An Internalization-Competent Influenza Hemagglutinin Mutant Causes the Redistribution of AP-2 to Existing Coated Pits and Is Colocalized with AP-2 in Clathrin Free Clusters†

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ABSTRACT: Image correlation spectroscopy and cross correlation spectroscopy were used to demonstrate that ∼25% of the internalization-competent influenza virus hemagglutinin mutant, HA+8, is colocalized with clathrin and AP-2 at the plasma membrane of intact cells, while wild-type HA (which is excluded from coated pits) does not colocalize with either protein. Clathrin and AP-2 clusters were saturated when HA+8 was overexpressed, and this was accompanied by a redistribution of AP-2 into existing coated pits. However, de novo coated pit formation was not observed. In nontreated cells, the number of clusters of clathrin or AP-2 colocalized with HA+8 was always comparable. Hypertonic treatment which disperses the clathrin lattices resulted in more clusters containing AP-2 and HA+8 than clathrin and HA+8. Less colocalization of HA+8 with clathrin was also observed after cytosol acidification, which causes the formation of deeply invaginated pits, where the HA+8 may be inaccessible to extracellular labeling by antibodies, and blocks coated vesicle budding. However, cytosol acidification elevated the number of clusters containing both HA+8 and AP-2, suggesting an increase in their level of association outside of the deep invaginations. Our results imply that AP-2 and HA+8 can colocalize in clusters devoid of clathrin, at least in cells treated to alter the clathrin lattice structure. Although we cannot ascertain whether this also occurs in untreated cells, we propose that AP-2 binding to membrane proteins carrying internalization signals can occur prior to the binding of AP-2 to clathrin. While such complexes can in principle serve to recruit clathrin for the formation of new coated pits, the higher affinity of the internalization signals for clathrin-associated AP-2 [Rapoport, I., et al. (1997) *EMBO J. 16,* 2240−2250] makes it more likely that once the AP-2−membrane protein complexes form, they are quickly recruited into existing coated pits.

Receptor-mediated endocytosis via clathrin-coated pits requires interactions between membrane receptors, clathrin, and the clathrin-associated adaptor protein complex (AP-2, reviewed in refs *1*−*7*). Clathrin is the structural component that forms the lattice lining the coated pit (*8*, *9*), while AP-2 binds to the internalization signals on the cytoplasmic tail of plasma membrane receptors (*2*, *6*, *10*, *11*), and recruits clathrin to the membrane (*12*−*17*).

There is strong evidence that AP-2 only binds to membrane receptors that contain a particular internalization signal. Binding studies in vitro show that the μ2 subunit of AP-2 interacts with specific amino acid sequences containing tyrosine which are found on the cytoplasmic tail of the receptor (YXXϕ, where X is any amino acid and ϕ is a hydrophobic amino acid; *18*, *19*), and experiments in vivo suggest that this interaction is important for concentrating receptors in coated pits (reviewed in refs *6*, *7*, *11*, and *20*−*23*). Co-immunoprecipitation studies show that AP-2 does bind to receptors such as members of the EGF receptor family (*24*−*29*), and to internalization-competent influenza virus hemagglutinin (HA)[1] mutants (*30*). The binding of AP-2 to clathrin in coated vesicles has been shown to enhance the interaction between AP-2 and peptides containing internalization sequences by as much as 10-fold (*31*). In turn, it is possible that interactions between membrane receptors and AP-2 increase the affinity of AP-2 for clathrin. In situ evidence for these intermolecular interactions at the plasma membrane of intact cells is not yet available.

To investigate the interactions between internalization-competent proteins and coated pits in situ, quantitative measurements of the distribution and colocalization of the internalization-competent HA+8 (*30*, *32*) and clathrin and AP-2 in CV-1 cells were carried out. The HA+8 protein

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[1] Abbreviations: CD, cluster density; HA, influenza virus hemagglutinin; FITC, fluorescein isothiocyanate; ICS, image correlation spectroscopy; ICCS, image cross correlation spectroscopy; mAb, monoclonal antibody; RhR, rhodamine red.

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contains an addition of eight amino acids at the cytoplasmic tail that includes an internalization sequence which induces its rapid endocytosis from the cell surface at a rate of 60% per minute (*32*). This internalization is mediated via coated pits, as indicated by its blockade following treatments known to disperse (hypertonic treatment) or "freeze" (cytosol acidification) the coated pit structure (*32*). On the other hand, wild-type HA (HA wt) is not internalized and does not interact with coated pits (*30*, *33*, *34*) and thus serves as a control.

Although it is known that clathrin and AP-2 interact to form coated pits and that AP-2 binds to certain internalization signals to concentrate cargo proteins into coated pits, the order in which these proteins interact in vivo is not known. In this study, we used image correlation spectroscopy (ICS) (*35*−*37*) and image cross correlation spectroscopy (ICCS) (*38*, *39*) to investigate this question. CV-1 cells have been used previously for studies of the internalization of transiently expressed proteins and are particularly well suited for image correlation measurements because they are large (∼13000 μm^2) and flat (<2 μm). Our findings demonstrate that a significant fraction of the internalization-competent HA+8 is associated with most of the clathrin and AP-2 clusters at the plasma membrane. On cells treated to disrupt the coated pit structure, the level of association between HA+8 and clathrin is reduced while the association between HA+8 and AP-2 remains intact. This provides new evidence for in situ clathrin-independent interactions between HA+8 and AP-2. This interaction with AP-2 can presumably be extended to include interactions between internalization-competent membrane receptors and AP-2 in general. This is consistent with the possibility that AP-2−receptor binding may precede the AP-2−clathrin interactions in the coated pit assembly process. Although the interactions between AP-2 and internalization signals are relatively weak (*18*, *19*, *40*, *41*) and may not play a key role in the initial recruitment of AP-2 to the plasma membrane, they are sufficient to mediate a redistribution of AP-2 to existing coated pits upon overexpression of HA+8, probably by enhancing the affinity toward clathrin of AP-2 bound to the internalization motif.

EXPERIMENTAL PROCEDURES

Antibodies. X22 mouse monoclonal antibodies (mAb) against the clathrin heavy chain were a gift from F. Brodsky (University of California, San Francisco, CA). AC1-M11 mouse mAb for the α-chain of AP-2 (*42*) were prepared using hybridoma cells donated by M. S. Robinson (University of Cambridge, Cambridge, U.K.). Monovalent Fab′ polyclonal rabbit antibodies to Japan HA (A/Japan/305/57 strain) were described previously (*30*, *34*). FITC-labeled goat anti-rabbit Fab′ was prepared from FITC-labeled $F(ab')_2$ obtained from Jackson Immuno-Research. FITC-labeled and unlabeled Fab specific goat anti-mouse antibodies, Fc specific goat anti-mouse antibodies, and normal goat IgG were from Sigma. The FluoReporter Rhodamine Red (RhR) Protein Labelling Kit was from Molecular Probes (Eugene, OR), and was used to label goat anti-mouse Fab and Fc specific antibodies. The fluorophore-to-protein ratios were determined to be 2.2:1 (Fab specific) and 2.7:1 (Fc specific).

