An image correlation analysis of the distribution of clathrin associated adaptor protein (AP-2) at the plasma membrane

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SUMMARY

Clathrin associated adaptor protein is involved in endocytosis at the plasma membrane (AP-2) and protein sorting at the Golgi membrane (AP-1). There is a great deal of information available on the structure, function and binding characteristics of AP-2, however, there is little quantitative data on the AP-2 distribution at the membrane. Image correlation spectroscopy is a technique which yields number counts from an autocorrelation analysis of intensity fluctuations within confocal microscopy images. Image correlation spectroscopy analysis of the indirect immunofluorescence from AP-2 at the plasma membrane of CV-1 cells shows that AP-2 is in a bimodal distribution consisting of large coated pit associated aggregates of ~60 AP-2 molecules, and smaller aggregates containing ~20 AP-2 molecules, which we propose are coated pit nucleation sites. Following hypertonic treatment 25% of the AP-2 molecules dissociate from the large AP-2 aggregates and form AP-2 dimers, leaving the remaining AP-2 as large aggregates with ~45 molecules. The smaller AP-2 aggregates completely dissociate forming AP-2 dimers. Dispersion of AP-2 with hypertonic treatment is not seen qualitatively because the number of large AP-2 aggregates is unchanged, the aggregates are just 25% smaller. Change in temperature from 37°C to 4°C has no affect on the number of AP-2 aggregates or the AP-2 distribution between the two populations. These data and estimates of the coated pit size suggest that coated pits cover ~0.9% of the cell membrane. Combination of image correlation spectroscopy analysis and measurements of the CV-1 cell surface area show that there are ~6·10⁵ AP-2 molecules per CV-1 cell with ~2·10⁵ AP-2 molecules within coated pit structures.

Key words: Adaptor protein (AP-2), Distribution, Clathrin

INTRODUCTION

Clathrin associated adaptor protein functions in endocytosis at the plasma membrane (AP-2) and internalization and sorting at the Golgi membrane (AP-1). A number of early studies discovered ~100 kDa polypeptides which copurified with clathrin coats (Pearse, 1978; Keen et al., 1979; Zaremba and Keen, 1983) these proteins were later determined to be components of proteins now known as adaptor proteins. The structures of AP-1 and AP-2 are well known (Ahle et al., 1988; Heuser and Keen, 1988; Heuser and Anderson, 1989; Matsui and Kirchhausen, 1990), both adaptor proteins are heterotetrameric in structure containing two larger subunits of approximately 100 kDa (α and β, for AP-2 and γ and β′ for AP-1), a medium subunit (μ2 for AP-2; μ1 for AP-1), and a small subunit (σ2 for AP-2; σ1 for AP-1; reviewed by Robinson, 1987, 1992; Hurtley, 1991; Kirchhausen, 1993; Traub, 1997). AP-2 was first proposed by Keen et al. (1979) to promote coat assembly and it is known to bind to clathrin likely recruiting that protein to the membrane in order to form the structural component of the coated pit (Pearse, 1978; Zaremba and Keen, 1983; Pearse and Robinson, 1984; Keen, 1987; Keen and Beck, 1989; Ahle and Ungewickell, 1989; Mahaffey et al., 1990; Prasad and Keen, 1991; Schroder and Ungewickell, 1991; Peeler et al., 1993; Goodman and Keen, 1995). AP-2 is present in both a soluble form and a membrane bound form. It was recently shown that the AP-2/clathrin interaction is likely regulated by phosphorylation of AP-2, with the phosphorylated AP-2 being found in the cytosol while the membrane and clathrin bound AP-2 is dephosphorylated (Wilde and Brodsky, 1996). AP-2 has also been shown to bind to the membrane likely via an AP-2 membrane receptor (Zaremba and Keen, 1983; Moore et al. 1987; Mahaffey et al., 1990; Chang et al., 1993; Seaman et al. 1993) and using a tilt series of EM micrographs, Vigers et al. (1986) demonstrated that AP-2 forms a layer between the membrane and the clathrin coat. AP-2 functions in binding certain transmembrane receptors bringing them into clathrin coated pits and the endocytosis process. AP-2/receptor binding has been shown in vitro using solubilized or immobilized proteins for a number of membrane receptors including: a number of receptor cytoplasmic tails (Pearse, 1988); the mannose-6-phosphate receptor and the insulin like growth factor receptor (Glickman et al., 1989); the asialoglycoprotein receptor (Beltzer and Spiess, 1991; Chang et al., 1993); the cytoplasmic domain of the lysosomal acid phosphatase receptor.
disperses the smaller clusters to dimers of AP-2. Decreases the size of the larger clusters by about 25% and among them. However, hypertonic treatment of the cells clusters of about one third the size. Temperature changes do AP-2 exists as large clusters, presumably associated with distribution. Specifically, we show that about one third of the order to get a quantitative representation of the AP-2 AP-2 distributions visualized via indirect immunofluorescence on this distribution. We use confocal microscopy images of AP-2 at the plasma membrane of CV-1 cells, and investigate membrane protein distributions (Petersen et al., 1993; Liu and Robinson, 1995). AP-2 is clearly a very important component of the internalization process, and although many of the structural and functional characteristics of AP-2 are now known, to our knowledge there has been no quantitative work done on how AP-2 is distributed at the plasma membrane. Previously, this was not possible because the methodology for such studies did not exist. Now, image correlation spectroscopy (ICS) provides an approach which enables the quantitative analysis of membrane protein distributions (Petersen et al., 1993; Wiseman, 1995). In this study we determine the distribution of AP-2 at the plasma membrane of CV-1 cells, and investigate the effects of hypertonic treatment, and changes in temperature on this distribution. We use confocal microscopy images of AP-2 distributions visualized via indirect immunofluorescence labelling, to obtain a qualitative representation of the AP-2 distribution. We perform an ICS analysis of these images in order to get a quantitative representation of the AP-2 distribution. Specifically, we show that about one third of the AP-2 exists as large clusters, presumably associated with clathrin in coated pit structures, with the remainder in smaller clusters of about one third the size. Temperature changes do not affect the size of the clusters or the distribution of AP-2 among them. However, hypertonic treatment of the cells decreases the size of the larger clusters by about 25% and disperses the smaller clusters to dimers of AP-2.

