tyr 543 vs. HA wt), temperature, and treatments that disrupt or alter coated pit structure.

Materials and Methods

Materials

TRITC was obtained from Molecular Probes, Inc. (Eugene, OR). N-4-nitrobenzo-2-oxa-1,3-diazolyl-phosphatidylethanolamine (N-NBD-PE) was from Avanti Polar Lipids (Pelham, AL). Restriction enzymes and T4-DNA ligase were from New England Biolabs (Beverly, MA). Trypsin, soybean trypsin inhibitor, cytochalasin D, colchicine, and amiloride hydrochloride were from Sigma Chemical Co. (St. Louis, MO). Polyclonal rabbit antiserum that recognizes all forms of HA (raised against virions of the A/Japan/305/57 strain) was used throughout. Monovalent Fab' fragments labeled with TRITC (TRITC-Fab') were prepared from the IgG fraction as described (24), following standard labeling procedures (5). The Fab' preparation was free of contamination by $F(ab')_2$ or IgG, as judged by SDS-PAGE under nonreducing conditions.

Recombinant Virus Stocks and Infection Procedures

The studies employed the following plasmids, based on SV-40 vectors containing pBR322 derivatives: pKSVEHA, which contains the full-length cDNA encoding the A/Japan/305/57 HA protein (38, 52); pKSVEHATyr543, containing the Tyr 543 mutant HA cDNA (38); and dl1055, helper virus DNA providing the late SV-40 genes for the recombinant virus stocks (50).

The plasmid DNAs containing the HA wt or Tyr 543 genes were digested with KpnI to remove the pBR322 derivative, and recircularized under dilute ligation conditions (3 μg/ml). The resulting SVEHA or SVEHATyr543 vectors were transfected into CV-1 monkey kidney cells in subconfluent monolayers, together with an equal amount of dl1055 DNA; the transfection and the development of high titer virus stocks were described (13). CV-1 cells (from the American Type Culture Collection, Rockville, MD) were grown in DME supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biological Industries, Beth-Haemek, Israel). For experiments, slightly subconfluent CV-1 cells were infected with 1:5–1:10 dilutions of second passage virus stocks (13, 38), and the biophysical or microscopic assays on the expressed proteins were performed 36–40 h later.

Immunofluorescence Microscopy

CV-1 cells were split and grown on glass coverslips 1 d before infection as described above. At 36–40 h postinfection, the cells were washed twice by HBSS with Earle's salts (Biological Industries) supplemented with 20 mM Hepes and 2% BSA (Sigma Chemical Co.) (HBSS/Hepes/BSA, pH 7.2), and incubated with anti-HA TRITC-Fab' (100 μg/ml, 60 min, 4°C). After rinsing three times with the same cold buffer, the cells were either taken live for the lateral mobility measurements (see following section), or fixed successively in methanol (−20°C, 5 min) and acetone (−20°C, 2 min) and mounted in a solution (85% glycerol, 14 mM Tris-HCl, pH 8) containing the antibleaching agent *n*-propyl-gallate (0.5% wt/vol; Sigma Chemical Co.) for fluorescence photography. Fluorescence micrographs were taken using standard rhodamine filters with a Zeiss Universal fluorescence microscope (63× oil immersion objective, Kodak TMAX film).

Fluorescence Photobleaching Recovery

Lateral diffusion coefficients (D) and mobile fractions (R_f) of HA and Tyr 543 on the surface of CV-1 cells were measured by FPR (3, 36) using an apparatus described previously (23). The bleaching conditions employed in the FPR studies were shown not to alter the lateral mobilities measured (36, 60). After labeling HA or Tyr 543 with anti-HA TRITC-Fab' (as described in the former section), the coverslip carrying the live cells was placed (cells facing downward) over a serological slide with a depression, filled with HBSS/Hepes/BSA equilibrated at the desired temperature. A thermostated microscope stage was used to keep the sample temperature throughout the FPR experiments, performed within 1 h of the labeling. The initial internalization rate of Tyr 543 at 37°C is ~4% per min, reaching 30% internalization after 10 min, and plateaus at a steady state of 40% internal and 60% at the plasma membrane after 40 min (38). Therefore, in experiments done at 37°C with the endocytosis-competent Tyr 543 protein, the coverslip was replaced within 10 min of the shift to 37°C, and areas on the cell that contained fluorescent vacuoles (which most likely represent internalized fluorophores, and which were immobile in the FPR studies) were avoided. The effectiveness of this procedure is evidenced by the similar lateral mobility parameters (both D and R_f) obtained throughout the 10-min incubation at 37°C.

The monitoring laser beam (Coherent Innova 70 argon ion laser; 529.5 nm,1 μW, for TRITC; 488 nm, 0.1 μW, for N-NBD-PE) was focused through the microscope (Zeiss Universal) to a Gaussian radius of (0.61 ± 0.02) μm (n = 25; beam size measured by the scan photobleaching method as described in ref. 49) with a 100× oil immersion objective. A brief pulse (5 mW, 30 ms, for TRITC; 0.5 mW, 60 ms, for NBD) 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 R_f were extracted from the fluorescence recovery curves by nonlinear regression analysis (49). Incomplete fluorescence recovery was interpreted to represent fluorophores that are immobile on the FPR experimental time scale ($D \leq 5 \times 10^{-12}$ cm^2/s).

Treatments that Interfere with Coated Pit Structure

The treatments employed were incubation of cells in a hypertonic medium (27), or acidification of the cytoplasm (12, 26, 56).

Following expression of HA wt or Tyr 543, the cells were incubated (15 min, 37°C) in a hypertonic medium (HBSS/Hepes/BSA, pH 7.2, supplemented with 0.45 M sucrose). This treatment causes the clathrin lattices underlying coated pits to disappear, thereby blocking endocytosis (27). The cells were kept in the sucrose-containing medium during the labeling with anti-HA TRITC-Fab' and throughout the FPR experiments.