Cell Culture and Infection. CV-1 cells were grown (*43*), and SV40 virus stocks for HA wt or HA+8 were prepared as described previously (*30*, *44*). Cells were infected in suspension for 45 min on ice with third-passage recombinant virus stocks, as described previously (*34*, *45*). Experiments were performed 36 h (HA wt) or 44 h (HA+8) postinfection. Longer infection times were used for the HA+8 because less than 20% of the protein is found at the cell membrane, and higher expression levels are needed to conduct immunofluorescence experiments.

Treatments. Treatments were carried out prior to labeling and fixation. For the hypertonic treatment, cells were left for 30 min at 37 °C in HBSS buffered with 20 mM Hepes (pH 7.2, HBSS/Hepes) containing 0.5 M sucrose. The cytosol acidification protocol has been described in detail elsewhere (*34*).

Immunofluorescent Labeling and Cell Fixation. CV-1 cells were washed twice with the appropriate ice-cold buffer: HBSS/Hepes, 0.5 M sucrose in HBSS/Hepes, or KA buffer (0.14 M KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride hydrochloride, and 20 mM HEPES) all at pH 7.2. An additional wash with the same buffer and 2% BSA was performed before labeling and between all labeling steps. Cells were labeled at 4 °C successively with (i) normal goat IgG (200 μg/mL, 30 min), (ii) rabbit anti-HA Fab′ fragments (100 μg/mL, 1 h), and (iii) FITC-labeled goat anti-rabbit Fab′ fragments (50 μg/mL, 30 min). For treated cells, antibodies were dissolved in the appropriate buffer. Following HA labeling, the cells were warmed to 20 °C for 10 min to allow association of the HA proteins with coated pits. Incubation at 20 °C rather than at 37 °C prevented significant internalization of HA+8.

Cells were fixed and permeabilized in methanol (5 min, −20 °C) followed by acetone (2 min, −20 °C). Cells were then labeled successively at room temperature with (i) normal goat IgG (200 μg/mL, 1 h), (ii) AC1-M11 against the α-chain of AP-2 (50 μg/mL) or X22 against the clathrin heavy chain (20 μg/mL) for 2 h, and (iii) RhR-labeled goat anti-mouse Fab specific antibody for 1 h (50 μg/mL for AP-2 and 20 μg/mL for clathrin labeling). After extensive washing, the coverslips were mounted on microscope slides using Airvol containing *n*-propylgallate and taken for ICS studies.

Image Collection. Dually labeled cells were visualized using a Biorad MRC 600 Confocal microscope equipped with an Ar/Kr mixed gas laser and using the appropriate filter sets for dual fluorophore imaging. Cells expressing appropriate HA levels were selected under mercury lamp illumination using a 60× (1.4 NA) objective and an inverted Nikon microscope. An area on the cell, removed from the nucleus, was zoomed in on (10×) and visualized. Fluorescein is more susceptible to photobleaching so the HA protein labeling was visualized first. The filter wheel was set for 488 nm laser excitation, and neutral density filters were used to attenuate the laser to 1% laser power. Twenty-five scans were accumulated on photomultiplier tube 2 (PMT2) in photon counting mode (to ensure linear scaling of the intensity). The filter wheel was then shifted to allow excitation with the 568 nm laser line, and five scans were accumulated on PMT1. In this way, only one fluorophore at a time is excited and cross talk between the two collection channels is minimized to <1% of the intensity of the primary fluorophore. The two photomultiplier tubes were set with the black level at 6.0 on the vernier scale, and the gain set at 10. After collection of each set of five images, images were collected using identical settings but with the shutter to the sample closed to obtain a measure of the dark current for each PMT.

Data Analysis and Interpretation. The principles of ICS and ICCS have been described previously (*35*−*39*), and their application to studies of membrane proteins has been demonstrated (*30*, *35*−*39*, *46*). Basically, in the ICS analysis, an autocorrelation function is determined from the intensity fluctuations within a confocal laser scanning microscope image and the amplitude of the function, $g(0,0)$, provides a quantitative measurement of the number of independent fluorescent clusters per square micrometer of cell membrane, i.e., the cluster density (CD):

$$\mathrm{CD} = \frac{1}{g(0,0)\pi\omega^2} \tag{1}$$

where ω is the e^{-2} radius of the laser beam. In turn, in the ICCS analysis, the amplitude of a cross correlation function is calculated from the intensity fluctuations within two separate images arising from two distinct proteins (immunofluorescently labeled with two distinct fluorescent probes) within the same area of the cell. In this case, the amplitude can be interpreted as a density of clusters containing both proteins. Following the methods of Rigler and co-workers (*47*), it can be shown that the density of colocalized clusters ($\mathrm{CD_{gr}}$) can be (*38*) determined with eq 2:

$$\mathrm{CD_{gr}} = \frac{g_{\mathrm{gr}}(0,0)}{g_{\mathrm{g}}(0,0)g_{\mathrm{r}}(0,0)\pi{\omega_{\mathrm{gr}}}^2} = \frac{\bar{N}_{\mathrm{gr}}}{\pi{\omega_{\mathrm{gr}}}^2} \tag{2}$$

where $g_{\mathrm{gr}}(0,0)$ is the amplitude of the cross correlation function between images of green and red labeled proteins and $g_{\mathrm{g}}(0,0)$ and $g_{\mathrm{r}}(0,0)$ are the amplitudes of the autocorrelation functions corresponding to images of the green and red labeled proteins, respectively.