Materials and Methods

Materials

Trypsin, n-propylgallate, bovine serum albumin (BSA), chlorpromazine, saponin, fluorescein isothiocyanate (FITC) labelled Fab specific goat anti-mouse secondary antibody, and normal goat IgG were from Sigma Chemical Company (St Louis, Missouri). Hanks’ balanced salt solution (HBSS), Dulbecco’s modified Eagle’s medium (DME), fetal bovine serum (FBS), and penicillin-streptomycin were all from Life Technologies (Gaithersburg, MD). CV-1 (monkey kidney) cells were obtained from the American Type Culture Collection (Rockville, MD) and tested negative for mycoplasma contamination using Hoechst Dye 33258. Airlol 205 was from Air Products and Chemical Inc. (Allentown, PA). AC1-M11 mouse monoclonal antibodies specific for the αα and αα chains of AP-2 (Robinson, 1987), originally prepared by Dr Margaret S. Robinson (University of Cambridge, UK), were obtained from Dr Yoav I. Henis (Tel Aviv University, Israel). AP6 mouse monoclonal antibodies to the α chain of AP-2 were a gift from Dr Francis Brodsky (UCSF, San Francisco).

Cell culture and cell fixation methods

The CV-1 cells were grown in DME containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (full-DME), at 37°C, 100% humidity and 5% CO2. The day before an experiment confluent CV-1 cells (passage 35-60) were removed from a 25 cm2 tissue culture flask by washing twice with about 1 ml of 0.05% trypsin (in PBS (Ca2+, Mg2+ free) containing EDTA) and leaving the flask at 37°C for about 5 minutes. 8 ml of full-DME were added to the flask, and 1 ml was placed in each of eight tissue culture dishes containing sterile 22 mm square glass coverslips, resulting in a 1:2 dilution of the cells. An additional 0.5 ml of full-DME was added to the CV-1 cells to a total of 1.5 ml per culture dish. The following day the tissue culture dishes containing the cells growing on the coverslips were taken and the cells were washed three times with 37°C HBSS containing 20 mM Hepes and fixed and permeabilized in one of two ways: (1) for AC1-M11 monoclonal antibody (mAb) labelling cells were fixed and permeabilized by placing the coverslips in a coverslip holder and submerging them in cold methanol for 5 minutes (~20°C) followed by cold acetone for 2 minutes (~20°C) and then air dried. (2) For AP6 mAb labelling cells were fixed for 10 minutes at RT by placing a few milliters of a 4% paraformaldehyde solution (made up in PBS containing 0.11 mg/ml lysine and 24 mg/ml sodium periodate at pH 7.4) in the tissue culture dishes containing the coverslips. The cells were then washed three times with HBSS/Hepes and permeabilized for 10 minutes at RT with a few milliters of 0.04% saponin in HBSS/Hepes (pH 7.4). After either fixation the coverslips were washed twice with HBSS/Hepes and once with HBSS/Hepes containing 2% BSA (HBSS/Hepes/BSA, pH 7.4) before immunofluorescence labelling.

Treatments

All treatments were carried out prior to fixation and labelling. For temperature studies the cells were incubated in HBSS/Hepes solution at 4°C for two hours or at 22°C or 37°C for 15 minutes. For hypertonic treatment cells were treated for 15 minutes at 37°C with HBSS/Hepes containing 0.5 M sucrose. In order to permeabilize the cells and wash away the cytosol they were permeabilized with 0.04% saponin in HBSS/Hepes (pH 7.4) at 4°C for 10 minutes and washed three times with cold HBSS/Hepes. Chlorpromazine treatment involved treating the cells with 100 μM chlorpromazine in HBSS/Hepes for 30 minutes, at 37°C, followed by washing with cold HBSS/Hepes.

Immunofluorescence labelling of AP-2

Fixed cells were labelled at room temperature with the following antibodies: (1) normal goat IgG (to block non-specific binding of the goat anti-mouse antibody; 200 μg/ml, one hour); (2) AC1-M11 mouse monoclonal antibodies specific for the αα and αα chains of AP-2, or AP6 mouse monoclonal antibodies specific for the α chain of AP-2 (50 μg/ml, two hours, this concentration was varied for antibody binding studies); (3) FITC labelled goat anti-mouse IgG Fab specific (20 μg/ml, one hour). After each labelling step the cells were washed twice with HBSS/Hepes and once with HBSS/Hepes/BSA; after the second and third labelling steps each wash was for ten minutes with rocking. After labelling, the fixed cells were mounted using Airlol containing n-propylgallate and taken for ICS studies.

Data analysis and collection

Fluorescence correlation spectroscopy refers to a family of techniques
which analyse fluorescence fluctuations in time or space to yield number counts and dynamic information such as diffusion or flow (Elson and Magde, 1974; Ehrenberg and Rigler, 1976; Eigen and Rigler, 1994). By providing number counts in defined volumes or areas, these techniques are capable of quantifying the state of aggregation and changes in aggregation due to the redistribution of fluorescent components (Elson and Magde, 1974; Weissman et al., 1976). Image correlation spectroscopy (ICS) relies on spatial intensity fluctuations in images collected on a confocal laser scanning microscope (described in detail by Petersen et al., 1993; Wiseman, 1995; Srivastava and Petersen, 1996). Typically, one generates an image of the distribution of the fluorescence intensity on the cell surface by scanning a fluorescently labelled cell with a laser beam, recording fluctuations in fluorescence intensity as a function of position in two dimensions (Petersen et al., 1993; Eigen and Rigler, 1994; Wiseman, 1995). The image analysis is based on calculating the autocorrelation function for these fluorescence intensity fluctuations, since it has been shown that the amplitude of this function contains the number count. To obtain dynamic information, analysis of many temporally spaced images are needed (Srivastava and Petersen, 1996).