Acidification of the cytoplasm to block internalization via clathrin-coated pits was performed following the procedure of Sandvig et al. (56). CV-1 cells expressing HA wt or Tyr 543 were incubated in Hepes-buffered DME (pH 7.2) containing 30 mM NH_4Cl (30 min, 37°C). This medium was replaced by warm potassium/amiloride (KA) buffer (0.14 M KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride hydrochloride, 20 mM Hepes, pH 7.2). After 5 min at 37°C, the cells were transferred to 4°C in the same buffer, which was also used (in the presence of 2% BSA) for the labeling with TRITC-Fab' and the FPR experiments. This treatment results in cytosol acidification, yielding cytoplasmic pH values of 5.6–5.8, and blocks coated pit-mediated internalization due to elimination of the pinching off of coated vesicles (26, 56).

Assays for Internalization

Cells expressing Tyr 543 or HA wt were pulsed with ^{35}S methionine and cysteine (15 min; Tran^{35}Slabel, ECN, Irvine, CA) and the radiolabeled proteins were chased to the cell surface in DME at 37°C (2 h). The labeled cells were subjected to one of the following treatments: (*a*) cytosol acidification, as described above; (*b*) cytochalasin D (10 μg/ml, 37°C, 15 min), a treatment that disrupts microfilaments and was shown to block uptake by mechanisms independent of clathrin (55); or (*c*) hypertonic medium treatment (described above). The cells were cooled to 4°C, and incubated (45 min) with 1% rabbit anti-HA serum in either medium without additives (control), KA buffer (treatment *a*), 10 μg/ml cytochalasin D (treatment *b*), or 0.45 M sucrose (treatment *c*). Unbound antibody was removed by washing the cells with the respective media in the cold, followed by chasing each preparation in the appropriate medium at 37°C for 0–32 min intervals. Cells were returned to 4°C and treated with DME containing trypsin (100 μg/ml) to cleave the HA0 form of Tyr 543 or HA remaining at the cell surface into HA1 and HA2 subunits. With the exception of cytosol-acidified cells, over 97% of the labeled protein present at the cell surface was cleaved by trypsin in cells incubated with antibodies in the cold and never chased at 37°C. Under these conditions, little antibody was lost after trypsin cleavage (shown in Fig. 4). At the end of the treatment, the cells were washed with medium

containing serum and soybean trypsin inhibitor (100 μg/ml). Cells were lysed in 50 mM Tris (pH 8.0) containing 1% NP-40, 0.1% SDS, 100 μg/ml soybean trypsin inhibitor, 0.1 U/ml aprotinin (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 10 mM EDTA. Insoluble material was removed by centrifugation (10,000 *g*, 10 min); each sample was precipitated with protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), and the proteins were analyzed by SDS-PAGE and autoradiography as described (37, 38). As a positive control for experiments employing cytosol acidification, cytochalasin, or hypertonic medium, internalization of the endogenous transferrin receptors of the CV-1 cells was assayed under each condition following the protocols described by Sandvig et al. (56) and by Sandvig and van Deurs (55). In these control experiments, cytochalasin D treatment had a clear effect on cell shape, but did not reduce the internalization of iodinated transferrin (two experiments), whereas cytosol acidification (two experiments) and treatment with a hypertonic medium (one experiment) resulted in 100% and 60% inhibition, respectively. For these experiments, human transferrin (Sigma Chemical Co.) was labeled with $Na^{125}I$ (NEN, Boston, MA) using iodogen (Pierce Chemical Co., Rockford, IL) according to the instructions of the manufacturer. As a negative control for experiments on the internalization of Tyr 543 under different conditions, cells expressing HA wt were assayed in parallel with those expressing Tyr 543. No internalization of HA wt was observed under any condition, including untreated cells (data not shown).

Results

Different Lateral Mobilities of HA wt and Tyr 543 in the Plasma Membrane of CV-1 Cells

Interactions of membrane proteins with immobile structures affect their lateral diffusion (14, 18, 25). Coated pits are essentially immobile in lateral mobility measurements by FPR (4). Thus, studies comparing the lateral mobility of a transmembrane protein that interacts with coated pits (Tyr 543) with that of a protein which is excluded from these structures (HA wt) have the capability of measuring the interactions with coated pits in the plasma membrane of live cells.

To enable measurements of the lateral mobilities of HA wt and Tyr 543 by FPR, CV-1 cells expressing these proteins were labeled in the cold by anti-HA TRITC-Fab′ monovalent antibody fragments (to avoid possible cross-linking that may affect the lateral diffusion). The labeling patterns observed were mainly homogeneous (Fig. 1), although on cells expressing Tyr 543 some patches were also detected occasionally. D and R_f of the labeled proteins in the plasma membrane were determined by FPR experiments. A typical FPR curve, depicting the lateral mobility of HA wt on the surface of a CV-1 cell, is shown in Fig. 2. The average results of many such experiments at several temperatures (37, 22, and 10°C) are depicted in Fig. 3. The D value of HA wt at 22°C was only about fivefold smaller than that of the lipid probe N-NBD-PE incorporated into the plasma membrane of the same cells ($(1.7 \pm 0.15) \times 10^{-9}$ cm²/s at 22°C; $n = 30$), in the range expected based on the size difference between the membrane protein and the lipid molecules and the weak (logarithmic) dependence of lateral diffusion in membranes on molecular size (54). The mobile fraction was essentially similar to that of N-NBD-PE (82 ± 4)%, among the highest observed for cell surface proteins (reviewed in 16, 22, 31). This suggests unrestricted lateral diffusion of HA wt. The D and R_f values obtained for HA wt at 22°C are in good agreement with the values found on a fibroblast cell line permanently expressing influenza HA (17). Furthermore, the high mobility measured for HA wt at 37°C ($D = 1.17 \times 10^{-9}$ cm²/s, $R_f = 87$%) is not a general property of the CV-1 cells or due to the experimental setup; this is demonstrated by the significantly lower R_f ((50 ± 5)%) and D ($(4.0 \pm 0.5) \times$