It is often more informative to describe colocalization in terms of the fraction of clusters of one protein which are associated with clusters of the other protein. The fraction of HA clusters (*H*, green) that are associated with clathrin (*C*, red) or AP-2 (*A*, red) clusters is defined by eq 3:

$$F(H|C) = \frac{\mathrm{CD_{gr}}}{\mathrm{CD_g}}; \quad F(H|A) = \frac{\mathrm{CD_{gr}}}{\mathrm{CD_g}} \tag{3}$$

Similarly, the fraction of clathrin or AP-2 clusters associated with HA clusters is defined by eq 4:

$$F(C|H) = \frac{\mathrm{CD_{gr}}}{\mathrm{CD_r}}; \quad F(A|H) = \frac{\mathrm{CD_{gr}}}{\mathrm{CD_r}} \tag{4}$$

In previous work, it was observed that the HA protein is only labeled on the top surface of the cell (for example, see Figure 1 in ref *30*, *38*), while both AP-2 and clathrin are labeled on the inside surface of the upper and lower membrane of the cell (*38*, *43*, *46*). The measured cluster density of AP-2 or clathrin ($\mathrm{CD_r}$) is therefore twice the "real" value per unit area of membrane. Only clathrin and AP-2 clusters on the top surface of the cell will contain "labeled" HA protein and contribute to the colocalization analysis. The proper fraction of colocalization is therefore obtained in these experiments when the fraction is multiplied by 2 (corresponding to dividing the $\mathrm{CD_r}$ by 2).

Because of imperfections in the microscope, the maximum of the cross correlation function is shifted slightly from the origin to coordinates x_o and y_o. In cases where there is significant cross correlation, these coordinate shifts are <10 pixels (<0.3 μm). In those cases where there is no significant cross correlation, spurious correlations are frequently found at random coordinates in the correlation function. The fraction of images which exhibit an average of x_o and y_o below 10 pixels provides a good measure of the fraction of cells in the population which exhibit significant colocalization of the two proteins.

Control Experiments. Cross correlation between unrelated images was examined as a negative control. The percentage of images which provided a fit at the origin of the cross correlation function was <1%. Those few that did fit at the origin yielded $F(H|A)$ values of ~0.03 and $F(A|H)$ values of ~0.13. Consequently, values for the fraction of colocalization of >0.15 are reliable.

Experiments in which clathrin is labeled simultaneously with two fluorophores (X22 mAb followed by simultaneous labeling with both FITC-labeled Fab specific antibody and RhR-labeled Fc specific antibody) served as a positive control. All images yielded cross correlation at the origin of the function; the fraction of FITC-labeled clusters associated with RhR-labeled clusters [$F(G|R)$] was 0.90, while the fraction of RhR-labeled clusters associated with FITC-labeled clusters [$F(R|G)$] was 1.07. The deviations of $F(G|R)$ and $F(R|G)$ from 1.0 are consistent with an energy transfer process, but clearly, the effect is small (<10%) even when the fluorophores are known to be close to each other. Since fluorescence is collected in separate channels using only one excitation beam at a time, the extent of crossover of fluorescence between channels is 1% or less in either direction.

Statistics. Standard error of the mean (SEM) values were calculated from the raw data at the 99% confidence level. One-tailed Student's *t* tests for the difference between means were conducted, and values were considered significantly different if $P < 0.01$. Comments within the text are based on these *t* test results, and *P* values are only quoted for selected data.

RESULTS

Native Distributions and Colocalization of the Proteins. ICS analysis of images of cell surfaces labeled by indirect immunofluorescence can provide information about the native distribution of the target antigens. HA wt has a high cluster density (CD, eq 1)[2] relative to the other proteins (Table 1), indicating that HA wt is more dispersed in the membrane, or that there are more independent clusters of HA wt per unit area. Note that in ICS a cluster can be a large aggregate or as small as a monomer, as long as it contributes to the correlation function as one independent fluctuation. HA+8 was more aggregated than HA wt (has a lower CD), presumably due to localization within coated pits. However, HA+8 was more dispersed than clathrin or AP-2, consistent with the idea that a fraction of HA+8 is free to diffuse in the membrane and is not associated with coated pits (*44*). The CD values for clathrin and AP-2 are similar

[2] Previous results were presented in terms of the degree of aggregation (DA; number of clusters per square micrometer) which takes into account variable HA expression levels (*30*). However, CDs are used here because it is the parameter needed for the cross correlation analysis.

Table 1: Summary of the Autocorrelation Results[a]

protein	$\langle i \rangle \pm$ SEM (arbitrary units)	$\langle \mathrm{CD} \rangle \pm$ SEM (clusters/μm^2)	N^b
wt HA	4.6 ± 0.4	18 ± 2	360
HA+8	2.3 ± 0.5	3.2 ± 0.7	303
clathrin	3.3 ± 0.3	0.9 ± 0.1	356
AP-2	3.5 ± 0.3	1.0 ± 0.2	277

[a] Autocorrelation results from all control experiments for the four proteins that were studied. $\langle i \rangle$ values were corrected for contributions from instrument noise, nonspecific secondary antibody binding, and autofluorescence (*37*, *43*). Cluster density (CD) values were calculated using the autocorrelation results and eq 2. [b] Number of cells imaged and analyzed.

to CD values previously determined with noninfected cells (Table 1; *38*, *43*, *46*).[3]

An ICCS analysis was conducted on cells labeled with antibodies to both the HA protein and clathrin or AP-2. Consistent with the fact that HA wt does not undergo clathrin-mediated endocytosis (*33*, *34*), there was very little colocalization between HA wt and clathrin or AP-2. Only ~20% of the cells expressing HA wt exhibited some degree of cross correlation, corresponding to ~2% of the HA wt clusters. The lack of colocalization between HA wt and clathrin is shown qualitatively in Figure 1A−C. Similar results were obtained with HA wt and AP-2 (not shown).

HA+8 exhibited a high level of colocalization with both clathrin (Figure 1D−F and Table 2) and AP-2 (Figure 1G−I and Table 2), and all of the cells that were visualized exhibited some level of colocalization. The fractions of HA+8 associated with clathrin and AP-2 were identical (eq 3 and Table 2), indicating that one-quarter of the HA+8 clusters are associated with clathrin and AP-2. Correspondingly, three-quarters of the HA+8 clusters are not associated with clathrin or AP-2, in accord with the finding that the fraction of laterally mobile HA+8 is 25% lower than that of HA wt (*44*). Indeed, there are some green spots which must correspond to clusters which only contain HA+8 (Figure 1F,I). Some of these clusters could be HA+8 localized within endosomes, but this contribution should be relatively small due to the short period of incubation at 20 °C. The fractions of clathrin and AP-2 associated with HA+8 were essentially the same (eq 4 and Table 2), suggesting that the clusters correspond to coated pits containing clathrin and AP-2. There were also a number of bright red spots corresponding to clathrin or AP-2 aggregates which did not colocalize with HA+8 (Figure 1F,I). This is expected because HA+8 is only labeled on the upper membrane of the cell, whereas clathrin and AP-2 are labeled on both membranes (see Data Analysis and Interpretation). Data are normalized for the total membrane area that is labeled within each image to take this into account (see Data Analysis and Interpretation). In other words, a fraction of 0.7 means that ~70% of the total clathrin and AP-2 aggregates contain HA+8, which is in agreement with our previous estimate that ~65% of the total clathrin protein is associated with coated pits in the periphery of CV-1 cells (*38*, *46*). CD_{gr} is the density of clusters containing both HA+8 and clathrin, or HA+8 and AP-2, and the value of ~0.6 cluster/μm^2 is in line with our previous estimate for the distribution of coated pits on CV-1 cells (*38*, *46*).