The decay of the autocorrelation function depends on the transverse intensity profile of the laser beam. Therefore, we fit the calculated autocorrelation function, \( g(\xi, \eta) \), to a two-dimensional Gaussian function as in Equation 1 (Petersen et al., 1993; Wiseman, 1995).

\[
g(\xi, \eta) = g(0, 0) e^{-\frac{\xi^2 + \eta^2}{2w^2}} + g_o,
\]

where \( \xi \) and \( \eta \) are the position lag coordinates (for the \( x \) and \( y \) axes, respectively) of the autocorrelation function, \( \omega \) is the \( e^{-2} \) radius of the laser beam, and \( g(0, 0) \) is the amplitude of the autocorrelation function upon extrapolation of \( \xi \) and \( \eta \) to zero; the offset, \( g_o \), is introduced to account for the finite sample size of the images, which can result in a decay of \( g(\xi, \eta) \) to a non-zero level at large lag coordinates. The \( g_o \), \( g(0, 0) \), and \( \omega \) values are extracted from the fitting procedure (Petersen et al., 1993). All calculations were performed on a Massively Parallel Computer (MP-2, MasPar Computer Corporation, Sunnyvale, CA).

After labelling and fixation of the cells as indicated above (Immunofluorescence Labelling of AP-2), fluorescence images were obtained using a Bio-Rad MRC-600 confocal microscope, illuminating at 488 nm (to excite FITC) with a 25 mW argon ion laser attenuated to 1% laser power (equivalent to a few hundredth of a mW). To ensure linear scaling of the intensity, images were collected in the attenuated to 1% laser power (equivalent to a few hundredth of a mW).

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The simplest interpretation of the \( g(0, 0) \) value is that it arises from a single population of fluorescently labelled molecules. In this case:

\[
g(0, 0) = \frac{1}{N_p},
\]

where \( g(0, 0) \) is the zero lag amplitude of the autocorrelation function, and \( N_p \) is the average number of independent fluorescently labelled particles in the observation area, i.e. the area of the laser beam (Petersen, 1986; Petersen et al., 1993). A ‘particle’ is any independent fluorescently labelled species, i.e. a fluorescent monomer is one particle, but an aggregate containing many monomers is also one particle. Therefore, aggregation leads to a reduction in \( N_p \) (larger and fewer particles), which is measured by the \( 1/g(0,0) \) value (Petersen et al., 1993; Wiseman, 1995). The average intensity of an image, \( <i> \), gives an estimate of the total number of fluorophores, \( N_m \), in the observation area, or the total number of fluorescently labelled proteins, and \( g(0,0) \) gives an estimate of the number of independent fluorescent particles, \( N_p \). If we have a single population then the product of these two parameters will give an estimate of the number of individual fluorescent proteins (monomers) in each particle, or cluster. This product is defined as the degree of aggregation (DA) in Equation 3.

\[
DA = g(0, 0) <i> = \alpha \frac{N_m}{N_p}.
\]

Here \( \alpha \) is a constant containing both instrumental and experimental constants. The number of particles, \( N_p \), is dependent on the beam area, however, the cluster density (CD), can be obtained simply by dividing this quantity by the beam area:

\[
CD = \frac{1}{g(0,0)\pi\omega^2};
\]

where \( \omega \) is the \( e^{-2} \) radius of the laser beam. The CD is the number of independent fluorescent particles per \( \mu^2 \) of cell membrane.

Petersen (1986) demonstrated that if a system contains fluorescently labelled molecules in many different populations then the \( g(0,0) \) value has a more complicated interpretation corresponding to Equation 5.

\[
g(0, 0) = \frac{1}{N_m} \sum_i N_i (\sigma_i^2 + \mu_i^2).
\]

The \( g(0,0) \) value is dependent on the average number of monomers (individual fluorophores or correspondingly individual proteins), \( N_m \), in the observation area. It is also dependent on the number of aggregates within each population, \( N_i \); the mean number of proteins in each type of aggregate, \( \mu_i \); and the variance in size of the aggregates within each population, \( \sigma_i^2 \). If one of the populations, \( i \), has a large mean, \( \mu_i \), then the variance term in Equation 5, \( \sigma_i^2 \), will often be small relative to the mean squared (e.g. for a Poisson distribution with \( \mu_i \geq 10 \), \( \sigma_i^2 \leq 10\% \) of the value of the bracketed term in Equation 5), and thus, the variance term may be ignored without much error, relative to the experimental uncertainty in \( g(0,0) \). On the other hand, if one of the populations, \( j \), has a large number of aggregates, \( N_j \), with a small mean, \( \mu_j \), then ignoring the variance, \( \sigma_j^2 \), may introduce as much as a 30\% error in the contribution from this population. If a system consists of a bimodal distribution of two populations each fitting into either of the conditions presented above, then Equation 5 can be simplified as follows:

\[
g(0,0) = \frac{1}{N_m^2} (N_j \mu_j^2 + N_i \mu_i^2 + \sigma_i^2 - \sigma_j^2).
\]

### Corrections for non-specific antibody binding

The fluorescence measured by confocal microscopy on whole cells invariably contains contributions from non-specific binding of the secondary antibody and from autofluorescence. As shown by St. Pierre and Petersen (1992), it is possible to correct the correlation data for these contributions. Provided that the sources of fluorescence are independent and not correlated with each other, then:

\[
g_m(0,0) = \frac{g_m(0,0) <i>_m^2 - g_d(0,0) <i>_d^2}{<i>_d^2},
\]

where \( g_m(0,0) \), \( <i>_m \), \( g_d(0,0) \), \( <i>_d \), \( g(0,0) \) and \( <i> \) are the zero lag amplitude and the average intensity of the autocorrelation function for the measured function (\( m \)), the contribution from specific labelling (\( s \)) and the contribution from non-specific labelling (\( n \)), respectively. It is...
presumed here that the measurements on controls for non-specific antibody binding include contributions from the autofluorescence of the cell. The $\langle i_{\text{p}} \rangle$ was also corrected for the contribution from the background intensity due to ‘dark current’ in the photomultiplier tube. This background intensity was measured under conditions where there was no exposure of the laser illumination to the sample. This intensity value was then subtracted off from the measured intensity to give the corrected value. The background intensity is totally random and thus does not contribute to the autocorrelation function.