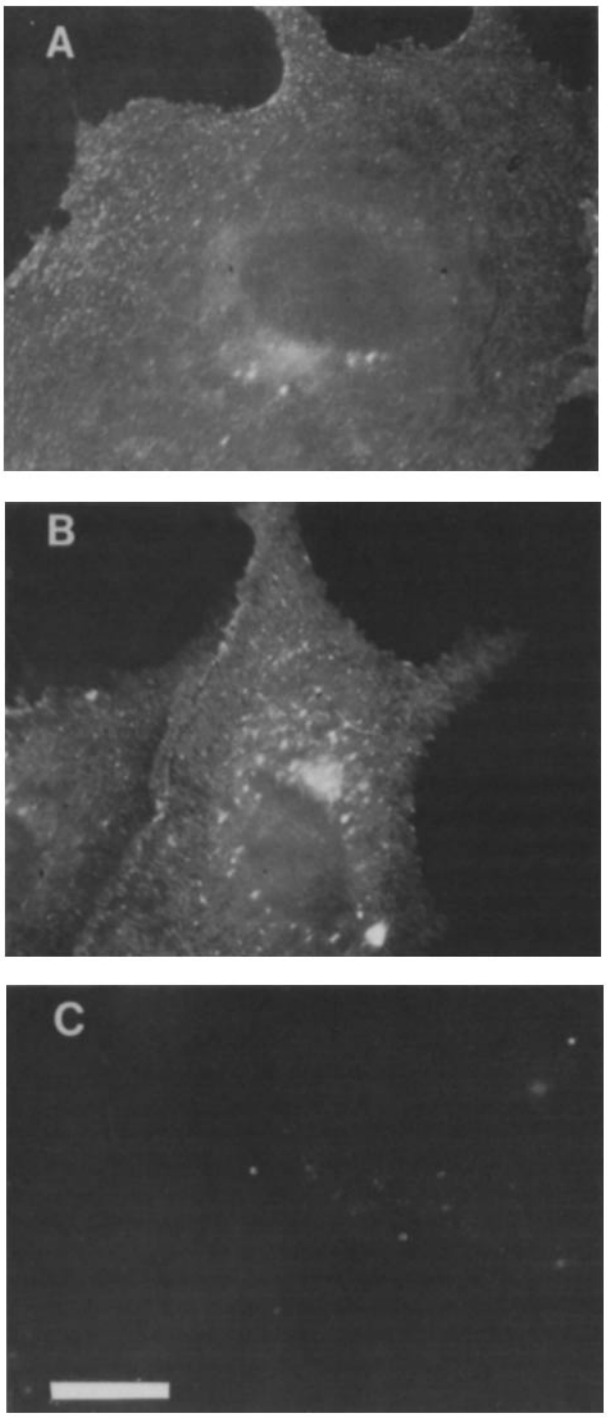


Figure 1. Immunofluorescence labeling of HA wt and Tyr 543 by monovalent Fab′ fragments on the surface of CV-1 cells. CV-1 cells expressing HA wt or Tyr 543 were grown on glass coverslips, and labeled at 4°C by anti-HA TRITC-Fab′ as described under the immunofluorescence microscopy section (Materials and Methods). The cells were fixed and photographed using rhodamine filters as detailed under Materials and Methods. (*A*) CV-1 cells expressing HA wt. (*B*) CV-1 cells expressing Tyr 543. (*C*) CV-1 cells mock-infected with the SV-40 vector devoid of the HA cDNA. Bar, 20 μm.

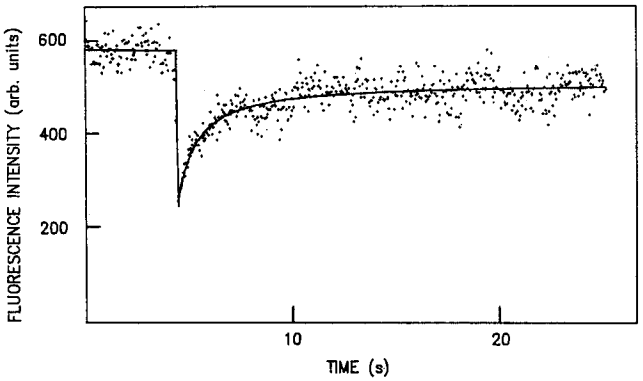


Figure 2. A representative FPR curve of the lateral motion of HA wt in the plasma membrane of a CV-1 cell. Cells were infected with the recombinant SV-40 virions, grown on glass coverslips, and labeled with TRITC-Fab' as described under Materials and Methods. The cells were wet-mounted in warm (37°C) HBSS/Hepes/BSA, and taken for the FPR measurement performed at 37°C. The dots represent the fluorescence intensity (photons counted over dwell times of 50 ms each). The solid line is the best fit to the lateral diffusion equation using nonlinear regression (49). The specific curve shown yielded $D = 1.33 \times 10^{-9}$ cm²/s, and $R_f = 82\%$.

10^{-10} cm²/s) values (mean ± SEM of 21 measurements) obtained under identical conditions for Con A receptors labeled with TRITC-succinyl-con A.

Unlike HA wt, Tyr 543 enters coated pits and undergoes endocytosis (38). The interactions of Tyr 543 with the coated pits, which are immobile on the FPR experimental time scale, can alter its mobility. In principle, interactions of a transmembrane protein with immobile structures may have one of two effects on its lateral mobility (14, 18, 25). (*a*) If it associates with the immobile entity to form complexes whose lifetime is long relative to that of the FPR experiment, R_f of the transmembrane protein is expected to drop, since a molecule associated with an immobile structure will remain bound for the entire duration of the measurement. (*b*) If the complexes have a lifetime comparable to or shorter than the characteristic diffusion time of the transmembrane protein ($\tau_D = w^2/4D$, where w is the Gaussian radius of the laser beam at the cell surface), each protein molecule will undergo several association–dissociation cycles with the immobile structures during the FPR measurement. This will reduce the effective D value rather than R_f, since each protein molecule will spend some time bound to the immobile entity, being free to diffuse the rest of the time.