Expression of HA+8 Causes a Redistribution of AP-2 into Existing Coated Pits. When a transfected system is studied, there is cell-to-cell variation in the expression level of the HA proteins. This makes comparing the density of HA+8 to the distribution of the coated pit components on an individual cell basis straightforward. The amount of HA expressed by a cell is estimated by the average intensity of fluorescence, $\langle i \rangle$, and the distribution of clathrin, or AP-2, on the same cell is measured by the CD value. Figure 2 shows the CD values for clathrin (Figure 2A) and AP-2 (Figure 2B), as a function of the $\langle i \rangle$ for either HA wt (white bars) or HA+8 (black bars). The distribution of clathrin was not affected by the expression level of HA wt (Figure 2A; compare the white bars) or HA+8 (Figure 2A; compare the black bars). Furthermore, the presence of HA+8 did not change the clathrin distribution relative to that of cells expressing HA wt (Figure 2A; compare the white and black bars). Thus, overexpression of HA+8 does not induce the formation of new clathrin clusters, or coated pits, which would result in a change in the CD value. Although the expression level of HA wt has no effect on the AP-2 distribution (Figure 2B; compare the white bars), the presence of HA+8 causes it to aggregate (decreases the CD value, Figure 2B; compare the white and black bars). Furthermore, the aggregation of AP-2 is further enhanced at higher HA+8 expression levels (Figure 2B; compare the black bars). Therefore, the expression of HA+8 must cause a redistribution of AP-2 into existing coated pit structures, but it does not cause de novo coated pit formation.

Every AP-2 and Clathrin Aggregate Is Associated with HA+8 at High Expression Levels. At low HA+8 expression levels, 60−70% of the clathrin and AP-2 clusters are associated with HA+8 (Table 3). However, at high expression levels, essentially all of the clathrin and AP-2 clusters contain some HA+8 (Table 3). If there was a significant amount of clathrin, or AP-2, free in the cytosol, it should not be colocalized with HA+8, and the fraction of clathrin and AP-2 associated with HA+8 should not equal 1. Thus, the contribution from free clathrin, or free AP-2, within the cytosol to the ICCS analysis is not significant at high HA+8 expression levels. The total number of clusters containing both HA+8 and clathrin, or HA+8 and AP-2, increases at high expression levels (Table 3; although the increase in CD_{gr} for the HA+8 and clathrin colocalization is not statistically significant). Therefore, at low expression levels, we are looking at cells that are expressing a moderate level of HA+8 and the coated pits are not saturated.

The fractions of HA+8 associated with clathrin or AP-2 at high expression levels are reduced, indicating that a smaller fraction of HA+8 is associated with clathrin and AP-2 aggregates (Table 3). At high expression levels, excess HA+8 molecules remain free in the membrane, resulting in an increase in the total number of HA+8 clusters, CD_g, from 2−3 to 8−10 clusters/μm^2. Therefore, there is a corresponding decrease in the fraction of HA+8 clusters associated with clathrin and AP-2 (eq 3). A small percentage of cells expressing HA wt exhibit some colocalization with clathrin or AP-2. However, there is no correlation between the HA wt expression level and the amount of colocalization with clathrin, or AP-2.

[3] Cells infected to produce HA proteins are flatter (*45*) so that contributions to the ICS data from cytosolic proteins are smaller, resulting in lower CD values for clathrin and AP-2 (Table 1) relative to previous measurements on noninfected cells (*38*, *43*, *46*).