**RESULTS**

**AP-2 aggregates are large**

It is important in any quantitative experiment that all of the molecules of interest are detected (St Pierre and Petersen, 1992). Therefore, antibody binding curves were produced using the ICS results from samples labelled with various concentrations of primary antibody, either mouse mAb AC1-M11 (methanol/acetone fixed samples), or AP6 (paraformaldehyde fixed cells) which are both specific for the $\alpha$ chain of AP-2.

The AC1-M11 mAb concentration was varied from 6.25 $\mu$g/ml to 200 $\mu$g/ml while the AP6 mAb concentration was varied from 5 $\mu$g/ml to 200 $\mu$g/ml. In either case the secondary FITC-conjugated goat anti-mouse antibody concentration was held constant at 20 $\mu$g/ml. All of the $g(0,0)$ values were corrected for the contributions from both non-specific binding of the goat anti-mouse antibody and cell autofluorescence using ICS results from images collected on cells labelled with FITC conjugated goat-anti mouse antibody, but with no primary AC1-M11 or AP6 mAb (as per Equation 7). At low primary antibody concentrations, the degree of aggregation (DA, Equation 3) increases linearly as more AP6 or AC1-M11 mAb is added (Fig. 1A,B). This increase in the DA is due to more and more individual antibodies binding to each aggregate as the antibody concentration is increased resulting in detection of a larger number of monomers per aggregate, i.e. a higher DA. This linear increase over a tenfold change in concentration supports the idea that the AP-2 aggregates are large. After 50 $\mu$g/ml of primary antibody has been added all of the AP-2 binding sites are saturated and the DA value levels off at a value of ~7 (Fig. 1A) The situation is a bit different for the AC1-M11 mAb labelling. In this case we see a decrease in the DA at high concentrations of antibody rather than saturation (Fig. 1B). We believe this is due to an increase in the non-specific antibody binding at these high concentrations. This could be due to more non-specific binding following methanol/acetone fixation than paraformaldehyde fixation, or because the AC1-M11 mAb tends to bind more non-specifically than AP6 mAb. Either way this increase in non-specific binding is likely due to the antibody binding to the many ‘sticky’ proteins which are exposed once the cells are permeabilized. These proteins would likely only bind individual antibody molecules which will increase the total number of independent particles (or aggregates), $\bar{N}_p$, and therefore result in a decrease in the DA value (Equation 3). This increase in non-specific labelling is not seen with the AP6 mAb or when similar antibody binding curves are performed on proteins labelled from outside the cell, i.e. without permeabilizing the cells (St Pierre and Petersen, 1992; Wiseman, 1995). The DA values are different for the two primary antibodies (~7 for AP6 and ~5 for AC1-M11) because it depends not only on the number of clusters of AP-2, but also on the labelling efficiency of both the primary and the secondary antibodies.

We chose to use 50 $\mu$g/ml of AC1-M11 mAb for the remainder of the experiments in order to ensure the saturation of the AP-2 aggregates, maximize the signal to noise ratio in the images and yet minimize the effect of non-specific antibody labelling (The AP6 mAb was obtained after most of these experiments were performed and was only used to confirm the results we obtained with the AC1-M11 mAb). In order to confirm that the antibody binding was specific for AP-2, cells were treated with 100 $\mu$M chlorpromazine. Wang et al. (1993) have shown that AP-2 redistributes from the membrane to internalized vesicles with this treatment and we also saw this characteristic redistribution (data not shown) supporting specific AP-2 labelling. The most significant observation from the data so far is that the dominant AP-2 aggregates we are detecting are large.
Hypertonic treatment decreases the average AP-2 aggregate size

In order to determine if the AP-2 aggregates are associated with coated pits, we performed experiments involving hypertonic treatment of the cells which inhibits formation of clathrin coated pit structures (Heuser and Anderson, 1989; Hansen et al., 1993). Electron microscopy studies show that following hypertonic treatment clathrin trimers form many small, membrane free, microcages on the cytoplasmic side of the plasma membrane, likely utilizing all of the soluble pool of clathrin so that new coated pits can no longer form (Heuser and Anderson, 1989). Previous fluorescence microscopy data have shown that AP-2 remains aggregated and associated with the membrane after hypertonic treatment (Hansen et al., 1993), and that membrane receptors still interact with AP-2 even after hypertonic treatment (Fire et al., 1997) or K+ depletion (Sorkin and Carpenter, 1993) which has similar effects as hypertonic treatment. Qualitative analysis of the immunofluorescence labelling of AP-2 does not show any significant difference in the distribution with or without hypertonic treatment (Hansen et al., 1993; compare Fig. 2A,D). However, the results from ICS analysis show that after hypertonic treatment for fifteen minutes the DA decreases from 4.6±0.9 monomers per aggregate to 1.8±0.4 monomers per aggregate (Fig. 3). The fact that hypertonic treatment affects the average size of the AP-2 aggregates supports the idea that they are in some way associated with clathrin coated pits. Hypertonic treatment disperses the AP-2 within 5 minutes at 37°C. Longer incubation (up to 45 minutes) has no further effect on the AP-2 distribution (data not shown).

Temperature does not affect AP-2 aggregates

To our knowledge there is no information available on the effect of temperature on the distribution of AP-2 at the cell membrane. While it has been suggested from thin section EM data that at 4°C there may be as many as twice the number of clathrin coated pits as there are at 37°C, these authors were not convinced that this temperature effect was ‘real’ (Anderson et al., 1977a,b). There is evidence from other ICS data that certain membrane receptors actually disperse at lower temperatures. These include the platelet-derived growth factor β (PDGF-β) receptor (Wiseman, 1995; Wiseman et al., 1997) and internalization competent influenza virus hemagglutinin (HA) mutants (Fire et al., 1997). If receptors disperse at low temperature this would suggest that the receptor AP-2 complex may break up to some extent at low temperature resulting in an apparent dispersion of the receptor molecules.

