Redistribution of Clathrin-coated Vesicle Adaptor Complexes during Adipocytic Differentiation of 3T3-L1 Cells

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Abstract. Mechanisms for intracellular retention of proteins are induced during adipocytic differentiation of 3T3-L1 cells. To investigate the potential role of clathrin lattices in these retention processes, we performed a morphological and biochemical analysis of coated vesicle components in 3T3-L1 cells. Optical sectioning and image restoration revealed a marked increase in the staining of clathrin and β adaptins in the perinuclear region of cells with differentiation. In addition, predominance of β (subunit of the AP-2, plasma membrane adaptor) over β' (subunit of the AP-1, Golgi adaptor) adaptin was observed in immunoblots of clathrin-coated vesicles purified from non-differentiated fibroblasts, and this ratio was reversed in coated vesicles purified from differentiated adipocytes. These results indicate that the relative abundance of TGN-derived clathrin lattices increases markedly during adipocytic differentiation. Subcellular fractionation indicated that cytosolic AP-1 and AP-2 adaptors comprised approximately 70% of the total cellular adaptor pool. Interestingly, neither the concentration nor the relative ratio of cytosolic AP-1 to AP-2 adaptors increased significantly during differentiation. These data suggest that the increase in TGN-derived lattices results from differentiation-induced mechanisms for enhanced assembly or stabilization of adaptors on Golgi membranes. Interestingly, double-immunofluorescence microscopy also revealed that whereas extensive colocalization between clathrin and beta adaptins occurred both in fibroblasts and adipocytes, structures stained only with anti-adaptin antibody could be detected. Taken together these results suggest that membranes coated with adaptors, but not clathrin, can exist in these cells.

3T3-L1 fibroblasts can be induced to differentiate into an adipocytic phenotype by the addition of dexamethasone, methylisobuylaxanthine, and insulin (Rosen et al., 1979; Green and Kehinde, 1975). The differentiation process is manifested morphologically by cell rounding and the accumulation of large lipid droplets, and functionally by the expression of many of the biochemical features of primary adipose tissue cells (Green and Kehinde, 1973, 1976). These cells have been useful in understanding the molecular basis of adipocyte functions.

One specific feature of adipose cells is that they respond to insulin with an acute stimulation of glucose transport (Resh, 1982; Karlsson, 1979). Key elements in the development of this response are the induction of high-affinity insulin receptors, and of the specific glucose transporter isoform, GLUT-4 (Rosen et al., 1979; Karlsson et al., 1979; Reed et al., 1977; Kaestner et al., 1989). Also important in the generation of insulin sensitivity is a gradual reduction in basal glucose transport activity (Yang et al., 1992). Both the down regulation of basal transport activity, and the acute stimulation of transport by insulin, involve mechanisms that control the cellular sorting of glucose transporters. For example, the decrease in basal glucose uptake is partially due to a gradual decrease in the proportion of cellular GLUT-1 distributed to the cell surface as cells reach confluence and progress through differentiation (Yang et al., 1992). In addition, GLUT-4 induced during differentiation is retained in a principally intracellular location. In response to insulin stimulation, both transporter isoforms are rapidly redistributed to the cell surface (Calderhead et al., 1990; Clancy and Czech, 1990; Holman et al., 1990). These observations indicate that a key feature in the development of insulin responsiveness during adipocytic differentiation of 3T3-L1 fibroblasts is the development of mechanisms that insure the intracellular sequestration of glucose transporters.

