Partitioning of Proteins into Plasma Membrane Microdomains

CLUSTERING OF MUTANT INFLUENZA VIRUS HEMAGGLUTININS INTO COATED PITS DEPENDS ON THE STRENGTH OF THE INTERNALIZATION SIGNAL

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Internalization of membrane proteins involves their recruitment into plasma membrane clathrin-coated pits, with which they are thought to interact by binding to AP-2 adaptor protein complexes. To investigate the interactions of membrane proteins with coated pits at the cell surface, we applied image correlation spectroscopy to measure directly and quantitatively the clustering of influenza hemagglutinin (HA) protein mutants carrying specific cytoplasmic internalization signals. The HA system enables direct comparison between isolated internalization signals, because HA itself is excluded from coated pits. The studies presented here provide, for the first time, a direct quantitative measure for the degree of clustering of membrane proteins in coated pits at the cell surface. The degree of clustering depended on the strength of the internalization signal and on the integrity of the clathrin lattices and correlated with the internalization rates of the mutants. The clustering of the HA mutants fully correlated with their ability to co-precipitate α-adaptin from whole cells, the first such demonstration for a membrane protein that is not a member of the epidermal growth factor receptor family. Furthermore, both the clustering in coated pits and the co-precipitation with α-adaptin were dramatically reduced in the cold, suggesting that low temperature can interfere with the sorting of proteins into coated pits. In addition to the specific results reported here, the general applicability of the image correlation spectroscopy approach to study any process involving the clustering or oligomerization of membrane receptors at the cell surface is discussed.

Receptor-mediated endocytosis constitutes a crucial part in the life cycle of cell surface proteins and provides a major mechanism for receptor down-regulation and signal termination (1–4). The clathrin-coated pits, which are the major port of entry into the endocytic pathway in many cell types, represent the best studied example of a sorting domain where a coat of proteins to the cytosolic face of the membrane selectively binds membrane proteins containing specific motifs that serve as internalization signals (5–7). Two classes of clathrin-associated assembly proteins (APs)1 have been identified, one specific for plasma membranes (AP-2) and one for the trans-Golgi network (AP-1) (5–10). AP-2 is known to function both in assembly of the clathrin lattice (10–13) and in binding to membrane proteins carrying internalization signals, which are concentrated in clathrin-coated pits for endocytosis (5, 14–22).

Recent evidence identifies several distinct classes of cytoplasmic internalization signals (reviewed in Refs. 3, 4, 23, and 24). The best characterized are those containing an essential aromatic residue, typically a tyrosine. They conform mostly to one of two major subclasses: NPXY, where Tyr is preceded by an asparagine-proline dipeptide and a random amino acid, or YXXZ, where Z is an amino acid with a hydrophobic side chain (3, 23–25). It was suggested that a type 1 tight turn conformation might constitute a general feature of tyrosine-based internalization signals (3, 23, 26–30). However, a recent study employing combinatorial selection methods on the binding of YXXZ-containing peptides to the μ2 chain of AP-2 indicated no requirement for a prefolded structure around the tetrapeptide signal (31). A different class of internalization signals contains a di-leucine motif (LL or LI) (24, 32–34).

Although much has been learned about clathrin-mediated endocytosis in recent years, currently it is not well understood how membrane proteins bind to coated pits at the surface of the intact cell. In vivo endocytosis studies generally measured the sequestration of proteins into vesicles or deeply invaginated pits and did not allow direct measurement of many events that occur earlier during coated vesicle formation (e.g. the selection of certain proteins for inclusion in coated pits and the exclusion of others). Furthermore, experimental evidence for interactions of the internalization signals of membrane receptors with AP-2 subunits has been limited mostly to in vitro assays employing solubilized and immobilized proteins (16–19, 21, 31, 35). These assays have detected only a subset of the sequences known or suspected to function as internalization signals in vivo (36, 37). In particular, association of AP-2 with receptors by co-immunoprecipitation from cells has thus far been demonstrated only for receptors from the epidermal growth factor (EGF) family (14, 15, 20, 21, 38). Even for these receptors, it is not clear whether this in vitro association is directly related to the efficiency of their internalization via coated pits (21, 39, 40). It is therefore important to explore the relationships between binding to AP-2, clustering in coated pits, and the efficiency of the