Visually, it is difficult to see if temperature has any effect on the distribution of AP-2 (compare Fig. 2A,B). Detailed analysis of the ICS data indicates that there is no change in the AP-2 distribution as the temperature is varied from 4°C to 37°C.

**Fig. 2.** Confocal images showing CV-1 cells either methanol/acetone fixed and immunofluorescently labelled with AC1-M11 anti-AP-2 mAb (A,B,D) or paraformaldehyde fixed, permeabilized and immunofluorescently labelled with AP6 anti-AP-2 mAb (C) as described in Materials and Methods. CV-1 cells on 22 mm coverslips were untreated (A,C), left for 2 hours in HBSS/Hepes buffer at 4°C (B), or treated for 15 minutes with 37°C HBSS/Hepes buffer containing 0.5 M sucrose (D), before fixation and labelling. Note: a new laser system was installed during the course of these experiments so the image in C, showing the AP6 mAb labelling, is slightly smaller than the other images. Bars: 50 μm (bar in D is also valid for A and B).
sample (treatment; give yielding an average of ~150 clusters per image which over 30 images (similar to Fig. 4B) were counted manually per image. The number of clusters visible as bright spots in we in fact measure 532 aggregates labelled on both the upper and lower membranes (Fig. 4B-D) but since we pick up intensity from AP-2 shown), therefore, each image measures an area of 266 mm². From confocal cross sections we know that under the conditions we collect our images we cannot combine all of the data from images collected from 439 (Fig. 3). The DA values remain essentially constant over this temperature range: 5.9, 6.2, and 5.7 at 4°C, 22°C, and 37°C, respectively, and the CD behaves correspondingly with little change. Hence, it must be the receptor molecules that change. Hence, it must be the receptor molecules that dissociate from the AP-2 aggregates at 4°C if they disperse within the membrane.

AP-2 exists in two aggregate populations
If we assume that the AP-2 distribution is the result of a single population of aggregates then Equation 2 should hold. If we combine all of the data from images collected from 439 individual cells, sampling a total surface area of over 10⁵ µm², we calculate an average cluster density (CD, Equation 3) of 1.4±0.2 clusters/µm². From confocal cross sections we know that under the conditions we collect our images we cannot resolve the upper and lower membranes of the cell (data not shown), therefore, each image measures an area of 266 µm² (Fig. 4B-D) but since we pick up intensity from AP-2 aggregates labelled on both the upper and lower membranes we in fact measure 532 µm² of cell membrane (Fig. 2). To obtain a CD of 1.4 we would expect to see ~750 AP-2 clusters per image. The number of clusters visible as bright spots in over 30 images (similar to Fig. 4B) were counted manually yielding an average of ~150 clusters per image which corresponds to a CD value of 0.28 clusters/µm². This fivefold difference in apparent cluster density can only be accounted for if we have more than one population of AP-2 aggregates. The other populations of AP-2 aggregates are not readily visible in the image, but are included in the more quantitative ICS analysis. If we then assume that the AP-2 aggregates are in a bimodal distribution, we can treat the data according to Equation 6.

Counting AP-2 clusters from visible intensity spots in 37 images of hypertonicity treated cells (similar to Fig. 4C), gives a cluster density of ~0.30 clusters/µm². Comparing this with the value of 3.7 measured by ICS suggests that a bimodal distribution still exists after hypertonic treatment, although the distribution has changed with respect to untreated cells resulting in a much higher cluster density.

The size of both AP-2 aggregates change upon hypertonic treatment
In order to determine if it was reasonable to assume that there is a second population of AP-2 aggregates we took a closer look at some cross sections of the intensity from two-dimensional images. Fig. 4F shows a representative cross section for AC1-M11 labelling of AP-2 and it is apparent that in addition to a series of large intensity peaks there are a number of broad low level intensity peaks. The larger peaks correspond to the visible clusters in the image and we presume the smaller peaks correspond to a population of smaller AP-2 aggregates. No broad low level intensity peaks are seen in the control sample for non-specific antibody binding (Fig. 4D,H). This gives further support for the idea that the AP-2 molecules are in a bimodal distribution. To ensure that this labelling pattern was not an artifact due to the methanol/acetone fixation or the AC1-M11 mAb in particular we repeated the same experiments using AP-6 mAb. The fluorescent labelling patterns for either mAb were similar at the cellular level (compare Fig. 2A,C), and at higher magnifications (compare Fig. 4A,B). Intensity profiles for the AP6 mAb labelling are similar to AC1-M11 mAb (compare Fig. 4E,F) and also show evidence of two populations of AP-2 (Fig. 4E). From the ICS analysis similar cluster densities were obtained using either AC1-M11 mAb and methanol/acetone fixation, or AP6 mAb and paraformaldehyde fixation (data not shown). Although the AP6 mAb does not bind as well following methanol/acetone fixation, as it does following paraformaldehyde fixation and permeabilization, we still obtained similar cluster densities using either fixation method (data not shown) demonstrating that the results we obtain are not an artifact of the fixation protocol.

After hypertonic treatment there must be a redistribution of the AP-2 molecules which results in a decrease in the CD value. There are still some intense peaks, but more of the broader intensity peaks are found just above the baseline (Fig. 4G). It also appears as if the baseline intensity in these images has increased slightly, suggesting that there may be a population of very small AP-2 clusters present after the treatment.

It should be noted that peaks with a maximum intensity of up to 90 were observed, but only on untreated cells. The intensity peaks in either case, including the low intensity peaks, are well above what would be expected from non-specific secondary antibody binding (compare Fig. 4E,F with Fig. 4H).

In order to determine the relative size of the AP-2 aggregates
before and after hypertonic treatment the ratio of the peak intensities was examined by looking at the peak intensities from four images prior to and after hypertonic treatment (images similar to those found in Fig. 4B,C). The clusters in each image were counted from the raw data, and then the number of peaks in each intensity range of 5-10 units were also counted from threshold images. It should be noted that the number of peaks in the lowest intensity range was calculated by difference from the lowest intensity threshold image and the number of clusters counted from the raw data. The average intensity (for over 700 intensity peaks for both treated and untreated cells) was then calculated for the peaks and an intensity ratio of 1:0.75 was determined for the untreated versus hypertonically treated cells. Thus, on average peaks in the hypertonically treated cells are only 75% as intense as in untreated cells, or the AP-2 aggregates are only 75% as large after hypertonic treatment.