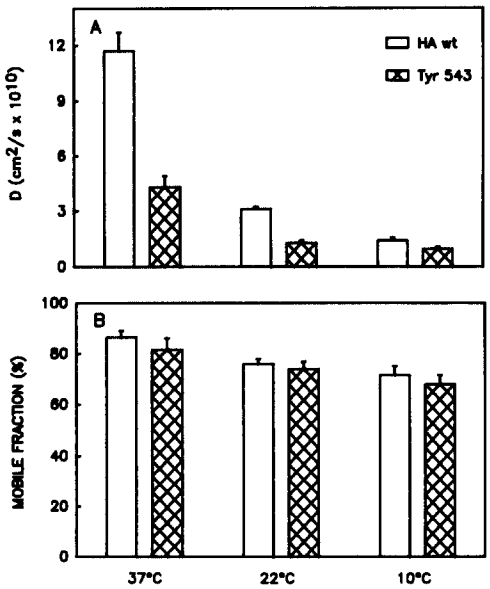


Figure 3. Lateral diffusion of HA wt and Tyr 543 on the surface of CV-1 cells. The experiments were conducted as in Fig. 2, on cells expressing either HA wt (*blank bars*) or Tyr 543 (*crossed bars*). The cells were labeled with anti-HA TRITC-Fab' 36–38 h postinfection. The mobility measurements were performed at either 37, 22, or 10°C. Each bar is the mean ± SEM of 30–40 measurements. (*A*) D values; (*B*) R_f values.

The data comparing the lateral diffusion of HA wt and Tyr 543 are presented in Fig. 3. At 37°C and 22°C, D of Tyr 543 was 2.5–2.7-fold smaller than that of HA wt. This effect cannot be due to binding of Tyr 543 by its cytoplasmic domain to soluble cytoplasmic proteins, in view of the very weak dependence of the lateral diffusion of membrane proteins on molecular size (54). The most likely explanation, which is supported by the studies described in the following section, is that Tyr 543 forms labile complexes (undergoes transient association–dissociation processes) with components that are immobile on the time scale of the lateral mobility measurement, resulting in the inhibition of its lateral diffusion rate relative to HA wt. The smaller difference (~1.4-fold) between the D values of Tyr 543 and HA wt at 10°C (Fig. 3) suggests that the mobility-restricting interactions are temperature-dependent, and become weaker at low temperatures. The similar R_f values of Tyr 543 and HA wt at all the measurement temperatures (Fig. 3) demonstrate the absence of detectable amounts of mutant proteins that form stable complexes with the immobile structures.

Treatments That Alter the Structure of Coated Pits Affect the Lateral Diffusion of Tyr 543 Relative to HA wt

Tyr 543 differs from HA wt in only one amino acid in the short cytoplasmic tail, and appears to be otherwise identical to HA wt in several assays of HA structure (38). In view of the ability of Tyr 543 to concentrate in coated pits and to be internalized, it is highly likely that the immobile structures which inhibit its lateral mobility relative to HA wt are coated pits and/or flat clathrin lattices reported recently to interact with human transferrin receptors (46). To test this point, we have treated CV-1 cells expressing Tyr 543 or HA wt under conditions that either disrupt or alter the structure of the coated pits, and examined the effects on the lateral mobility of the wild-type and mutant HA proteins.

Incubation in a hypertonic medium was shown to disassemble the clathrin lattice structure underlying coated pits in human and chicken fibroblasts (27). This treatment is effective also in CV-1 cells, as shown by the blockade of the endocytosis of Tyr 543 following a 15-min exposure of the cells to medium containing 0.45 M sucrose. Thus, prolonged incubation at 37°C shifted the homogeneous distribution of TRITC-Fab'-Tyr 543 (but not of Fab'-labeled HA wt) into discrete patches in untreated CV-1 cells, but failed to do so in cells treated by the hypertonic medium (Fig. 4, *A–C*). The inhibition of Tyr 543 internalization by hypertonic medium was quantified by a trypsin accessibility assay (Fig. 4 *D*). Tyr