1 The abbreviations used are: AP, assembly protein; EGF, epidermal growth factor; HA, influenza virus hemagglutinin; ICS, image correlation spectroscopy; HBSS, Hanks’ balanced salt solution; Sulfo-NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate; FITC-GAR Fab’, fluorescein-coupled affinity purified Fab’ of goat IgG directed against rabbit Fab’2; wt, wild type; BSA, bovine serum albumin.
internalization process as a function of the internalization signal; this manuscript presents measurements to that end.

Our studies were performed on a series of influenza hemagglutinin (HA) mutants carrying specific cytoplasmic internalization signals (37, 41, 42). This system is advantageous, because wild-type HA (HA wt) lacks internalization signals and serves as a natural control, enabling investigation of specific internalization sequences introduced into its cytoplasmic tail (41, 43, 44). Using this system, we employed comparative studies of the lateral mobility of these mutants to characterize the mode of their interactions with coated pits at the surface of intact cells (43, 44). However, in these studies, measurement of the binding of internalization-competent HA mutants to immobilized surfaces presumed to represent coated pits was indirect and was inferred from the reduction in the lateral diffusion rate or mobile fraction of the mutants relative to HA wt. Because numerous factors (and not only interactions with coated pits) may inhibit the lateral mobility of membrane proteins, it was important to measure the association of the HA mutants with coated pits at the cell surface in a direct and independent manner.

The current studies, which are potentially applicable for studying any type of clustering or oligomerization at the cell surface, provide a direct demonstration and measure for internalization signal-dependent clustering of HA mutants in cell surface coated pits. Together with the findings on signal-dependent co-precipitation of AP-2 with the various HAs in strict correlation with the ICS data, our results suggest that the degree of clustering in coated pits for a given protein depends on the strength of the association of its internalization signal with the clathrin-associated adaptor complexes. In turn, this clustering plays an important role in determining the endocytosis rate and restricts the lateral mobility of the internalization-competent proteins at the cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and T4-DNA ligase were from New England Biolabs (Beverly, MA). Trypsin, amiloride hydrochloride, protein A-Sepharose 4B, BSA, and N-propylgallate were from Sigma. Hanks’ balanced salt solution (HBSS) was from Sigma or Life Technologies, Inc. Protein A-peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Sulfosuccinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin) and streptavidin-peroxidase were from Pierce, and ECL reagent (Renaissance) was from NEN Life Science Laboratories (West Grove, PA). Protein A-peroxidase was from Jackson ImmunoResearch Laboratories, Inc. The Flourescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) and from normal goat IgG as described (45). The FITC-GAR Fab'9' preparations were used in the current experiments. When a single fluorescently labeled component is analyzed (as in the current measurements), the autocorrelation function, $g(\xi, \eta)$, is derived and fitted to a two-dimensional Gaussian function determined by the intensity profile of the laser beam (49),

$$g(\xi, \eta) = g(0,0) e^{-(\xi^2 + \eta^2)/g_0^2}$$  

(Eq. 1)

where $\xi$ and $\eta$ are the position coordinates (for the $x$ and $y$ axes, respectively) of the autocorrelation function, $g(0,0)$ is the value of the autocorrelation function at $(0,0)$, $\xi$ and $\eta$ are the position coordinates of the fluorescent particle, and $g_0$ is a characteristic decay length. 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 antibody incubation and subsequent steps. In most cases (except where indicated), the labeled cells were warmed to $22 \, ^\circ\mathrm{C}$ for 10 min prior to fixation in methanol ($-20 \, ^\circ\mathrm{C}$, 5 min) and acetone ($-20 \, ^\circ\mathrm{C}$, 2 min) to enable stronger interactions with coated pits while avoiding significant internalization of some mutants at $57 \, ^\circ\mathrm{C}$ (43, 44). The fixed cells were mounted in Slowfade solution (Molecular Probes, Eugene, OR) or in Airvol 205 containing N-propylgallate and taken for the ICS studies.