It has recently been proposed that clathrin lattices play an important role in the intracellular sequestration or retention of specific membrane proteins (Seeger and Payne, 1992*a*,*b*). This hypothesis is based on the finding that clathrin-deficient yeast cells display an increase in the cell surface concentration of proteins which are ordinarily found in the Golgi apparatus. In animal cells, this hypothesis is supported by the finding that the fungal toxin Brefeldin A (BFA)[1], which selectively disrupts clathrin lattices derived from the TGN, causes a redistribution of IGF-II/mannose-6-phosphate re-

1. *Abbreviations used in this paper*: AP, assembly polypeptides; BFA, Brefeldin A; CCD, charged-coupled device; PM, plasma membrane.

ceptors, which are concentrated in TGN-derived clathrin-coated vesicles, to the cell surface (Damke et al., 1991; Wood et al., 1991). We have recently found that GLUT-4 can be found in clathrin-coated vesicles isolated from adipose cells, and that BFA increases the concentration of transporters at the cell surface. Thus, the association of glucose transporters with clathrin lattices may be an important element in securing their intracellular retention. If so, changes in the ability of clathrin lattices to mediate intracellular retention of transporters and other proteins may occur during differentiation of 3T3-L1 cells. In this paper, we have begun to investigate this hypothesis by analyzing whether the process of differentiation of 3T3-L1 fibroblasts into the adipocyte phenotype is accompanied by changes in the functional properties of elements of the clathrin-coated vesicle pathway.

Two types of clathrin-coated vesicles exist in animal cells, one type derived from the plasma membrane (PM), and the second derived from the intracellular TGN. Whereas the former are involved in the endocytosis and targeting of proteins from the cell surface to intracellular compartments, the latter are thought to be involved in the targeting of lysosomal enzymes to prelysosomal compartments and of secretory proteins into specialized granules (Geuze et al., 1985; Tooze and Tooze, 1986). Both types of coated vesicles are composed of two major protein complexes: clathrin, which is thought to play a mechanical role in the budding process, and assembly polypeptides (APs) or adaptors (Pearse and Crowther, 1987). Two types of APs (AP-1 and AP-2) are selectively localized to the TGN or the PM, respectively (Ahle et al., 1988; Robinson and Pearse, 1986), thus revealing the existence of structural differences between coated vesicles derived from these compartments. In addition, functional differences exist between the clathrin cycles at the PM and TGN, as evidenced by the selective effects of BFA to disrupt TGN- but not PM-derived lattices (Robinson and Kreis, 1992; Wong and Brodsky, 1992).

Both the AP-1 and AP-2 assembly polypeptides are heterotetrameric complexes, composed of two polypeptides of 100–110 kD, one medium polypeptide of 47 or 50 kD and one small polypeptide of 17 or 19 kD (Ahle et al., 1988; Keen, 1987). The large polypeptides of the AP-1 assembly complex are known as γ and β', whereas those of the AP-2 complex are known as α and β. Whereas β and β' appear to be structurally and immunologically related, α and γ display limited sequence identity (Robinson, 1990). In this study, we have used mAb 100.1 (Ahle et al., 1988) which recognizes the β and β' subunits of AP-2 and AP-1, respectively, to measure the expressed levels and assembly states of these complexes during differentiation of 3T3-L1 cells. Our results suggest that the differentiation process is accompanied by pronounced changes in the cellular distribution of clathrin-coated vesicle components.

Materials and Methods

Cells

3T3-L1 fibroblasts (American Type Culture Collection) were seeded and fed every 2 d in DME supplemented with 0.75 mg/ml glutamine, nonessential aminoacids (GIBCO-BRL, Gaithersburg, MD), 50 U/ml penicillin, 50 μg/ml streptomycin and 10% FCS (Hyclone Labs, Logan, UT), and grown under 10% CO_2. At confluence, differentiation was started by addition of culture medium containing 0.25 μM dexamethasone (Sigma Immunochemicals, St. Louis, MO), 0.5 mM isobutyl methylxanthine (Sigma Immunochemicals), and 1 μM insulin. After 48 h, this was replaced with fresh medium containing 1 μM insulin, which was removed after 48 h. Lipid droplets were observed in 90–95% of the cells 4 d after initiating differentiation.