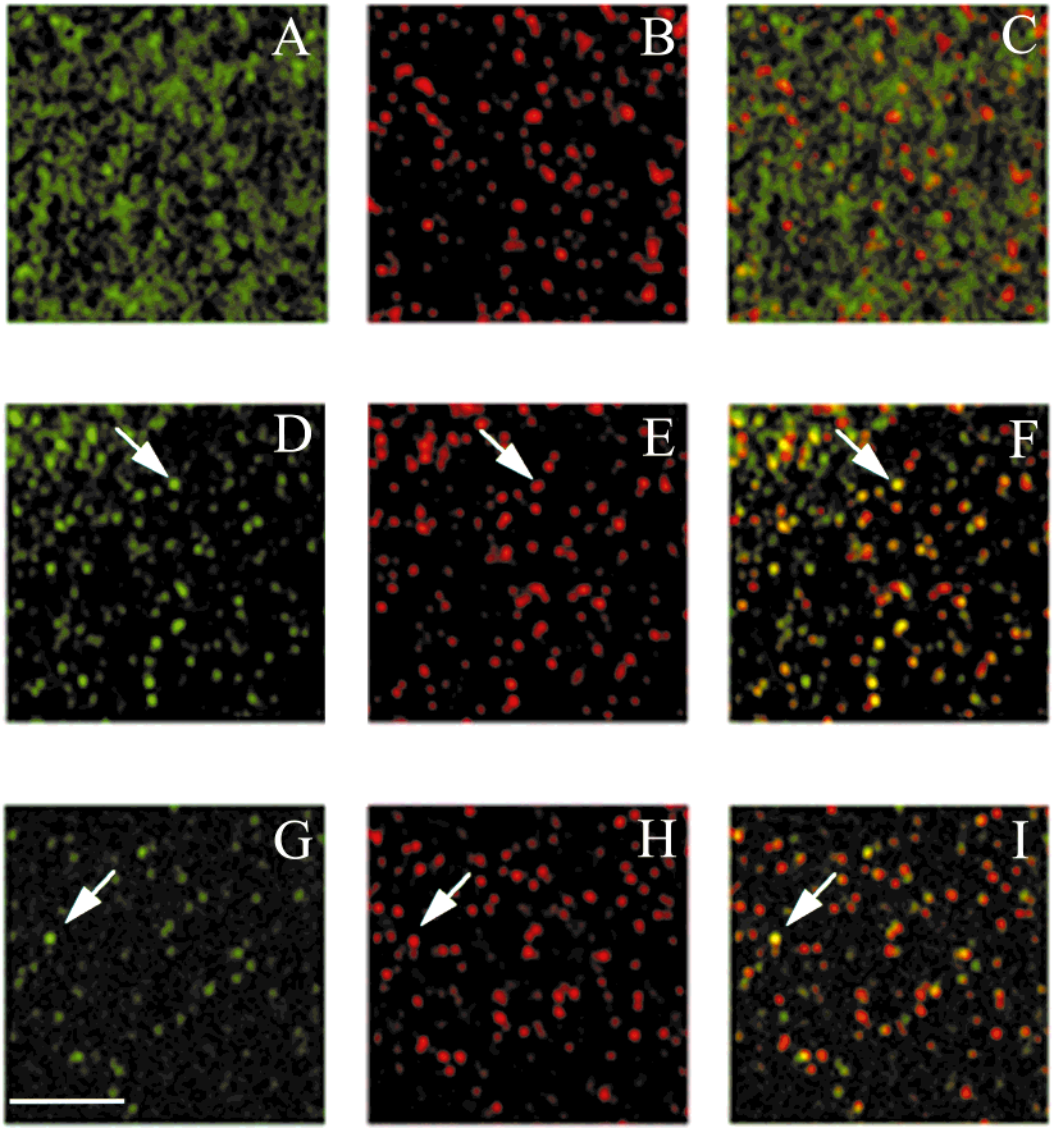

FIGURE 1: Indirect immunofluorescent labeling of the HA proteins, clathrin, or AP-2. CV-1 cells were immunofluorescently labeled for the HA protein fixed with methanol and acetone and then labeled for clathrin or AP-2 (see Experimental Procedures). Images are of FITC-labeled HA wt (A) or HA+8 (D and G) or of rhodamine-labeled clathrin (B and E) or AP-2 (H). Background intensity was subtracted off, and images were contrast enhanced and overlapped (C, F, and I) using Adobe Photoshop 4.0. The scale bar is 5 μm.

Table 2: Cross Correlation Results for Nontreated Cells[a]

protein	$F(H\|C)$ or $F(H\|A) \pm$ SEM	$F(C\|H)$ or $F(A\|H) \pm$ SEM	CD_{gr} (colocalized clusters/μm^2)	N[b]
clathrin (C)	0.25 ± 0.02	0.72 ± 0.06	0.59 ± 0.06	175
AP-2 (A)	0.25 ± 0.03	0.69 ± 0.07	0.57 ± 0.06	127

[a] Cross correlation results from experiments where HA+8 and clathrin (six experiments) or HA+8 and AP-2 (five experiments) were dually labeled as explained in Experimental Procedures. The fractions of HA+8 colocalized with clathrin, $F(H|C)$, or AP-2, $F(H|A)$, were calculated from the ratio of the autocorrelation CD values (eq 2) for the HA+8 and the CD_{gr} value (eq 3) calculated from the cross correlation results. The fraction of clathrin, $F(C|H)$, or AP-2, $F(A|H)$, associated with HA+8 was calculated as the ratio of the autocorrelation CD values for clathrin or AP-2 (eq 2), and the CD_{gr} value (eq 4) calculated from the cross correlation analysis. [b] Number of cells imaged and analyzed.

Hypertonic Conditions Reduce the Extent of HA+8 Colocalization with Clathrin but Increase the Extent of Colocalization with AP-2. Hypertonic conditions are known to inhibit clathrin-mediated endocytosis by disrupting the coated pit structure (*48*). Under these conditions, there is a dispersion of clathrin ($CD = 1.7 \pm 0.07$) but some large aggregates of clathrin are still present (for further details, see ref *46*). ICS and ICCS analyses demonstrate that under these conditions there is a dissociation of HA+8 from coated pits or residual clathrin aggregates. This is shown by (i) a decrease in the fraction of HA+8 associated with clathrin indicating that there are fewer HA+8 molecules in each coated pit (Table 4) and (ii) a reduction in the number of clusters containing both HA+8 and clathrin (Table 4), indicating that there are some residual clathrin aggregates, which no longer contain HA+8 following hypertonic treatment. The slight decrease in the fraction of clathrin associated with HA+8 following hypertonic treatment is not statistically significant ($P > 0.10$).

ICS analysis showed that both HA+8 (not shown; *30*) and AP-2 ($CD = 1.49 \pm 0.08$; *46*) disperse following hypertonic treatment. However, the ICCS analysis showed that following hypertonic treatment there was an increase in the fraction of AP-2 associated with HA+8, and in the total number of clusters containing both HA+8 and AP-2 (Table 4; $P < 0.0005$ for both $F(A|H)$ and CD_{gr}) so that essentially all of

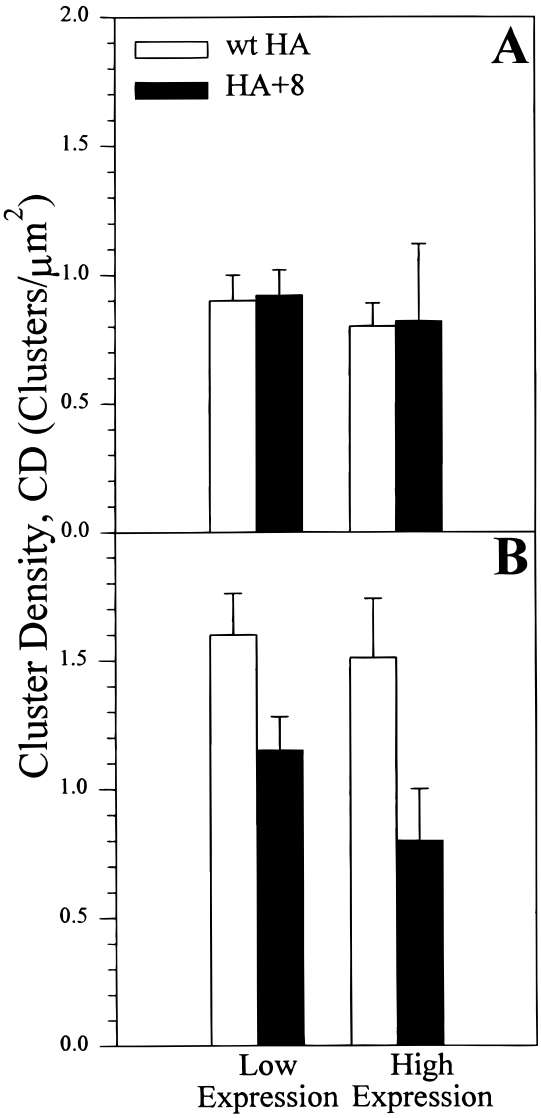

FIGURE 2: Distributions of clathrin and AP-2 as a function of the HA protein expression level. Autocorrelation results from five to six experiments comparing the cluster density (CD, eq 2) of clathrin (A) or AP-2 (B) to the expression level of HA wt (white bars) or HA+8 (black bars). The high expression level for HA was arbitrarily set to any cell with an $\langle i \rangle$ of HA labeling of more than 5 intensity units. Error bars represent the standard error of the mean.