**Image Correlation Spectroscopy**—ICS (described in detail in Refs. 49 and 50) is an adaptation of the fluorescence correlation spectroscopy method (51–53) used to analyze fluorescence images collected on a confocal laser scanning microscope. It is sensitive to and capable of quantifying differences in the aggregation state and distribution of fluorescently labeled components at the cell surface. In fluorescence correlation spectroscopy, one examines the volume or area illuminated by a laser beam, usually in a microscope. In ICS, instead of observing fluorescent particles as they diffuse in and out of a fixed laser beam, one generates an image of the distribution of the fluorescence intensity on the cell surface and monitors the fluorescence intensity fluctuations. When a single fluorescently labeled component is analyzed (as in the current measurements), the autocorrelation function, $g(\xi, \eta)$, is derived and fitted to a two-dimensional Gaussian function determined by the intensity profile of the laser beam (49),

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The sequences of the short cytoplasmic domains of each HA mutant are shown. The dots in HA-Y543 and HA+4 stand for amino acids similar to those in wt HA or HA−8, respectively. An asterisk is a stop codon.

**RESULTS**

**ICS Demonstrates Selective Increase in the Clustering of Internalization-competent HA Mutants at the Cell Surface**—ICS measures the spatial fluorescence intensity fluctuations arising from fluorescently labeled membrane components distributed across the surface of the cell and employs autocorrelation analysis to derive a quantitative measure of the density of independent fluorescent particles from which the degree of aggregation can be determined (Ref. 49; see "Experimental Procedures"). Proteins internalized via the clathrin-coated pit pathway have internalization signals that are thought to interact with AP-2 adaptors (5, 14–22, 31, 35, 38) and thus lead to preferential localization in plasma membrane coated pits. The resulting change in surface distribution of such internalization-competent proteins is essentially a clustering phenomenon, which could be measured by ICS. To demonstrate the capability of ICS to measure association with coated pits at the cell surface, we compared the surface distribution and clustering of HA wt and several HA mutants that carry different cytoplasmic internalization signals and are internalized at widely different rates.

CV-1 cells expressing different HA mutants (Table I) were fluorescently labeled in the cold by monovalent Fab' fragments to avoid any possibility for IgG-mediated cross-linking that might affect the clustering. The labeled cells were incubated (10 min) at 22 °C prior to fixation, because earlier work (45, 44) has shown that interactions of HA mutants with coated pits were greatly reduced at lower temperatures; warming to 37 °C was avoided due to the fast internalization of HA+8 at this temperature (42). The fixed cells were taken for ICS experiments. Typical confocal images of a protein that is excluded from coated pits (HA wt) and a mutant that is internalized at a moderate rate (HA-Y543; Refs. 41 and 42) are depicted in Fig. 1, A and B. This clustering disappears in cells preincubated under hypertonic conditions (Fig. 1C; this effect is discussed later). To quantify these differences, images of flat 16.3 × 16.3-μm² areas on the cell surface were processed by autocorrelation analysis to determine the g(0,0) values, from which 1/g(0,0) (which gives a direct measure of Np) can be calculated (Table I).
those of HA wt and HA+4 (Fig. 2, A and B), the frequency distributions of these values among the cells are fairly broad. This could be caused, at least in part, by cell-to-cell differences in the expression levels of the HA proteins at the cell surface. The reason for the dependence of \(1/g(0,0)\) on the surface density of the labeled protein is that a higher density can increase the number of the labeled molecules (in the current study, HA proteins) per particle. An increase in the surface density of labeled protein molecules, with no changes in the aggregation state (i.e. the particle size is unaltered) will elevate \(N\) (Eq. 2)

\[ DA = g(0, 0) \times (i) = a(N_m / N_p) \]

where \((i)\) is the average fluorescence intensity of the same image used to generate the specific autocorrelation function. The proportionality constant \(a\) depends on instrumental parameters and cancels out if relative DA values (ratios) are used. Because \((i)\) is directly proportional to the average number of fluorescently labeled molecules in the area illuminated by the beam \(N_m\) and \(g(0,0)\) is equal to \(1/N_p\), their product \((DA)\) is proportional to \(N_m/N_p\), the average number of fluorescently labeled molecules (in the current study, HA proteins) per particle. An increase in the surface density of labeled protein molecules with no changes in the aggregation state (i.e. the particle size is unaltered) will elevate \(N_m\) and \(N_p\) by the same factor, leaving \(DA\) constant. However, if more protein molecules are driven into aggregates, the average number of molecules per particle will increase, and the higher aggregation will lead to a higher \(DA\) value.