Preparation of Clathrin-coated Vesicles

Coated vesicles were isolated essentially by the differential centrifugation procedure descried by Campbell et al. (1984). We adapted this method to obtain a more rapid preparation of coated vesicles from smaller amounts of tissue (Corvera and Capocasale, 1990). Three 150 mm plates of either 3T3-L1 fibroblasts or adipocytes were used per condition. All the procedures were performed at 0–4°C in a buffer (MES buffer) composed of 100 mM 2-(N-morpholino) ethanesulfonic acid, 1 mM EGTA, 0.5 mM magnesium chloride, 0.02% sodium azide, 50 mM sodium fluoride, 100 μM sodium vanadate, 1 mM 1,10-phenanthroline, 10 μg/ml leupeptin, and 1 mM benzamidine adjusted to pH 6.5. Cell monolayers were scraped into 2 ml of the buffer and broken by the addition of 0.1% Triton X-100 and vortexing. The homogenates were placed in 1.5 ml microcentrifuge polyallomer tubes (Beckman Instruments, Palo Alto, CA) and centrifuged for 12 min at 15,600 *g* in a TLA 100.3 rotor (Beckman Instruments). The supernatant was removed, and centrifuged for 21 min at 36,350 *g*, yielding a supernatant (cytosol), and a pellet. The pellet was resuspended in 200 μl of buffer, and an equal volume of a solution composed of 12.5% (wt/vol) sucrose and 12.5% (wt/vol) Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) was added. After vortexing, the suspension was centrifuged for 12 min at 36,350 *g*. The supernatant was diluted with 5 vol of ice cold buffer, and centrifuged for 21 min at 72,000 *g*. The pellet from this centrifugation contained 70–80% pure clathrin-coated vesicles.

Preparation of Assembled and Unassembled Fractions and Analysis on Sucrose Gradients

The separation of the pools of assembled and unassembled clathrin was performed essentially as described by Goud et al. (1985). Cells were grown in 60 mm dishes, and harvested by scraping with a rubber policeman into 500 μl of MES buffer and broken by passing through a 26 g needle for 10 times, or by adding Triton X-100 at a final concentration of 0.1%. Similar results were obtained by both procedures. The extracts were vortexed and then centrifuged at 350,000 *g* for 15 min in a table top ultracentrifuge (TJ-100; Beckman Instruments). The supernatants (unassembled fraction) were collected, and the cell pellets (assembled fraction) were resuspended in 500 μl of buffer. For sucrose gradient analysis, unassembled fractions were prepared from 100 mm dishes. The unassembled fraction was concentrated by centrifugation on Centricon filters to approximately one third of the original volume, and analyzed by sucrose gradient centrifugation basically as described (Ahle and Ungewickell, 1989). Approximately 0.5 mg of protein (300 μl) from the concentrated unassembled fraction were loaded on a 5 ml linear 5–20% sucrose gradient made in a buffer composed of 20 mM TAPS, pH 9.0, 2.5 mM $MgCl_2$, 2.5 mM EGTA, 50 mM sodium fluoride, 100 μM sodium vanadate, 1 mM 1-10 phenanthroline, 1 mM benzamidine, 1 mM PMSF and 10 μg/ml leupeptin. Samples were centrifuged for 18 h at 42,000 rpm in a rotor (SW50.1; Beckman Instruments) at 4°C. Fractions (200 μl) were collected from the top of the tube, and analyzed by electrophoresis on 7.5% polyacrylamide gels and immunoblotting. Gradients were calibrated using aldolase (Sigma Immunochemicals) and BSA (Intergen, Purchase, NY).