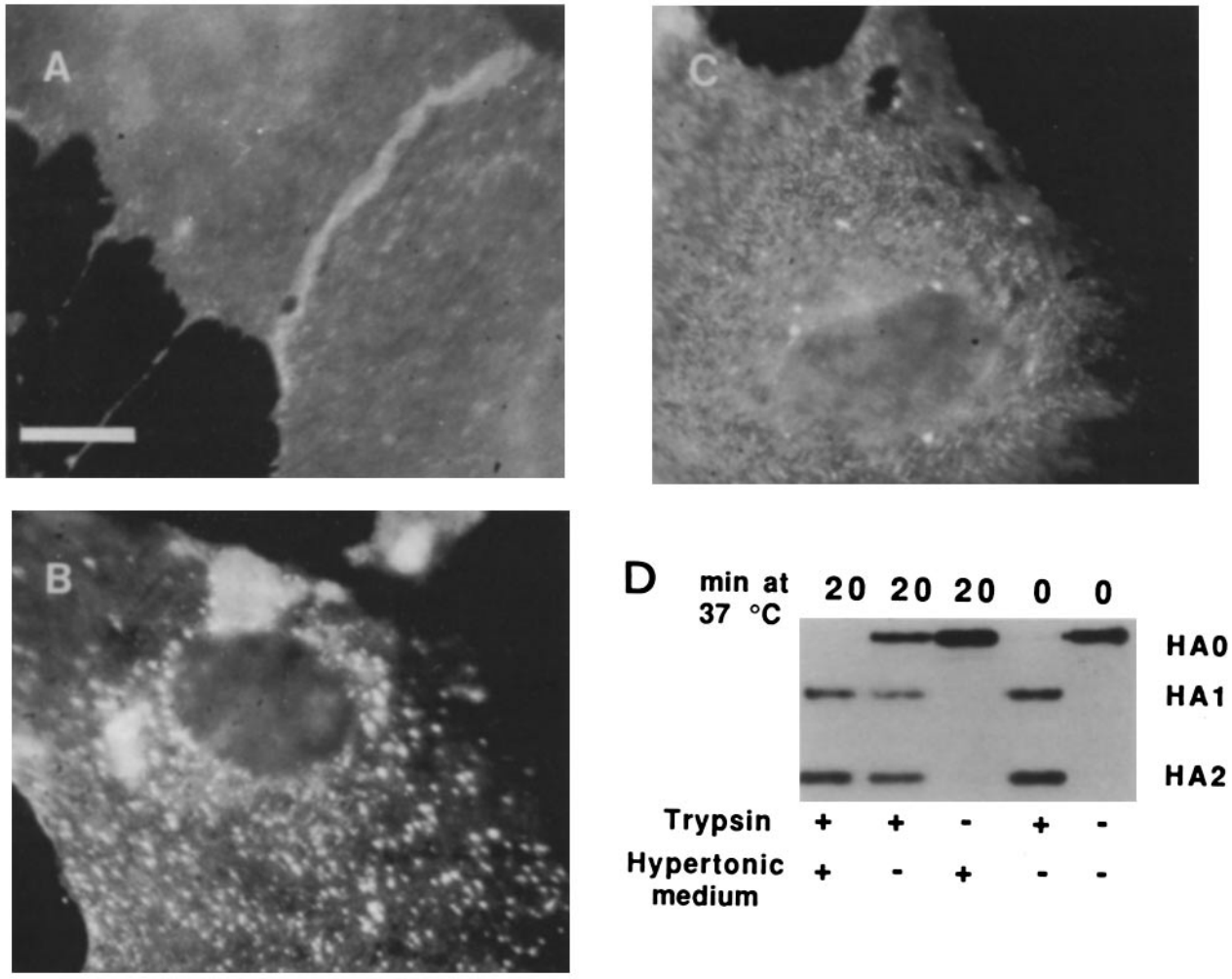


Figure 4. Effect of hypertonic medium treatment on the internalization of Tyr 543. Treatment of cells with the hypertonic medium was performed as described under Materials and Methods. In the fluorescence microscopy experiments (*A–C*), the cells were treated as described in the immunofluorescence microscopy section (Materials and Methods), except that the incubation with anti-HA TRITC-Fab′ (100 μg/ml) was carried out for 30 min at 37°C to allow endocytosis. The fluorescence pattern obtained upon labeling in the cold is shown in Fig. 1. (*A*) Fluorescence labeling pattern on untreated CV-1 cells expressing HA wt. The labeling pattern remained homogeneous, as expected in the absence of endocytosis. (*B*) Fluorescence labeling pattern on untreated cells (normal medium) expressing Tyr 543. The labeling shifts into discrete patches after the incubation at 37°C. (*C*) Hypertonic medium inhibits the 37°C-induced shift in the fluorescence labeling pattern on cells expressing Tyr 543. (*D*) Effect of hypertonic medium on the internalization of Tyr 543 as determined by the trypsin accessibility assay. Cells expressing ^{35}S-labeled Tyr 543 at the surface were prepared for the experiments as described in Materials and Methods. Tyr 543 at the cell surface was tagged with anti-HA antibodies, and the percentage of the antibody-tagged molecules that become inaccessible to extracellular trypsin was determined after 20 min at 37°C (Materials and Methods). In hypertonic medium, Tyr 543 was quantitatively cleaved into HA1 and HA2 subunits, indicating that all of the protein remained at the cell surface. In normal medium, 40% of the protein became inaccessible to trypsin (remaining in the HA0 form). Control experiments demonstrated that recovery of Tyr 543 in the immunoprecipitation procedure is not affected by the presence of trypsin or by prior treatment with hypertonic medium. Bar, 20 μm.

543 molecules prelabeled by antibody at the cell surface at 4°C in hypertonic or normal medium were allowed to be internalized at 37°C for 20 min in the respective media, and then treated with trypsin at 4°C to cleave the Tyr 543 molecules remaining at the cell surface into HA1 and HA2 subunits. In hypertonic medium, no Tyr 543 became inaccessible to trypsin under these conditions, whereas ~40% of the protein became protected from extracellular trypsin in normal medium.

Fig. 5 depicts the effects of hypertonic medium treatment on the lateral diffusion of Tyr 543 and HA wt. The presence of sucrose in the medium resulted in a minor decrease (20–30%) in the lateral diffusion rate of HA wt. The effect on D of Tyr 543 was much more striking, as this value increased significantly and became identical to that of HA wt under the same conditions.

Acidification of the cytosol arrests the pinching-off of coated pits, causing the clathrin lattices to remain at their plasma membrane sites, albeit at an altered lattice morphology (12, 26, 56). It was therefore of interest to examine the effects of cytosol acidification on the mobility-restricting interactions experienced by Tyr 543. As in the case of the hypertonic medium treatment, cytosol acidification resulted in a blockade of the endocytosis of Tyr 543 labeled with

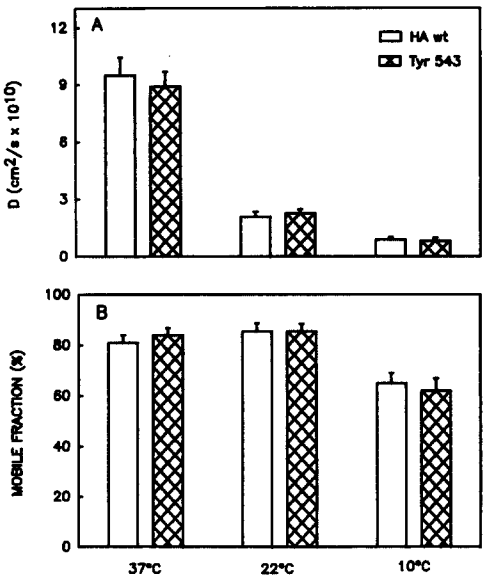


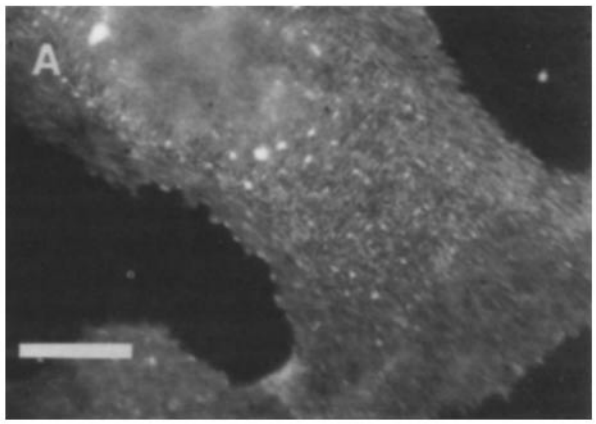
Figure 5. Effect of hypertonic medium treatment on the lateral diffusion of Tyr 543 and HA wt. CV-1 cells expressing HA wt or Tyr 543 were pretreated with a hypertonic medium as described under Materials and Methods. The FPR experiments were performed at 37, 22, or 10°C as in Fig. 2, except that HBSS/Hepes/BSA supplemented with 0.45 M sucrose was used throughout. Each bar is the mean ± SEM of 30–40 measurements. (*A*) *D* values; (*B*) R_f values.