To internally calibrate the measurements for possible variations between separate experiments, each experiment included a “standard” sample of HA wt at 22 °C. This sample (chosen due to HA wt being excluded from coated pits) was given the \(DA\) value of one, and the \(DA\) values of all the other samples were calibrated relative to this value. The results are depicted in Fig. 3. It is apparent that at 22 °C, the internalization-competent HA mutants display significantly higher \(DA\) values. HA+8, which is internalized at a high rate and shows stable interactions with coated pits in lateral mobility studies (42, 44), yields the highest \(DA\) value. HA-Y543, which is internalized at a moderate rate and appears to interact transiently with coated pits in lateral mobility experiments (42, 43), shows an intermediate \(DA\) value; and HA+4, whose internalization is very slow and which does not interact appreciably with coated pits (42, 44), has a \(DA\) value essentially identical to that of HA wt. The higher \(DA\) values of HA+8 and HA-Y543 suggest that they appear in aggregates containing, on the average, higher numbers of HA molecules than HA wt or HA+4. This phenomenon most likely reflects the tendency of HA+8 and HA-Y543 to cluster in coated pits (or bind to AP-2 aggregates) at the cell surface. This interpretation is strongly supported by the effects of disrupting the clathrin lattice structure on the \(DA\) values of the HA mutants and by the coprecipitation of \(\alpha\)-adaptin with HA+8 and HA-Y543 (see Figs. 4A and 5). Interestingly, the
increase in the DA values of HA+8 and HA-Y543 relative to HA wt was much weaker in the cold (Fig. 3), suggesting that the observed clustering and the interactions leading to it are temperature-dependent and are elevated at higher temperatures. This notion is supported by the significant reduction in the co-precipitation of α-adaptin with the internalization-competent mutants (for example, see Fig. 1C), indicating that at 22 °C the differences between the pairs HA+8/HA wt and HA-Y543/HA wt are highly significant (p < 0.0005), whereas HA+4 is not significantly different from HA wt (p < 0.025). At 4 °C, only HA+8 was significantly different from HA wt incubated at the same temperature (p < 0.0005), and even this difference was only slightly above the 99% confidence level employed in the analysis of the ICS data. For the other pairs (HA+4/HA wt and HA-Y543/HA wt), the differences were not significant (p < 0.025 and p < 0.150, respectively).

Effects of Treatments That Disperse or Alter Coated Pit Structure on the Surface Distribution of HA Mutants—To obtain further support for the notion that the increased DA of the internalization-competent HA proteins (HA+8 and HA-Y543) is due to their preferred localization in coated pits, we have tested the effects of treatments demonstrated previously to alter coated pit structure on the DA values of HA+8, HA-Y543, and HA+4 relative to HA wt. Two independent treatments that have drastic but different effects on the clathrin lattice structure were employed. The first was incubation in a hypertonic medium to disperse the clathrin lattices with differing coated pits (56, 58). The second was acidification of the cytosol, which has been demonstrated to alter the morphology of the clathrin lattices, rendering them associated with the plasma membrane at a "frozen" state (56, 58, 60). Both treatments are highly effective in inhibiting coated pit-mediated internalization in CV-1 cells (42, 43).

The hypertonic treatment dispersed the clusters of the internalization-competent HA mutants (for example, see Fig. 1C). The effects of this treatment on the clustered distribution of the HA proteins at 22 °C as measured by ICS are depicted in Fig. 4A. The disruption of the clathrin lattices had a dramatic effect on the DA values of HA+8 and HA-Y543, which were reduced significantly as compared with untreated cells, becoming essentially equal to DA of HA wt on untreated cells. The decrease in the DA value is specific for the internalization-competent HA proteins and is not observed for HA+4 and HA wt, demonstrating that it is not due to a general change in cell shape or membrane properties. These results suggest that when the coated pits are dispersed, HA mutants that associate with them in untreated cells (HA+8 and HA-Y543) lose the clustered distribution similar to HA proteins that do not interact with coated pits to begin with.