Immunoblotting

Serial dilutions of each fraction (containing ~10–100 μg of protein from crude fractions, and 1–10 μg of protein from coated vesicles) were electrophoresed on 7.5% polyacrylamide gels, and electrophoretically transferred onto nitrocellulose paper. The blots were probed with anti-clathrin heavy chain mAb Chc5.9 (ICN Radiochemicals, Irvine, CA), monoclonal antibody 100.1, or mAb 100.2 at a final concentration of 2.5, 2, and 5 μg/ml, respectively. The primary antibodies were detected with polyclonal goat anti-mouse immunoglobulins coupled to horseradish peroxidase, and enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). The intensity of the bands was quantified using an Ultroscan XL laser densitometer (LKB Instruments, Gaithersburg, MD). Analysis of serial dilutions of each sample insured that the signals obtained from each fraction were within the same linear range.

Immunofluorescence

Cells were grown and differentiated on glass coverslips, washed rapidly twice with cold 2 ml PBS, and fixed by immersion in −20°C methanol for 6 min. The methanol was removed, and coverslips briefly air dried, and blocked in PBS containing 1% FCS. After 30 min, coverslips were transferred into buffer containing mAb Chc5.9 (ICN Radiochemicals) or mAb 100.1 at a final concentration of 10 μg/ml. Primary antibodies were detected using a rhodamine-coupled goat anti-mouse IgM (Tago Inc., Burlingame, CA), and a FITC-conjugated anti-mouse IgG (Tago Inc.). To simultaneously label cellular compartments containing terminal N-acetylglucosamine, cells were incubated with 5 μg/ml of FITC-coupled Lens-culinaris lectin (E-Y Laboratories, San Mateo, CA) in PBS containing 1% FCS for 30 min at room temperature. Samples were visualized on a Zeiss IM-35 microscope (Carl Zeiss, Oberkochen, Germany), using a Nikon Apo 60/1.4 oil immersion lens (Nikon Inc., Garden City, NY), as indicated. For image deconvolution, 35 serial two-dimensional images were recorded at 0.2-μm intervals using a thermoelectrically cooled charged-coupled device (CCD) camera (Photometrics Ltd., Tucson, AZ). Each image was corrected for lamp intensity variations and photobleaching. Blurring of fluorescence from regions above and below the plane of focus was reversed using an iterative constrained deconvolution algorithm based on the theory of ill-posed problems (Carrington et al., 1990). The series of optical sections were then analyzed individually or summed to provide clear two-dimensional images of the distribution of fluorescence within the cell.

Results

The typical features of the cells used in the studies described in this paper are illustrated in Fig. 1. It can be seen that the process of differentiation of 3T3-L1 cells involves pronounced rounding and accumulation of large lipid droplets, which can be distinguished by their refractive nature. Maximal accumulation of lipid is observed between 10 and 15 d after initiation of the differentiation process. Only preparations in which over 90% of the cells exhibited this morphology were used in the studies described below.

Localization of Clathrin and β Adaptins Analyzed by Optical Sectioning and Image Reconstruction

We sought to examine whether the process of differentiation of 3T3-L1 cells is accompanied by changes in the dynamics of the clathrin cycle at the PM or TGN. However, comparative immunofluorescence analysis of 3T3-L1 cells is hampered by the pronounced differences in general morphology of nondifferentiated and differentiated cells. The latter are present in a tightly packed monolayer, and are much rounder and thicker than fibroblasts due to the accumulation of large lipid droplets (Fig. 1). To obtain images that would more accurately represent the distribution of clathrin in different regions of these cells we employed optical sectioning microscopy and image reconstruction. Staining of undifferentiated 3T3-L1 fibroblasts with mAb Chc5.9 resulted in a punctate staining pattern which is typical of clathrin-coated pits and vesicles. The punctate pattern was homogeneously distributed throughout the cell, with an area of concentration in a juxtanuclear region which corresponds to the TGN (Fig. 2, *top left*). This area comprised a relatively small proportion of the total staining, the majority of which was observed in more peripheral regions, and probably represents plasma membrane-derived clathrin-coated pits and vesicles (Ahle et al., 1988; Robinson and Pearse, 1986). The staining pattern observed after 6 d of differentiation differed from that observed in fibroblasts in that a larger proportion of the total clathrin signal was detected in the perinuclear region, rather than in the peripheral regions of the cell which surround the emerging lipid droplets (not shown). The intensity of clathrin in the perinuclear region increased, becoming maximal after 10–14 d of differentiation (Fig. 2, *top right*).