Table 3: Cross Correlation Results as a Function of HA+8 Expression Level[a]

protein	expression level	$F(H\|C)$ or $F(H\|A) \pm$ SEM	$F(C\|H)$ or $F(A\|H) \pm$ SEM	CD_{gr} (colocalized clusters/μm^2)	N[b]
clathrin	low	0.26 ± 0.02	0.69 ± 0.06	0.57 ± 0.07	158
(C)	high	0.14 ± 0.05	1.03 ± 0.26	0.74 ± 0.23	17
AP-2	low	0.27 ± 0.03	0.61 ± 0.05	0.54 ± 0.06	111
(A)	high	0.08 ± 0.04	1.26 ± 0.31	0.81 ± 0.32	15

[a] Cross correlation results from experiments where HA+8 and clathrin (six experiments) or HA+8 and AP-2 (five experiments) were dually labeled as described in Experimental Procedures. The fractions of colocalized proteins and the CD_{gr} values were calculated as described in Table 2. High expression levels of the HA+8 were arbitrarily set to any cell with an $\langle i \rangle$ value of more than 5. [b] Number of cells imaged and analyzed.

the AP-2 clusters contain some HA+8. This indicates that although HA+8 dissociated from clathrin-coated pits, it remained associated with AP-2, and previously unassociated AP-2 must have also become associated with HA+8. Unlike control conditions where the CD_{gr} is the same for HA+8 association with clathrin or AP-2 (Table 2), the hypertonic treatment resulted in more AP-2 clusters containing HA+8 than clathrin clusters (Table 4). This means that there must be AP-2 clusters containing HA+8 that are not associated with clathrin. It should be noted that some of the decrease in the fraction of HA+8 associated with AP-2 is due to the dispersion of HA+8 which causes an increase in the total number of HA+8 clusters at the membrane (Table 4). Following hypertonic treatment, there is still very little colocalization between HA wt and clathrin or AP-2.

Table 4: Cross Correlation Results and Hypertonic Treatment[a]

protein	treatment	$F(H\|C)$ or $F(H\|A) \pm$ SEM	$F(C\|H)$ or $F(A\|H) \pm$ SEM	CD_{gr} (colocalized clusters/μm^2)	N[b]
clathrin	control	0.26 ± 0.03	0.74 ± 0.10	0.65 ± 0.10	90
(C)	hypertonic[c]	0.10 ± 0.02	0.66 ± 0.16	0.47 ± 0.10	42
AP-2	control	0.27 ± 0.05	0.70 ± 0.17	0.74 ± 0.17	22
(A)	hypertonic[c]	0.09 ± 0.03	1.20 ± 0.17	1.30 ± 0.40	30

[a] Cross correlation results from two experiments. Cells were labeled for HA+8 and clathrin or HA+8 and AP-2 as explained in Experimental Procedures. The fraction of colocalized proteins and CD_{gr} values were calculated as described in Table 2. [b] Number of cells imaged and analyzed that demonstrated significant cross correlation. [c] Hypertonic treatment as described in Experimental Procedures.

Table 5: Cross Correlation Results and Cytoplasmic Acidification[a]

protein	treatment	$F(H\|C)$ or $F(H\|A) \pm$ SEM	$F(C\|H)$ or $F(A\|H) \pm$ SEM	CD_{gr} (colocalized clusters/μm^2)	N[b]
clathrin	control	0.31 ± 0.06	0.54 ± 0.13	0.40 ± 0.09	30
(C)	acidic[c]	0.05 ± 0.03	0.32 ± 0.12	0.20 ± 0.05	33
AP-2	control	0.26 ± 0.06	0.50 ± 0.10	0.50 ± 0.10	45
(A)	acidic[c]	0.10 ± 0.06	0.30 ± 0.13	0.49 ± 0.16	37

[a] Cross correlation results from two experiments. Cells were labeled for HA+8 and clathrin or HA+8 and AP-2 as explained in Experimental Procedures. The fraction of colocalized proteins and the CD_{gr} values were calculated as described in Table 2. [b] Number of cells imaged and analyzed that demonstrated significant cross correlation. [c] Cytosol acidification as described in ref *34*.

Cytosol Acidification Reduces the Extent of HA+8 Colocalization with Clathrin but Increases the Extent of Colocalization with AP-2. Cytosol acidification inhibits endocytosis by "freezing" clathrin-coated pits in a highly invaginated state (*49*, *50*). Under these conditions, there is an aggregation of clathrin ($CD = 0.65 \pm 0.02$), presumably because there is more clathrin associated with each highly invaginated coated pit. There is also an aggregation of the free clathrin in the cytosol (*46*, *50*). Cross correlation analysis reveals that only ~60% of the cells had a significant level of cross correlation between HA+8 and clathrin. On those cells, the density of clusters that contained both HA+8 and clathrin was about half that of control values (CD_{gr}, Table 5). There was a corresponding decrease in the fraction of HA+8 colocalized with clathrin and the fraction of clathrin colocalized with HA+8 (Table 5). This result is unexpected since coated pits are still present following cytosol acidification, and internalization is only inhibited because these structures cannot bud from the membrane (*50*). There is every reason to think that HA+8 would still be present in these highly invaginated coated pits. In fact, this may still be the case, and the apparent lack of correlation between the HA+8 and clathrin may be due to the inaccessibility of HA+8 molecules in deeply invaginated coated pits to the extracellular labeling of the cells by primary and secondary Fab′ fragments. It has previously been shown that in cells in which the cytosol is

acidified, 7−15% of the surface population of another internalization-competent HA mutant, HA-Y543, are inaccessible to extracellular trypsin (comparable in size to Fab′; *34*). Since HA+8 is more tightly associated with coated pits than HA-Y543 (*34*, *44*), it is likely that a higher percentage of HA+8 molecules at the cell surface would be inaccessible to the Fab′ fragments. Thus, in cytosol-acidified cells, the HA+8 population located outside of highly invaginated coated pits could be preferentially labeled, leading to the apparent dispersal of HA+8, and to a reduced level of colocalization with clathrin.