The effects of cytosol acidification on the degree of aggregation of the HA proteins at the cell surface are shown in Fig. 4B. Unlike the hypertonic treatment, cytosol acidification does not disperse the clathrin lattices (58–60). If the enhanced aggre-
the cell surface. A and D, immunoprecipitation (see "Experimental Procedures") with anti-HA (see "Experimental Procedures." The level of each HA mutant at the cell surface was determined by surface biotinylation and immunoprecipitation (see "Experimental Procedures") with anti-HA (C and D) and quantified by densitometry. Based on this calibration, the lanes in A and B were loaded with equal amounts of each HA mutant at the cell surface. A, immunoprecipitation with anti-HA, blotting with AC1-M11 (anti-α-adaptin) followed by protein A-peroxidase. The experiment is representative of four carried out. Densitometric analysis showed that the amount of α-adaptin co-precipitated with HA-Y543 at 22 °C was 16% of that precipitated with HA + 8. Essentially no co-precipitation was detected with HA + 4 or HA wt in this experiment; in some cases (e.g. see Fig. 6) trace amounts of α-adaptin were precipitated with these proteins (up to 5% relative to the level precipitated with HA + 8). B, immunoprecipitation and blotting were as in A. The pairs of HA - 4 and 22 °C were taken from the same infected batch of cells. The amounts of α-adaptin co-precipitated at 4 °C were lower than for the same mutant at 22 °C (18% for HA + 8 and 30% for HA-Y543). C and D, immunoprecipitation with anti-HA and blotting with streptavidin-peroxidase.

Co-immunoprecipitation of α-adaptin with the various HA mutants is indeed due to preferential clustering in coated pits, it is expected to persist (possibly with some modifications) following cytosol acidification. Indeed, the DA values of both HA + 8 and HA-Y543 continue to be higher than those of HA wt and HA + 4 following this treatment. These findings are in accord with our former lateral mobility measurements, which suggested that the interactions of internalization-competent HA mutants with coated pits are not disrupted and in some cases are enhanced following cytosol acidification (43, 44). The DA of HA wt, which is excluded from coated pits, increased somewhat following cytosol acidification. The reason for this elevation is unknown, and it could indicate an increase in its aggregation (unrelated to coated pits) under these conditions.

Co-immunoprecipitation of α-adaptin with HA Mutants Carrying Cytoplasmic Internalization Signals—The results described above suggest that the clustering of internalization-competent HA mutants at the cell surface is due to their preferred association with coated pits. If this were the case, these mutants should interact with AP-2 adaptor complexes. To examine this issue, we measured the extent of coprecipitation of α-adaptin (which is specific to the plasma membrane AP-2) with the various HA proteins. After detergent solubilization and immunoprecipitation of the HA proteins, the cell extracts were analyzed for α-adaptin by SDS-polyacrylamide gel electrophoresis and immunoblotting (Fig. 5), calibrating the samples for similar levels of HA proteins at the cell surface by a surface biotinylation assay (based on HA immunoprecipitation following blotting with streptavidin; see "Experimental Procedures"). The calibration was performed according to the level of HA expression at the cell surface, because the percentage of the internalization-competent mutants (especially HA + 8) at the cell surface is significantly lower (42). Because clathrin and AP-2 appear to dissociate rapidly from endocytic vesicles and remain soluble in the cytoplasm until being recruited again into coated pits (46, 63–65), one should compare the different mutants based on their level at the plasma membrane rather than on their total expression level. As can be seen in Fig. 5A, when the immunoprecipitation was performed after a 10-min incubation at 22 °C, a high amount of α-adaptin co-precipitated with HA + 8, and a moderate amount accompanied HA-Y543. Only trace amounts of α-adaptin co-precipitated with HA wt and HA + 4. The co-precipitation of α-adaptin with HA + 8 and HA-Y543 was significantly reduced when the cells were preincubated at 4 °C, and the 10-min incubation at 22 °C was omitted (Fig. 5B). These results are in full agreement with the ICS experiments, and the rank order for association with α-adaptin in the coprecipitation studies is similar to that of the clustering at the cell surface in the ICS studies.