To determine whether the observed changes in juxtanuclear staining intensity observed with anti-clathrin antibody were not due to general changes in cell morphology, we double-stained cells with FITC-coupled Lens culinaris lectin, which stains all cellular compartments containing terminal N-acetylglucosamine. Golgi compartments, which are enriched in N-acetylglucosamine, are visualized in the juxtanuclear region of the cell (Tisdale et al., 1992). Whereas staining of the perinuclear region with anti-clathrin antibody was much more intense in differentiated cells (Fig. 2, compare *top right* and *left*), Lens culinaris staining in the juxtanuclear region of cells was similar before and after differentiation (Fig. 2, compare *bottom right* and *left*). These

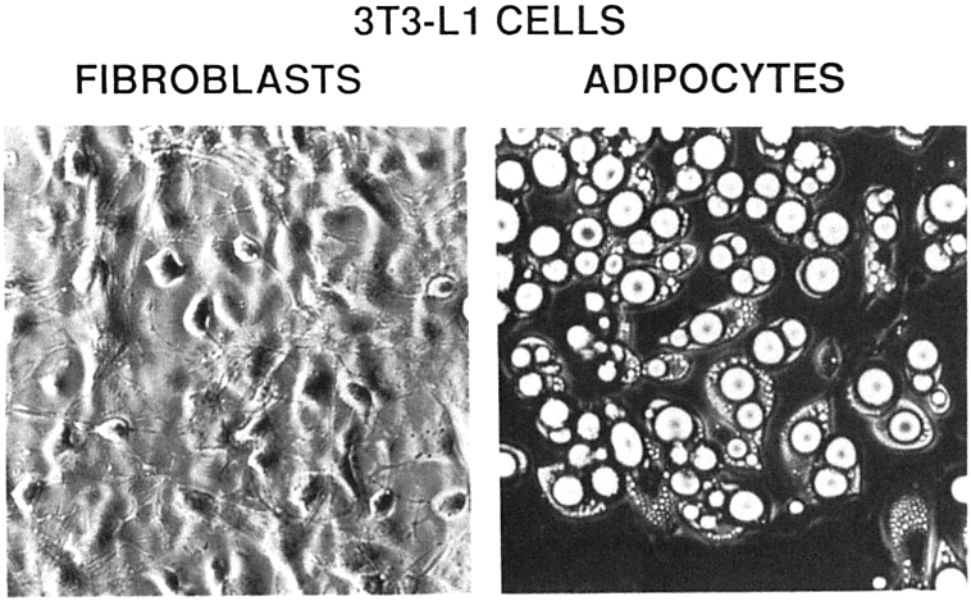

Figure 1. Phase contrast micrograph of 3T3-L1 cells before and after differentiation. 3T3-L1 cells were grown to confluence on glass coverslips and differentiated for 10 d. Cells were fixed and observed by phase contrast microscopy.