TRITC-Fab' (Fig. 6 *A*) or whole antibodies (Fig. 6 *B*). The trypsin accessibility assay (Fig. 6 *B*) demonstrated that Tyr 543 is internalized rapidly in normal medium, and at a slightly decreased rate but to a similar extent in cells treated with cytochalasin D, which blocks the nonclathrin mediated internalization pathway (55). No internalization was observed in cells whose cytosol was acidified, confirming that Tyr 543 is internalized by the pathway sensitive to the immobilization of clathrin that occurs at a low cytoplasmic pH (see reference 26). In contrast to the situation in untreated, hypertonic medium-treated (Fig. 4 *D*), or cytochalasin-treated cells (Fig. 6 *B*), where Tyr 543 that was never allowed to be internalized (no incubation at 37°C) was completely accessible to extracellular trypsin at 4°C, in cytosol-acidified cells 7–14% of Tyr 543 (three experiments) remained inaccessible to extracellular trypsin in the absence of 37°C chase (Fig. 6 *B*). This could represent Tyr 543 molecules trapped in deep coated pits in response to the acidified cytosol (26). Similar deep pits formed in cells depleted of ATP have recently been shown to be accessible to small peptides such as glutathione, but not to large proteins (58).

The effect of acidifying the cytosol on the lateral mobility of Tyr 543 was different from that of the hypertonic medium treatment. Acidification of the cytoplasm had no effect on *D* of HA wt, and only a minor reduction in its R_f value was detected at 37°C. However, the lateral mobility of Tyr 543 was drastically altered: its *D* values increased and became indistinguishable from those of HA wt, while at the same time its R_f dropped significantly (Fig. 7). This is indicative of the existence of two Tyr 543 populations—one which diffuses freely in the plasma membrane as does HA wt, and the other which is laterally immobile on the experiment time scale. On the other hand, disruption of the microfilaments by cytochalasin D (10 μg/ml) or of the microtubules by colchicine (10 μM) using the conditions described in Materials and Methods (under Assays for Internalization) did not alter significantly the lateral diffusion of Tyr 543 (or HA wt) measured at 37°C. The *D* and R_f values of Tyr 543 varied by <15% and 8%, respectively (two experiments with each drug, n = 30), in cells treated with either cytochalasin D or colchicine.

Discussion

The influenza HA protein is especially suitable for studies



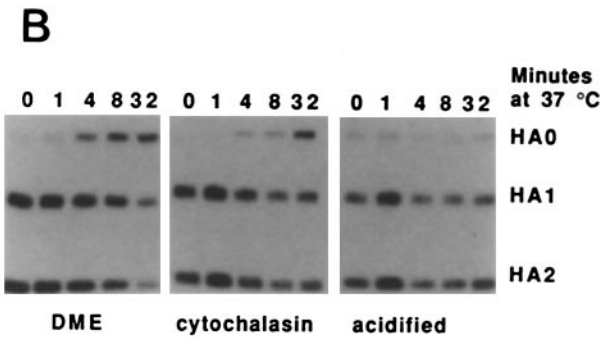


Figure 6. Internalization of Tyr 543 in cells treated to acidify their cytoplasm and in cytochalasin-treated cells. The treatments were carried out on cells expressing Tyr 543 as described in Materials and Methods. (*A*) Acidification of the cytoplasm prevents the 37°C-induced shift of TRITC-Fab' labeling to discrete patches. Experiments were performed on cells whose cytosol was acidified using the same protocol described in Fig. 4. The temperature-dependent change in the labeling pattern on cells expressing Tyr 543 in normal medium is shown in Fig. 4 *B*. (*B*) Trypsin accessibility internalization assay. The experiments were performed as detailed under Materials and Methods and in the legend to Fig. 4 *D*. The portion of Tyr 543 molecules at the surface that become inaccessible to trypsin (uncleaved HA0) as a function of the time of incubation at 37°C provides a measure of the internalization rate. In cells that were not subjected to cytosol acidification, either with or without cytochalasin D, less than 3% of the antibody-tagged Tyr 543 at the cell surface were protected from cleavage by trypsin to HA1 and HA2 in the absence of 37°C chase (0 chase lanes here in in Fig. 4 *D*). This percentage increased to ~40% (three experiments) at the long chase times. In contrast, in cytosol-acidified cells a small but significant percentage of Tyr 543 (7–14%; three experiments) was inaccessible to trypsin even when internalization was blocked by low temperature, and this percentage did not increase during a subsequent chase at 37°C. Bar, 20 μm.

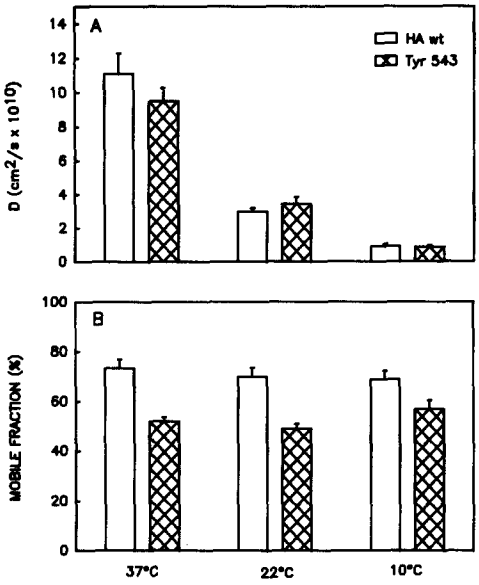


Figure 7. Lateral mobility of Tyr 543 and HA wt on the surface of cytosol-acidified CV-1 cells. Cells expressing HA wt or Tyr 543 were subjected to the cytosol acidification treatment (Materials and Methods). The FPR experiments were conducted in the KA/BSA buffer at the indicated temperatures. The bars represent the mean ± SEM of 30–40 measurements in each case. (*A*) *D* values; (*B*) R_f values.

on the nature of the interactions between transmembrane proteins and coated pits, since the HA wt is excluded from coated pits, while a point mutation (generating the Tyr 543 mutant) results in a positive signal enabling endocytosis and recycling (38). In this communication, we describe measurements on the lateral mobility of HA wt and Tyr 543 on the surface of untreated cells or of cells treated to alter the structure of their clathrin coats. These studies, conducted on live cells in culture, provide evidence for dynamic (association–dissociation) interactions between Tyr 543 and coated pits. Furthermore, they indicate that alterations in the clathrin lattice structure can either cancel or enhance these interactions.