The effects of hypertonic and cytoplasmic acidification treatments on the co-precipitation of α-adaptin with the HA mutants are shown in Fig. 6. Dispersal of the clathrin lattices by hypertonic treatment markedly reduced the co-precipitation of α-adaptin with HA + 8 or HA-Y543, in correlation with the loss of their clustered distribution on the cell surface (Fig. 4A) and the increase in their lateral mobility parameters (43, 44) following this treatment. Thus, in the absence of clathrin lattices, the internalization-competent HA mutants are not limited to specific microdomains at the cell surface and can disperse laterally throughout the plasma membrane. On the other hand, and again in accord with the ICS (Fig. 4B) and lateral mobility results (43, 44), the co-precipitation of α-adaptin with HA + 8 or HA-Y543 was not reduced following cytosol acidification (which "freezes" but does not disperse clathrin lattices), and in the case of HA-Y543 it was even enhanced (Fig. 6B).

DISCUSSION

The first step in receptor-mediated endocytosis involves the recruitment of membrane proteins destined for internalization into coated pits. Their preferred localization in these domains should alter their surface distribution due to clustering. This
can be measured directly and quantitatively by ICS, employed here to directly assess the clustering in coated pits of a series of influenza HA mutants carrying different cytoplasmic internalization signals. The fact that HA wt is devoid of any such signal provides an internal control of a protein that is excluded from coated pits and enables a direct comparison between specific internalization signals of variable strength.

The ICS data on untreated cells (Fig. 3) demonstrate a striking dependence of the degree of aggregation on the internalization signal introduced at the HA cytoplasmic tail. The rank order of the DA values at 22 °C was similar to that of the internalization rates of the HA mutants (42), with HA +8 (which undergoes fast endocytosis) displaying a higher DA value than the moderately internalized HA-Y543, whereas HA +4 and HA wt (whose internalization is very slow) exhibit lower and equal DA values. These results are fully compatible with the notion that HAs bearing internalization signals cluster in specific regions on the cell surface (most likely coated pits) and that the level of clustering depends on the strength of the interactions with these domains. The identification of these domains as coated pits is supported by several observations: (a) the correlation between the DA values at 22 °C (Fig. 3) and the internalization rates of the HA mutants (42); (b) the reduction in DA of HA +8 and HA-Y543 following hypertonic treatment to the level measured for the noninteracting HA wt and HA +4 (Fig. 4A); (c) the persistence of the selective clustering of the internalization-competent mutants under conditions that freeze the coated pits at the plasma membrane (cytosol acidification; Fig. 4B); (d) the selective co-precipitation of α-adaptin with the internalization-competent HAs at the same rank order of potency found for the DA values (Fig. 5); and (e) the specific effects of treatments that affect the structure of the coated pits on the above co-precipitation (Fig. 6).

The co-precipitation experiments on untreated cells (Fig. 5) provide direct evidence that the HA mutants bearing internalization signals interact with AP-2 adaptors. To our knowledge, such interactions were thus far demonstrated by co-immunoprecipitation from whole cells only for EGF receptor family members (14, 15, 20, 21, 38). The co-precipitation data (Fig. 5) are in accord with surface plasmon resonance studies on the binding of purified adaptors to immobilized peptides corresponding to the cytoplasmic tails of HA wt and HA-Y543, where significant binding was observed only for the latter (35). Although the interactions might also be with AP-2 complexes that are not associated with coated pits, as proposed for the EGF receptor (21), the dissolution of the clusters of the internalization-competent HAs following hypertonic treatment argues against this possibility. Under such conditions, AP-2 aggregates devoid of clathrin remain at the plasma membrane (58), but the selective clustering of the HA mutants disappears concomitantly with the dispersal of the clathrin lattices. The weaker interactions with AP-2 upon dispersal of the clathrin lattices differ from the reports that K⁺ depletion, which is analogous to the hypertonic treatment in dispersing the clathrin lattices underlying coated pits (56, 58, 66), enhanced the association of EGF receptors with AP-2 (15, 21). The latter effect may therefore be specific to EGF family receptors, whose endocytosis may involve several signals and multiple pathways, as suggested by the ability of a mutant EGF receptor that lacks AP-2 binding to undergo internalization (39) and by the involvement of the GRB2 protein in EGF receptor endocytosis in association with dynamin (67). This notion is in line with the report on specific requirements for the recruitment of EGF receptors (versus transferrin receptors) into coated pits (68).