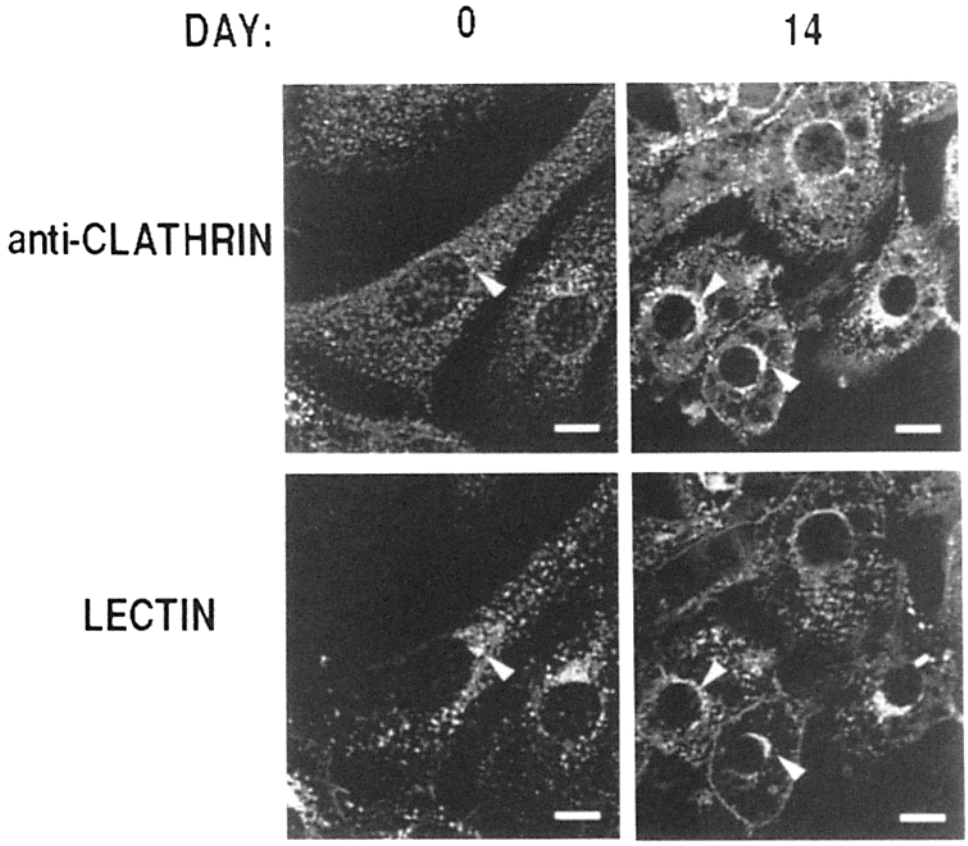

Figure 2. Analysis of the clathrin heavy chain during differentiation. Cells were fixed after 0 (*left*) or 14 (*right*) days of differentiation, and stained with anti-clathrin mAb Chc5.9 (*top*) and Lens culinaris lectin (*bottom*). These images comprise an individual optical section from the middle of the cell, where the most intense signal for clathrin was observed both before and after differentiation. The staining with Lens culinaris lectin of the same optical section is shown. Arrowheads indicate the region of most intense staining for clathrin, localized in the juxtanuclear region, and the corresponding stain with Lens culinaris lectin. Bar, 10 μm.