To investigate the nature of the proposed interactions between Tyr 543 and coated pits, the lateral mobilities of Tyr 543 and HA wt were compared. These experiments take advantage of the coated pits being immobile on the FPR experimental time scale (4), and of the fact that interactions of membrane proteins with immobile structures may affect either *D* or R_f of the membrane protein, depending on the lifetimes of the complexes formed relative to the lateral diffusion rate of the protein (14, 18, 25). The lateral mobility of HA wt in the plasma membrane of CV-1 cells was characterized by high mobile fractions (essentially similar to those of the lipid probe N-NBD-PE) and relatively high *D* values (only fivefold below *D* of N-NBD-PE) (Figs. 2 and 3; see Results). This indicates that the lateral mobility of HA wt is relatively unrestricted, unlike the situation with most membrane proteins, whose lateral diffusion is constrained by factors beyond the viscosity of the membrane lipid bilayer (16, 31). As discussed under Results, the slower diffusion of Tyr 543 relative to HA wt (Fig. 3) is most likely due to interactions of the mutated cytoplasmic domain of Tyr 543 with structures that are immobile on the FPR experimental time scale. Obvious candidates for such structures are coated pits, since Tyr 543 (but not HA wt) is internalized via the clathrin-coated pits pathway (38; Figs. 4 and 6) and therefore must interact with them. To explore this possibility, we investigated the effects of treatments known to disrupt or alter the coated pits on the lateral mobility of Tyr 543 relative to that of HA wt (Fig. 5 and 7). The data are entirely consistent with the simple interpretation that Tyr 543 mobility is restricted by binding to coated pits and that HA wt is unable to compete for space in the pit simply due to its lack of binding to these structures. Another interpretation for the failure of HA wt to bind to coated pits is active exclusion of this protein (but not of Tyr 543) from the coated pit region; however, this requires that HA wt would be excluded from coated pits by an unknown interaction that does not restrict its lateral mobility, an interaction for which there is currently no data.

Interactions of Tyr 543 with flat clathrin lattices, which were recently reported to bind human transferrin receptors in transfected chick embryo fibroblasts (46), are also possible. Thus, the term "interaction with coated pits" in further discussion will not attempt to discriminate between coated invaginations and flat clathrin lattices. It was proposed that slow directed motion of some transferrin receptor aggregates on the cell surface reflects the motion of flat lattices (46). However, such motion of Tyr 543-flat lattice complexes cannot contribute significantly to the apparent *D* values measured for the mutant protein, since the characteristic time for the directed motion is nearly 20-fold slower than the characteristic diffusion time.[2] Furthermore, only a minor fraction of the receptors underwent this type of motion (46, 59). An alternative explanation for the reduced diffusion rate of Tyr 543 is interactions with cytoskeletal elements; although this is still a formal possibility, there is no data to support it. The finding that disruption of the microfilaments (by cytochalasin D) or microtubules (by colchicine) does not elevate *D* of Tyr 543 further reduces the likelihood of this possibility.

At all the temperatures examined, *D* of Tyr 543 was lower than that of HA wt, while the R_f values of the two proteins were similar (Fig. 3). These differences disappeared following the disruption of coated pits by hypertonic medium (Fig. 5), reinforcing the dependence of the mobility inhibition on coated pits. As discussed under Results, the reduction in *D* (rather than in R_f) clearly indicates dynamic association-dissociation interactions between Tyr 543 proteins and immobile structures on the time scale of the FPR experiment. If these structures are indeed coated pits, this requires that all of the Tyr 543 molecules at the cell surface would be able to reach coated pits several times during the FPR measurement. The capture time (t_c) required for all molecules of a given transmembrane protein to reach coated pits is given by (21, 34):

$$t_c = (b^2/2D)\,[\ln(b/R) - 0.231], \qquad (1)$$

2. The contribution of directed motion to lateral diffusion measurement by FPR can be assessed by comparing the characteristic times of the two processes, τ_V and τ_D (3). For directed motion, $\tau_V = w/V$, where w is the Gaussian radius of the laser beam and V is the velocity of the directed motion (3). Introducing 1 μm/min for V (46, 59) and $w = 0.61$ μm (the radius of the laser beam in the current studies) yields $\tau_V = 36$ s. The lowest *D* value for Tyr 543 at 37°C was measured on untreated cells (4.3×10^{-2} μm²/s.). Introducing this value along with $w = 0.61$ μm in the expression for τ_D ($\tau_D = w^2/4D$; ref. 3) yields $\tau_D = 2.16$ s. Thus, τ_V is 17-fold higher than τ_D.

where b is the radius defining the average membrane area associated with a single coated pit, R is the radius of a coated pit, and D is the lateral diffusion coefficient of the transmembrane protein outside of the coated pit region. Introducing the appropriate values[3] in Eq. 1 yields t_c = 3.7 s at 37°C. This time is short relative to both the time scale of the FPR experiment (30 s for HA wt at 37°C) and the average lifetime of a coated pit in the membrane (~1–2 min at 37°C). Inclusion of the area covered by flat clathrin lattices in this calculation will reduce b and lead to even shorter t_c.