Potential artifacts within the ICS data
It is possible that some of the variability in the AP-2 distribution could be due to variable antibody accessibility. If this was the case we would expect to see an increase in accessibility after dispersion of AP-2 with the hypertonic treatment, but the average intensity of fluorescent labelling does not change (<i>_i_ = 2.4 vs 2.3 prior to treatment) so we do not feel accessibility is a major factor. We would also expect that different antibodies and different fixation protocols would lead to variations in accessibility, however, we obtain comparable results with either AC1-M11 or AP.6 mAb and with AP.6 mAb with either paraformaldehyde or methanol/acetone fixation.

Fig. 4. Representative zoom 10 images of square areas of the cell membrane immunofluorescently labelled for AP-2 with AP.6 mAb (A) or AC1-M11 mAb (B,C,D) as described in Materials and Methods, either in untreated (A,B), hypertonically treated cells (C), or in cells labelled with no primary AC1-M11 mAb present (D). The bright spots (large AP-2 aggregates) in A and B are believed to be clathrin coated pit associated AP-2 aggregates. In C these large aggregates may be associated with residual clathrin lattices. Intensity spots in these and other images were counted in order to determine the density of AP-2 aggregates. Similar, large aggregates are not visible in cells labelled for non-specific secondary antibody binding (D). The distribution of peak intensities with and without hypertonic treatment was determined from these among other images using image thresholding. The intensity ratio was determined to be 1:0.75, or the spots in C are, on average, 75% as intense as the spots in B. Intensity profiles from one line (shown as a white line) of the 512x512 images A, B, C and D are shown in E, F, G and H, respectively. Intensity values were corrected for non-specific binding by subtracting the average intensity value calculated from 25 images collected on cells which were labelled as in Materials and Methods but without the primary AC1-M11 mAb labelling step. Intensity values were also corrected for ‘dark current’ from the photomultiplier tube by subtracting the average intensity from an image collected with the sample shutter closed. Intensity peaks as high as 90 were observed but only for untreated cells. There were occasionally intense peaks observed in the non-specifically labelled cells, but these are corrected for in the calculations. Bar, 5 μm (4.7 μm in A, which is slightly smaller than B,C,D. See note in Fig. 2 caption).
order to clarify the steps involved in the endocytosis process. obviously important to understand all of the characteristics of in the cytosol, to form endosomes (Beck et al., 1992). It is even evidence Sosa et al., 1993; Boll et al., 1995; Nesterov et al., 1995; Sorkin and Spiess, 1991; Chang et al., 1993; Sorkin and Carpenter, 1993; 1990; Prasad and Keen, 1991; Schroder and Ungewickell, 1991; Keen, 1987; Keen for binding dynamin, a protein which is involved in the budding Peeler et al., 1993; Goodman and Keen, 1995). It is also known clathrin coated pit mediated endocytosis. It functions in binding and recruiting clathrin to the membrane (Pearse, 1978; Zaremba and Keen, 1983; Pearse and Robinson, 1984; Keen, 1987; Keen and Beck, 1989; Ahle and Ungewickell, 1989; Mahaffey et al., 1990; Prasad and Keen, 1991; Schroder and Ungewickell, 1991; Peeler et al., 1993; Goodman and Keen, 1995). It is also known to bind to dynamin, a protein which is involved in the budding of highly invaginated coated pits from the plasma membrane to form coated vesicles (Wang et al., 1995). AP-2 also binds to the internalization signals of a number of membrane receptors (or their cytoplasmic tail) drawing them into coated pits and into the endocytosis process (Pearse, 1988; Glickman et al., 1989; Beltzer and Spiess, 1991; Chang et al., 1993; Sorkin and Carpenter, 1993; Sosa et al., 1993; Boll et al., 1995; Nesterov et al., 1995; Sorkin et al., 1995, 1996; Vincent et al., 1997). There is even evidence that AP-2 may be involved in the aggregation of coated vesicles, in the cytosol, to form endosomes (Beck et al., 1992). It is obviously important to understand all of the characteristics of such a pivotal protein including its distribution at the membrane, in order to clarify the steps involved in the endocytosis process.

**DISCUSSION**

It has been well established that AP-2 is a critical component in clathrin coated pit mediated endocytosis. It functions in binding and recruiting clathrin to the membrane (Pearse, 1978; Zaremba and Keen, 1983; Pearse and Robinson, 1984; Keen, 1987; Keen and Beck, 1989; Ahle and Ungewickell, 1989; Mahaffey et al., 1990; Prasad and Keen, 1991; Schroder and Ungewickell, 1991; Peeler et al., 1993; Goodman and Keen, 1995). It is also known to bind to dynamin, a protein which is involved in the budding of highly invaginated coated pits from the plasma membrane to form coated vesicles (Wang et al., 1995). AP-2 also binds to the internalization signals of a number of membrane receptors (or their cytoplasmic tail) drawing them into coated pits and into the endocytosis process (Pearse, 1988; Glickman et al., 1989; Beltzer and Spiess, 1991; Chang et al., 1993; Sorkin and Carpenter, 1993; Sosa et al., 1993; Boll et al., 1995; Nesterov et al., 1995; Sorkin et al., 1995, 1996; Vincent et al., 1997). There is even evidence that AP-2 may be involved in the aggregation of coated vesicles, in the cytosol, to form endosomes (Beck et al., 1992). It is obviously important to understand all of the characteristics of such a pivotal protein including its distribution at the membrane, in order to clarify the steps involved in the endocytosis process.