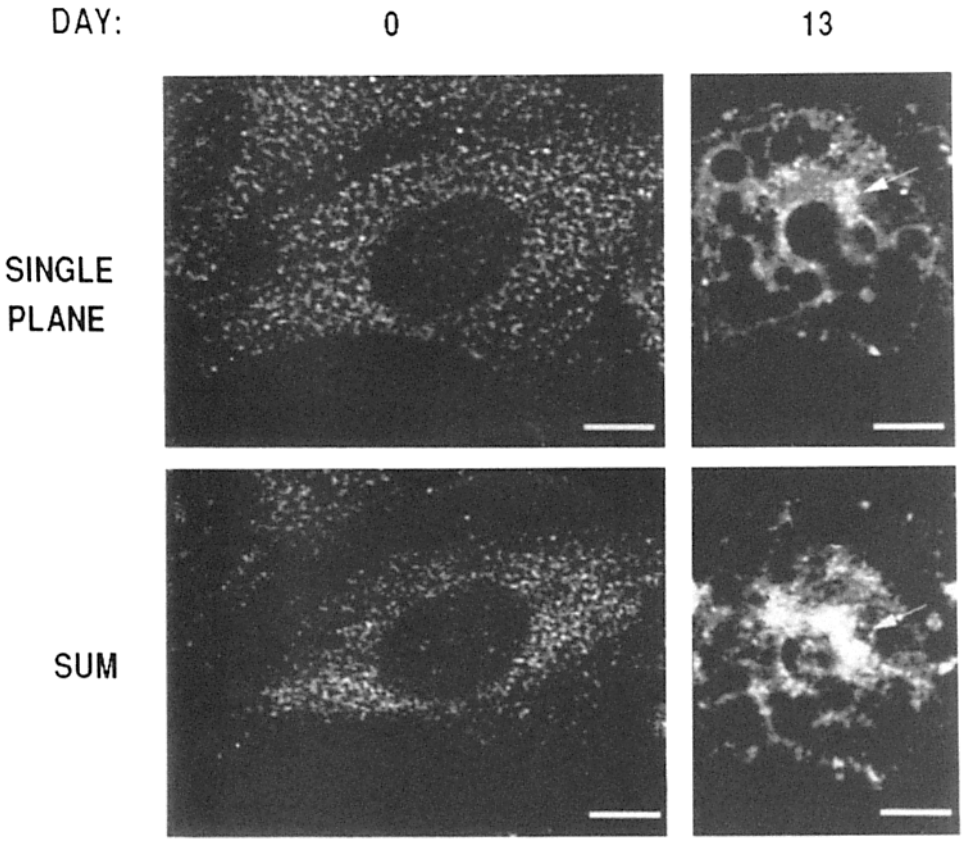

Figure 3. Analysis of β adaptins during differentiation. Cells were fixed after 0 (*left*) or 13 (*right*) d of differentiation, and stained with anti-β-adaptin mAb 100.1. The images shown in the top panels (*SINGLE PLANE*), comprise an individual optical section from the middle of the cell, whereas the images shown in the bottom panels (*SUM*) comprise the summed information from all optical sections throughout the cell. Arrows point out the marked perinuclear staining observed in differentiated cells, which was virtually absent before differentiation. Bar, 10 μm.