About 80% of the cells treated to acidify the cytosol exhibited a significant level of colocalization between HA+8 and AP-2, and the fractions of colocalized proteins were both reduced relative to control cells (Table 5). This reduction is in part due to the fact that both HA+8 (not shown) and AP-2 ($CD = 2.5 \pm 0.1$; *46*) disperse when the cytoplasm is acidified. This results in an increase in the total number of HA+8 or AP-2 clusters, and a corresponding decrease in the fraction of colocalized protein. Nevertheless, there is no change in the total number of clusters containing both HA+8 and AP-2 ($CD_{gr} \sim 0.5$ cluster/μm^2). Analogous to the hypertonic treatment, this indicates that there are clusters containing AP-2 and HA+8 that do not contain clathrin. Following cytosol acidification, only ∼3−12% of the cells expressing HA wt exhibited any significant amount of colocalization with clathrin, or AP-2.

DISCUSSION

ICS and ICCS were used to demonstrate and quantify the colocalization of the internalization-competent protein, HA+8 (*30*, *32*), with clathrin and AP-2 in situ, in intact cells. Our results show that HA+8 was colocalized with clathrin and AP-2 at the plasma membrane and strongly suggest that the colocalization occurred within clathrin-coated pits. When the cells were treated to disrupt the coated pit structure, the interactions between HA+8 and clathrin or AP-2 were altered. These findings demonstrate for the first time in whole cells that following treatments that disrupt endocytosis an internalization-competent protein can interact with AP-2 in clusters devoid of clathrin, at least in cells treated to alter their clathrin lattice structure.

We have previously proposed a model for the distribution of AP-2 at the cell surface based on ICS data (*43*, *46*). The model proposed two populations of AP-2 clusters: (i) large clusters associated with clathrin in coated pits and (ii) smaller clusters which could represent coated pit nucleation sites. Following hypertonic treatment or cytosol acidification, the smaller clusters dissociate to some extent, causing a dispersion of AP-2 (*43*, *46*). Here we show that this is accompanied by a shift to more clusters containing HA+8 and AP-2 than HA+8 and clathrin (Tables 4 and 5). Thus, it can be concluded that following the treatments described above HA+8 can interact with AP-2 in clathrin-independent clusters, and that this interaction does not depend on the smaller AP-2 clusters remaining intact.

Although there is an increase in the percentage of HA+8 interacting with AP-2 after hypertonic treatment (Table 4), the association between them may become weaker, probably due to the absence of clathrin (*31*, *46*). This is indicated by the reduced amount of co-immunoprecipitation of the two proteins after hypertonic treatment (*30*). A weaker HA+8−AP-2 association in hypertonically treated cells is in accord with earlier lateral mobility studies which demonstrated a shift of HA+8 from stable to transient interactions with immobile structures, and this relatively weak (transient) interaction after hypertonic treatment could be with the remaining AP-2 clusters (*44*). A major difference between the conditions of the ICCS studies and co-immunoprecipitation experiments is that the latter involve detergent solubilization, which could disrupt the clathrin-independent HA+8−AP-2 complexes. This emphasizes the advantage of performing experiments in situ where the use of detergents is not necessary.

The current data demonstrate that an internalization-competent protein can interact with AP-2 in clusters devoid of clathrin in cells treated to alter their clathrin lattice structure. In untreated cells, the higher affinity of the internalization signal for AP-2 in complex with clathrin (*31*) would favor the association of HA+8−AP-2 complexes with either existing coated pits or free clathrin. Under such conditions, clathrin free HA+8−AP-2 clusters, if they form at all, would be short-lived and their steady-state level is expected to be very low. Although we cannot ascertain whether such clusters do form in untreated cells, the ability to detect them in cells treated with hypertonic medium, which actually decreases the affinity of AP-2 for HA+8 (*30*), suggests that this interaction can occur. This notion is further supported by the detection of HA+8−AP-2 clusters that do not colocalize with clathrin in cytosol-acidified cells (Table 5), where the preferential labeling of HA+8 molecules located outside of deeply invaginated pits creates conditions that enable their detection. We propose that the binding of membrane proteins carrying internalization signals to AP-2 may precede the AP-2−clathrin association in the coated pit assembly process. Once formed, these complexes either can serve to recruit clathrin from the cytosol or, more likely, can be recruited to and associate with existing coated pits, due to the enhanced affinity in the clathrin-containing complex (*31*).

This proposal is supported by a second finding of our work, that even low expression levels of HA+8 caused a redistribution of AP-2 to existing coated pits (Figure 2). At higher levels of HA+8 expression, this redistribution of AP-2 was even more apparent (Figure 2). The effect is specific to HA+8, since expression of high levels of HA wt did not change the AP-2 distribution (Figure 2). This HA+8-mediated redistribution of AP-2 could not occur if coated pits were already saturated with AP-2 and were simply trapping HA+8 as it diffused into them; it also suggests that an increased extent of association of AP-2 with internalization-competent proteins enhances the binding of AP-2 to clathrin. We have evidence from a number of cell types that AP-2 is more aggregated on large cells where there should also be an increased demand for endocytosis (*38*). Taken together, this suggests that in response to an increased demand for endocytosis the cell may increase the amount of AP-2 associated with each coated pit without changing the distribution of coated pits. The net result will be an increase in the number of receptors bound in each coated pit, increasing the rate of endocytosis without the need for de novo coated pit formation.

Previous studies have provided evidence that there is an increase in the amount of membrane-associated clathrin when transferrin receptor is highly overexpressed (*51*, *52*). It is

possible that at very high expression levels of proteins carrying strong internalization signals the cell responds by recruiting cytosolic clathrin to increase the coated pit density, but it appears that at levels below or slightly above saturation of the coated pits (see Figure 2) the cellular response is to redistribute AP-2 so that each coated pit can bind a higher number of receptors. This is in accord with the report of Santini et al. (*53*), where no correlation was found between the level of constitutively internalized membrane receptors (at levels just high enough to saturate the endocytosis pathway) and the number of coated pits or the amount of membrane-associated clathrin or AP-2. Since small clusters of AP-2 devoid of clathrin cannot be detected by eye in fluorescence images (*38*, *46*), these former studies could not elucidate small changes in the distribution of membrane-associated AP-2, which can be detected by the more sensitive ICS analysis employed here.

In summary, using ICS and ICCS, low levels of, and subtle changes in, the distribution and colocalization of proteins are easily extracted from images where it would be difficult, at best, to determine changes in these parameters visually. Using these methods, we were able to detect for the first time clathrin free clusters containing HA+8 and AP-2 in whole cells. In addition, we were able to detect subtle changes in the distribution of AP-2 in the presence of increased levels of an internalization-competent protein. On the basis of these results, we propose that the binding of AP-2 to internalization signals on membrane proteins may occur prior to the association of AP-2 with clathrin, and likely plays an important role in the redistribution of AP-2 to preexisting coated pits.