The data in Fig. 3 suggest that the interactions of Tyr 543 with coated pits depend on the temperature. While the strongest reduction in D of Tyr 543 relative to HA wt was observed at 37°C, the retardation of Tyr 543 mobility at 10°C was significantly weaker (Fig. 3). Under conditions where the chemical reaction (association–dissociation) is fast compared to the lateral diffusion rate of a membrane protein (B) that forms labile complexes with an immobile component (A), the effective D of the protein (D_e) is given by (18):

$$D_e = D_B \times \{1/(1 + K[A])\} \quad (2)$$

where D_B is the D value of the protein B in the absence of interactions with A, K is the equilibrium association constant for the binding of B to A, and [A] is the equilibrium concentration of free (unoccupied) binding sites on A; $1/(1 + K[A])$ is the free (unbound) fraction of B. Thus, while the question of whether D or R_f of B will be affected by binding to A is determined by the binding kinetics relative to the diffusion rate, the extent of the effect in either case depends on the affinity. The simplest explanation for the weaker effect on Tyr 543 mobility at 10°C is therefore that the affinity of the mutant protein molecules to coated pits is reduced at lower temperatures; such a pattern of temperature dependence is typical of entropy-driven association processes (e.g., hydrophobic interactions).

The inhibition of Tyr 543 lateral mobility relative to HA wt appears to be due to interactions with coated pits, since it is eliminated when the membrane-associated coated pits are dispersed by the sucrose-containing medium (Fig. 5). In this context, it was of interest to examine the effects of other treatments that affect coated pit structure. To this end, we employed acidification of the cytoplasm by prepulsing the cells with NH_4Cl. This treatment alters the structure of the clathrin lattice underlying the plasma membrane, and blocks endocytosis by preventing the pinching-off of coated vesicles (26, 56). Following this treatment, which was highly effective in blocking the internalization of Tyr 543 (Fig. 6), the mutant protein exhibited a reduced mobile fraction, while its D value increased to the level of HA wt (Fig. 7). The reduction in R_f was by ~30% of the original R_f value, suggesting that ~1/3 of the surface Tyr 543 molecules become associated with immobile structures (most likely coated pits) for the entire duration of the measurement, while the diffusion of the remaining Tyr 543 molecules is not restricted beyond that of HA wt. The simplest interpretation of this observation is that the alteration in the clathrin lattice structure mediated by the acidification of the cytoplasm (26) alters the association–dissociation kinetics of Tyr 543 binding to the lattices, so that no significant exchange between bound and free Tyr 543 molecules occurs on the time scale of the FPR experiment. Under such conditions (where the chemical reaction rate is slow relative to the diffusion rate), the mobile fraction of a membrane protein (B) that binds to an immobile component (A) depends on K, much like the D_e value (Eq. 2) does when the relation between the rates of the chemical reaction and the diffusion is reversed (18):

$$R_f(B) = 1/(1 + K[A]) - M \quad (3)$$

where M is the "background" immobile fraction (which is not due to the binding equilibrium under study). Therefore, a lower affinity (smaller K) will produce a weaker effect on R_f. Indeed, the reduction in R_f of Tyr 543 in the NH_4Cl prepulsed cells at 10°C is lower than at 37 or 22°C (Fig. 7), in the same direction as the weaker effect on the D value of the mutant protein at 10°C in untreated cells (Fig. 3). These observations support the notion that the affinity of Tyr 543 to coated pits decreases at low temperatures in both untreated and cytosol-acidified cells.

The data presented in this communication suggest that Tyr 543 molecules interact in a transient manner with coated pits (attach to and detach from coated pits continuously). Interestingly, the other end of the spectrum of membrane protein-coated pit interactions (i.e., permanent entrapment) can also be observed in the current study in cells pre-treated to acidify their cytoplasm (Fig. 7). In this case, the effect is most likely due to the alteration in clathrin lattice structure following cytosol acidification (26). However, the diversity in the cytoplasmic domains of various receptors can also lead to quantitative differences in the kinetic and/or affinity constants characterizing their binding to coated pits. These parameters may also be modulated for certain receptors, such as those for specific growth factors, by ligand binding and/or receptor aggregation (15, 43, 57). In this context it should be noted that in a previous study of the EGF receptor, the mobile fraction of endocytosis-defective mutants lacking most of the cytoplasmic domain was as high as that of a lipid probe (N-NBD-PE) in the same cells, and showed a small but significant increase (from R_f = 60% to nearly 80%) relative to the native receptor (see Table II in ref. 41). At the same time, the D values of the mutant receptors remained similar to those of the wild-type receptor. These observations are entirely consistent with entrapment in coated pits of nearly 20% of the native receptors, but not the truncated mutants, for the entire duration of the FPR measurement. The relatively low percent increase in R_f of the mutant compared to the native receptor in these studies may reflect the high concentration of EGF receptors at the surface of the transfected COS cells used (about fivefold higher than the EGF receptor concentration on A431 cells, which is in the range of 1–4 million receptors/cell). Under these conditions,

3. The capture time (t_c) required for all the molecules of a given transmembrane protein to reach coated pits is given by Eq. 1. The coated pits occupy ~2% of the cell surface area (designated A), and the radius of a coated pit (R) is ~0.1 μm. Thus, the density of the coated pits in the plasma membrane is: $[0.02A/(\pi \times 0.1^2)]/A = 0.64$ pits/μm^2. The reciprocal of this value is the average membrane area containing a single coated pit; defining this area as a circle with a radius b, we obtain $b = 0.706$ μm. Introducing b and R in Eq. 1 (see text) yields t_c, providing D is known. If we assume that outside of the coated pit D of Tyr 543 is similar to that of HA wt (as indicated by the similar D values of HA wt and Tyr 543 after disruption of the coated pits; Fig. 6), the D value to be used for 37°C is 11.7×10^{-2} μm^2/s, yielding t_c = 3.7 s. Even if one employs instead the D value obtained for Tyr 543 (4.3×10^{-2} μm^2/s, which already includes the effect of the time spent by the molecule within coated pits), t_c is still relatively short (10 s).

coated pits would be expected to accommodate only a fraction of the native EGF receptors.

The labile nature of Tyr 543 binding to coated pits in untreated cells contrasts with the behavior expected on the basis of one of the models for coated pit or flat lattice formation, which proposes that transmembrane and coat proteins are added simultaneously at the edges of the growing lattice (6, 30, 46, 48). This obviously does not hold for the Tyr 543 protein, which under such conditions would be expected to exhibit a significant fraction which is not readily exchangeable with Tyr 543 molecules outside of the clathrin-coated regions. It should be noted that other transmembrane proteins, which may form stable complexes with clathrin lattices, could still function in lattice formation.

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