If we use Equation 6 and we assume that the large visible AP-2 clusters are coated pits we can utilize the following equation in order to determine the underlying bimodal AP-2 distribution (Petersen, 1986):

$$g(0,0) = \frac{1}{N_m^2} (N_m \mu_c + N_f \mu_f), \quad (8)$$

where $g(0,0)$ is the zero lag amplitude of the autocorrelation function, $N_m$, $N_f$ are the average number of monomeric AP-2 proteins, coated pits, and ‘free’ protein aggregates, respectively, per μm² of cell membrane. Also, $\mu_c$ and $\mu_f$ are the mean number of proteins per coated pit or ‘free’ protein aggregate, respectively. From counting intensity spots we determined that there are on average 0.28 coated pits per μm² of cell membrane which we assign as the value of $N_f$. Heuser and Anderson (1989) estimate that there are an average of ~120 clathrin triskelion per coated pit, and studies on isolated coated vesicles give an estimate of 1 AP-2 bound per 2 clathrin triskelion (Vigers et al., 1986; Keen, 1987; Heuser and Keen, 1988; Kirchhausen, 1993). Taken together these estimates give a value for $\mu_f$ of ~60 AP-2 proteins bound per coated pit. Using these two values and estimates of the values of $N_m$ and $\mu_f$ we can determine $N_f$ from Equation 8. The values determined for these parameters must also satisfy the fact that the total number of monomers must be present in either coated pits or ‘free’ aggregates, i.e. $N_m=N_f \mu_c+N_f \mu_f$. The only solution which satisfies both equations and give ‘reasonable’ values for all three parameters yields: $N_m=-50; \mu_f=20$; and $N_f=-1.5$. This means there are 5 smaller aggregates containing ~20 AP-2 molecules for each coated pit containing ~60 AP-2 molecules. These smaller aggregates are about 1/3 of the average size of the coated pit aggregates, which would in turn correspond to an average peak intensity of less than 8 units, which would be difficult to see visually above the background intensity in the images (Fig. 4B,F). These results clearly demonstrate that the information we can extract from ICS data far surpasses the quantitative abilities of any other analytical cell membrane techniques to date.

It is possible that there could also be contributions from the
soluble cytosolic fraction of AP-2 and AP-2 bound to coated vesicles. However, these contributions must not be large because if the cells are permeabilized with 0.04% saponin and the cytosol is washed away before fixation and labelling we see essentially the same labelling pattern and comparable ICS data (data not shown). Furthermore, the region of the cell we work on is very flat so the volume of cytosol within the images should be small. It has been shown that coated vesicles uncoat fairly quickly after internalization (Hansen et al., 1993) and as a consequence presumably lose their AP-2 content. Finally, Robinson (1987) demonstrated that AP-2 is found predominantly at the plasma membrane.

It was first hypothesised by Unanue et al., (1981) that AP-2 acts as a nucleation site for coated pit assembly. Our ICS data show that AP-2 is in a bimodal distribution between coated pits and smaller aggregates. It is tempting to speculate that the smaller AP-2 aggregates (of ~20 AP-2 molecules) serve to recruit membrane receptors and then clathrin, thereby, acting as clathrin coated pit nucleation sites (Fig. 5).

This AP-2 distribution, or labelling pattern, is not an artifact of the AC1-M11 mAb or the fixation method, because we see similar labelling patterns and obtain comparable ICS data (not shown), using either the AC1-M11 mAb (methanol/acetone fixation) or the AP6 mAb (paraformaldehyde fixation, compare Fig. 2A,C and Fig. 4A,B). We also see similar results using the AP6 mAb following either paraformaldehyde fixation and permeabilization or methanol/acetone fixation (data not shown). Finally, we obtain comparable results with a number of cell lines including NIH 3T3 cells, Ag1523 fibroblasts and L6 myoblasts (data not shown).

The hypertonic data emphasize the importance of using ICS in order to accurately quantify protein distributions. Visually there doesn’t appear to be any difference in the distribution of AP-2 with or without hypertonic treatment (compare Fig. 2A,D), and others have concluded from immunofluorescence data that there is no difference (Hansen et al., 1993). However, quantitative analysis of the ICS data reveals that the AP-2 aggregate distribution is different after hypertonic treatment (CD = 3.7 versus 1.4 prior to hypertonic treatment). Since the measured average intensities are constant (\( \sigma^2 = 2.4 \) vs 2.3 prior to treatment) it is reasonable to assume that the hypertonic treatment does not alter the total amount of membrane associated AP-2. Hence, this increase in the number of AP-2 aggregates must be due to a break up of the coated pit associated aggregates and/or of the smaller AP-2 aggregates. Counting AP-2 clusters from the images directly shows that there are about the same number of large AP-2 clusters (0.30 clusters/\( \mu m^2 \) compared to 0.28 prior to treatment), but from a closer analysis of the peak intensities we determine that the large AP-2 clusters are 75% smaller after the treatment, consisting of ~45 rather than ~60 AP-2 molecules. It is possible that some of these AP-2 aggregates are associated with residual clathrin lattices which have been shown to be present after hypertonic treatment (Heuser and Anderson, 1989). The other 25% of the AP-2 must dissociate from the coated pit aggregates to form smaller AP-2 aggregates. This observation would explain why the difference in the number of AP-2 aggregates after hypertonic treatment is difficult to see qualitatively because there are the same number of large aggregates, they just contain 25% fewer molecules.

The AP-2 distribution after hypertonic treatment can be analysed more closely using Equation 6. From the data presented above \( N_c \) remains essentially unchanged at a value of 0.30, but \( \mu_c \) is only ~45 AP-2 molecules per aggregate (previously coated pit associated aggregates). As argued above, the total amount of membrane associated AP-2 is unchanged, so \( N_m \) remains at a value of ~50 AP-2 molecules per \( \mu m^2 \) of cell membrane. With these values of \( N_c, \mu_c, \) and \( N_m \) the only pair of values \( \sigma_f \) and \( \sigma_m \) (Equation 4) which also satisfies the fact that \( N_m = N_c \mu_c + \frac{1}{2} \sigma_f \mu_f \) (as explained above), is \( \sigma_f = 17 \) and \( \mu_f = 2 \). Therefore, we conclude that the smaller aggregates dissociate to form AP-2 dimers. As discussed in Materials and Methods (Data Interpretation), the variance term for a distribution with a small mean can introduce large errors if it is omitted from Equation 5. The CD value was recalculated including the \( \sigma_f^2 \) term and it would only contribute ~3% to the CD value. Therefore, the assumption that the variance term could be omitted simplifying Equation 5 to Equation 6 appears reasonable even though our second AP-2 population has a small mean.