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REFERENCES

1. Pearse, B. M. F., and Robinson, M. S. (1990) *Annu. Rev. Cell Biol. 6*, 151−171.
2. Schmid, S. L. (1992) *BioEssays 14*, 589−596.
3. Watts, C., and March, M. (1992) *J. Cell Sci. 103*, 1−8.
4. Kirchhausen, T. (1993) *Curr. Opin. Struct. Biol. 3*, 182−188.
5. Robinson, M. S. (1994) *Curr. Opin. Cell Biol. 6*, 538−544.
6. Mellman, I. (1996) *Annu. Rev. Cell Dev. Biol. 12*, 575−625.
7. Schmid, S. L. (1997) *Annu. Rev. Biochem. 66*, 511−548.
8. Heuser, J. E., and Kirchhausen, T. (1985) *J. Ultrastruct. Res. 92*, 1−27.
9. Brodsky, F. M. (1988) *Science 242*, 1396−1402.
10. Pearse, B. M. F. (1989) *Methods Cell Biol. 31*, 229−246.
11. Marks, M. S., Ohno, H., Kirchhausen, T., and Bonifacino, J. S. (1997) *Trends Cell Biol. 7*, 124−128.
12. Keen, J. H., and Beck, K. A. (1989) *Biochem. Biophys. Res. Commun. 158*, 17−23.
13. Matsui, W., and Kirchhausen, T. (1990) *Biochemistry 29*, 10791−10798.
14. Prasad, K., and Keen, J. H. (1991) *Biochemistry 30*, 5590−5597.
15. Peeler, J. S., Donzell, W. C., and Anderson, R. G. W. (1993) *J. Cell Biol. 120*, 47−54.
16. Goodman, O. B., Jr., and Keen, J. H. (1995) *J. Biol. Chem. 270*, 23768−23773.
17. Shih, W., Gallusser, A., and Kirchhausen, T. (1995) *J. Biol. Chem. 270*, 31083−31090.
18. Ohno, H., Stewart, J., Fournier, M., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) *Science 269*, 1872−1875.
19. Boll, W., Ohno, H., Songyang, Z., Rapoport, I., Cantley, L. C., Bonifacino, J. S., and Kirchhausen, T. (1996) *EMBO J. 15*, 5789−5795.
20. Trowbridge, I. S. (1991) *Curr. Opin. Cell Biol. 3*, 634−641.
21. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annu. Rev. Cell Biol. 9*, 129−161.
22. Sandoval, I. V., and Bakke, O. (1994) *Trends Cell Biol. 4*, 292−297.
23. Kirchhausen, T., Bonifacino, J. S., and Riezman, H. (1997) *Curr. Opin. Cell Biol. 9*, 488−495.
24. Sorkin, A., and Carpenter, G. (1993) *Science 261*, 612−615.
25. Boll, W., Gallusser, A., and Kirchhausen, T. (1995) *Curr. Biol. 5*, 1168−1178.
26. Gilboa, L., Ben-Levy, R., Yarden, Y., and Henis, Y. I. (1995) *J. Biol. Chem. 270*, 7061−7067.
27. Nesterov, A., Kurten, R. C., and Gill, G. N. (1995) *J. Biol. Chem. 270*, 6320−6327.
28. Sorkin, A., McKinsey, T., Shih, W., Kirchhausen, T., and Carpenter, G. (1995) *J. Biol. Chem. 270*, 619−625.
29. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L., and Beguinot, L. (1996) *J. Biol. Chem. 271*, 13377−13384.
30. Fire, E., Brown, C. M., Roth, M. G., Henis, Y. I., and Petersen, N. O. (1997) *J. Biol. Chem. 272*, 29538−29545.
31. Rapoport, I., Miyazaki, M., Boll, W., Duckworth, B., Cantley, L. C., Shoelson, S., and Kirchhausen, T. (1997) *EMBO J. 16*, 2240−2250.
32. Zwart, D. E., Brewer, C. B., Lazarovits, J., Henis, Y. I., and Roth, M. G. (1996) *J. Biol. Chem. 271*, 907−917.
33. Lazarovits, J., and Roth, M. G. (1988) *Cell 53*, 743−752.
34. Fire, E., Zwart, D. E., Roth, M. G., and Henis, Y. I. (1991) *J. Cell Biol. 115*, 1585−1594.
35. Petersen, N. O., Hoddelius, P. L., Wiseman, P. W., Seger, O., and Magnusson, K. (1993) *Biophys. J. 65*, 1135−1146.
36. Wiseman, P. W. (1995) Ph.D. Thesis, Department of Chemistry, University of Western Ontario, London, ON.
37. Wiseman, P. W., and Petersen, N. O. (1999) *Biophys. J. 76*, 963−977.
38. Brown, C. M. (1998) Ph.D. Thesis, Department of Chemistry, University of Western Ontario, London, ON.
39. Srivastava, M., and Petersen, N. O. (1996) *Methods Cell Sci. 18*, 47−54.
40. Ohno, H., Fournier, M. C., Poy, G., and Bonifacino, J. S. (1996) *J. Biol. Chem. 271*, 29009−29015.
41. Page, L. J., and Robinson, M. S. (1995) *J. Cell Biol. 131*, 619−630.
42. Robinson, M. S. (1987) *J. Cell Biol. 104*, 887−895.
43. Brown, C. M., and Petersen, N. O. (1998) *J. Cell Sci. 111*, 271−281.
44. Fire, E., Gutman, O., Roth, M. G., and Henis, Y. I. (1995) *J. Biol. Chem. 270*, 21075−21081.
45. Naim, H. Y., and Roth, M. G. (1994) in *Methods in Cell Biology*, pp 113−136, Academic Press, San Diego, CA.
46. Brown, C. M., and Petersen, N. O. (1999) *Biochem. Cell Biol.* (in press).
47. Schwille, P., Meyer-Almes, F.-J., and Rigler, R. (1997) *Biophys. J. 72*, 1878−1886.
48. Heuser, J. E., and Anderson, R. G. (1989) *J. Cell Biol. 108*, 389−400.
49. Sandvig, K., Olsnes, S., Peterson, O. W., and van Deurs, B. (1987) *J. Cell Biol. 105*, 679−689.
50. Heuser, J. E. (1989) *J. Cell Biol. 108*, 401−411.
51. Iacopetta, B. J., Rothenberger, S., and Kuhn, L. C. (1988) *Cell 54*, 485−489.
52. Miller, K., Shipman, M., Trowbridge, I. S., and Hopkins, C. R. (1991) *Cell 65*, 621−632.
53. Santini, F., Marks, M. S., and Keen, J. H. (1998) *Mol. Biol. Cell 9*, 1177−1194.

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