The interactions of the internalization-competent HAs with coated pits appear to be temperature-dependent, becoming weaker in the cold. This is evident for the clustering of HA +8 and HA-Y543 as measured by ICS (Fig. 3), which is dramatically reduced at 4 °C, and for their reduced association with α-adaptin in the co-precipitation experiments (Fig. 5). This conclusion is in accord with the loss of the constraints on the lateral mobility of these HA mutants in the cold (43, 44). Lower interactions with AP-2 in the cold were also reported for the EGF receptors, where warming to 37 °C was required for efficient co-precipitation (15, 21). The notion that low temperature affects the association of the internalization signal with AP-2 rather than the coated pit structure is supported by reports that clathrin-coated pits persist at 4 °C (69, 70) and by the similar DA values obtained for α-adaptin distribution at 37 and 4 °C.²

The ICS measurements described here and the lateral mobility approach to measure interactions with coated pits (43, 44) measure different parameters and are complementary in many respects. The ICS data are in excellent correlation with lateral mobility studies on the same HA mutants (43, 44). Specifically, the lateral mobility data (44) demonstrate that 20–25% of the HA +8 population is stably entrapped in coated pits, whereas the remainder are free to diffuse at the cell surface. The ICS data show that HA +8, which has the strongest internalization signal, also yields the highest DA value (4-fold higher than HA wt; Fig. 3). From the lateral mobility data it is apparent that this average DA value consists of contributions from both the HA +8 molecules entrapped in coated pits (i.e. reside in clusters) and the 75–80% that are dispersed throughout the membrane. Therefore, the actual increase in HA +8 cluster size relative to HA wt is most likely significantly higher than the 4-fold elevation in the average DA value. HA-Y543, which has a weaker internalization signal, exhibits only a moderate increase in the DA value (Fig. 3), in accord with the lateral mobility studies (43) which demonstrated that HA-Y543 interacts transiently with coated pits. This in turn suggests that in the dynamic equilibrium characterizing HA-Y543 association with coated pits, the number of HA-Y543 molecules that reside at any given time in clusters is fewer than in the case of HA +8. The HA +4 mutant, whose internalization signal is truncated in half and which is internalized very slowly, has no detectable interactions with coated pits by either method (Fig. 3 and Ref. 44). The correlation also holds for treatments that affect the structure of coated pits; both methods indicate that the interactions with coated pits are disrupted by the hypertonic treatment that disperses the clathrin lattices, but not by the cytosol acidification which freezes them underneath the plasma membrane (Fig. 4 and Refs. 43 and 44).

The ICS results provide insight into the factors that may affect the lateral mobility experiments. In the latter studies, reduction in the mobile fraction or in the lateral diffusion rate may arise from any of several types of interactions (71–73). The present correlation between reduced lateral mobility and enhanced clustering indicates that the internalization-competent HA mutants are trapped in coated pits and that this entrapment is the cause for their reduced mobility at the cell surface (43, 44).

In summary, the studies presented here demonstrate directly and quantitatively the clustering of internalization-competent membrane proteins in coated pits at the cell surface. In combination with complementary approaches, these studies provide a deeper insight into the interactions that lead to the sorting of membrane receptors into coated pits. The ICS approach presented can be extended to explore various processes

² C. M. Brown and N. O. Petersen, unpublished observations.
involving the aggregation and interactions of plasma membrane proteins, such as determining the extent to which signaling molecules at the cell surface are clustered in caveoli or in glycolipid-rich domains.

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