To date, there is no accurate technique with which to determine the amount of membrane associated AP-2. From our ICS data we determined that, on average, there are ~50 AP-2 molecules per \( \mu m^2 \) of cell membrane, \( N_m \). From measurements of 115 individual adhering CV-1 cells we measured an average cell area of 13,000 \( \mu m^2 \). Taken together we calculate ~6\times10^5 membrane associated AP-2 molecules for each large, flat CV-1 cell. This can be broken down further to ~2\times10^5 AP-2 molecules associated with coated pits (0.28 coated pits/\( \mu m^2 \times ~60 \) AP-2 molecules/coated pit \( \times 13,000 \mu m^2/\)cell), or 33% of the membrane associated AP-2 is within coated pit structures. The remaining ~4\times10^5 AP-2 molecules are found in smaller aggregates of ~20 AP-2 molecules. This correlates well with estimates of the total cellular AP-2 concentration of approximately 5\times10^5 AP-2 molecules for each NIH 3T3 cell, which are smaller than CV-1 cells (Sorkin et al., 1995).

The data presented here provide enough information to propose a model for the AP-2 distribution at the cell membrane and changes in this distribution resulting from hypertonic treatment of the cells. There are two populations of AP-2 in untreated cells, one population in large aggregates of ~60 AP-2 which are associated with coated pits (Fig. 5). The other population is made up of smaller AP-2 aggregates of ~20 AP-2 molecules (Fig. 5). We can then speculate that it is these smaller aggregates which first bind to membrane receptors and then recruit clathrin from the cytoplasmic pool acting as coated pit nucleation sites. After hypertonic treatment 25% of the AP-2 previously associated with coated pits dissociates into small aggregates which are dimeric on average (Fig. 5). The remaining ~45 AP-2 molecules remain associated, possibly through AP-2 self association (Beck and Keen, 1991; Chang et al., 1993), and/or association with residual clathrin coats (Heuser and Andersen, 1989; Fig. 5). It is possible that the AP-2 molecules around the edges of the large aggregates are no longer held within the aggregate as tightly because of the lack of clathrin polymerization. The hypertonic treatment may also reduce the strength of AP-2 self-aggregation causing the smaller aggregates to break up forming dimeric AP-2 complexes (Fig. 5). It has been shown that upon hypertonic treatment LDL receptors dissociate from coated pit structures and are found dispersed about the membrane as dimers (Heuser and Anderson, 1989). It is possible that the remaining large
AP-2 aggregates loose their affinity for certain membrane receptors, but that the more abundant dissociated dimeric AP-2 can still associate with the receptors. Overall, hypertonic treatment corresponds to a shift from 33% of the AP-2 being associated with coated pits and 66% associated in potential coated pit nucleation sites of ~20 AP-2 molecules to 19% of the AP-2 molecules in large aggregates of ~45 AP-2 molecules and the remaining 81% of the AP-2 molecules as dimers. Although the idea of AP-2 dimers is attractive it is possible that AP-2 dissociates to a monomeric form rather than dimeric, however, this would also require an increase in the total number of AP-2 proteins and changes in the ‘normal’ AP-2 distribution.*

Many researchers have determined the number of coated pits in various different cell lines. It has been shown that clathrin coated pits make up 1-2% of the cell membrane surface (Anderson et al., 1977a,b). Here we determined that there are ~0.30 coated pits per μm² of cell membrane. If we assume coated pits are circular with an average diameter of 0.20 μm then this would correspond to ~0.9% of the cell membrane being covered by coated pits, agreeing well with previous estimates.

It is interesting to note that change in temperature has little or no effect on the AP-2 distribution in the membrane (Figs 2, 3). There is evidence which shows that PDGF-β receptor (Wiseman, 1995; Wiseman et al., 1997) and internalization competent influenza virus HA mutants (Fire et al., 1997) both undergo some sort of dispersion process as the temperature of the cell system is decreased to 4°C. This suggests that the AP-2/receptor interaction is temperature sensitive, with a weaker binding at low temperatures. This observation is also supported by co-immunoprecipitation results which demonstrate a weaker association between AP-2 and internalization competent HA mutants at 4°C (Fire et al., 1997). Additionally, co-immunoprecipitation studies have shown even in the presence of EGF, some heating to 37°C is needed for the growth factor induced association of the EGF receptor and AP-2 (Sorkin and Carpenter, 1993; Nesterov et al., 1995).

In summary, using an ICS analysis we are the first group to have quantified the amount of AP-2 protein at the cell membrane, and characterized the AP-2 distribution between coated pits and other non-coated pit associated aggregates. From a detailed analysis of the data we were able to propose a model for the AP-2 distribution at the cell membrane, and also show how hypertonic treatment of the cells changes this distribution.

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REFERENCES


*If the population of AP-2 was monomeric this would mean that we had ~80 rather than ~50 AP-2 proteins per μm². This would correspond to 230 AP-2 monomers for each aggregate of ~45 AP-2 proteins. It would follow that under ‘normal’ conditions the smaller AP-2 aggregates would contain ~10 rather than ~20 AP-2 proteins. Our model would have to be modified having 7 aggregates of ~10 AP-2 proteins for each coated pit of ~60 AP-2 proteins. Finally, this would increase the number of AP-2 proteins per cell by 60%. Interpreting the smaller population as AP-2 trimers is inconsistent with our ICS data. If the AP-2 was trimeric this would reduce the number of AP-2 proteins to ~36 AP-2 proteins per μm². Under ‘normal’ conditions this would force the smaller aggregates to be comparable in size to the coated pits so we would expect to be able to see them visually, and this is not the case.