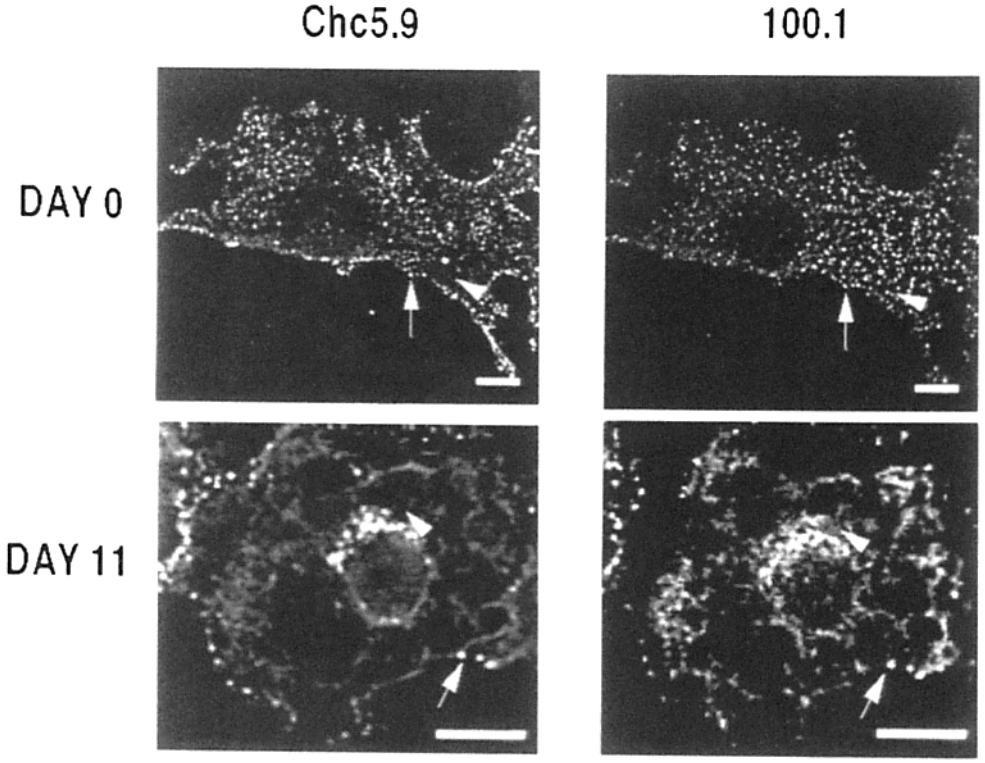

Figure 4. Double staining for clathrin and β adaptins during differentiation. Cells were fixed after 0 (*top*) or 11 (*bottom*) d of differentiation, and stained with mAb Chc5.9 against clathrin heavy chain (*left*) and with mAb 100.1 against β and β′ adaptins (*right*). Monolayers were then incubated with goat anti-mouse IgG coupled to FITC and with goat anti-mouse IgM coupled to rhodamine. Images comprise individual optical sections from the middle of the cell. Arrows point out to vesicular structures which co-stain with both antibodies. Arrowheads point out to regions which stain with mAb 100.1, but not with Chc5.9. Bars, 10 μm.

results suggest that the increased perinuclear staining observed with anti-clathrin antibodies reflects a specific increase in the proportion of clathrin cages at the TGN, and not a differentiation induced change in the general morphology or staining properties of the Golgi/TGN region.

To determine the cellular localization of clathrin-coated vesicle adaptor proteins during differentiation, we used a mAb which recognizes the β adaptins from the AP-1 and AP-2 adaptor complexes (mAb 100.1). The properties of this antibody have been well characterized using coated vesicle preparations from bovine brain (Ahle et al., 1988). Fig. 3 illustrates the reconstructed images of cells fixed before or after differentiation. The top panels represent the single plane in each cell which contained the most intense fluorescence signal. The bottom panels represent the sum of all the planes throughout the cell. Images were scaled independently to minimize signal saturation in areas of high adaptin concentration. In fibroblasts, staining with mAb 100.1 resulted in a punctate pattern distributed homogeneously throughout the cell (Fig. 3, *left*). In contrast, in differentiated adipocytes the signal was highly concentrated in the perinuclear region (Fig. 3, *right*). These results are consistent with those obtained with anti-clathrin antibodies, and suggest that the formation of structures coated both with adaptins and clathrin at the TGN is enhanced upon differentiation into the adipocyte phenotype.

Simultaneous Detection of Clathrin and Adaptins

Comparison of various images of nondifferentiated and differentiated cells after staining with anti-clathrin or anti-β adaptin antibodies suggested subtle differences in the staining patterns; the cell periphery, in the vicinity of the plasma membrane, seemed to stain more intensely with anti-clathrin antibody Chc5.9 than with anti-β adaptin antibody 100.1. To confirm these apparent differences, we double-stained cells for both clathrin and β adaptin using isotype specific secondary antibodies. To test the feasibility of this technique, cells were incubated with mAb Chc5.9 alone, mAb 100.1 alone or both mAbs Chc5.9 and 100.1 together. Monolayers were then incubated with goat anti-mouse IgG coupled to FITC, or with goat anti-mouse IgM coupled to rhodamine. No signals were detected in cells incubated with mAb 100.1 and goat anti-mouse IgM, nor with mAb Chc5.9 and goat anti-mouse IgG (not illustrated), but clear punctate staining was detected in cells incubated with all primary and secondary antibodies (Fig. 4). In both fibroblasts and adipocytes, extensive co-localization of clathrin and β adaptins could be observed (Fig. 4, *arrows*). However, punctate staining with anti-adaptin antibody was observed in some areas in which no clathrin could be detected (Fig. 4, *arrowheads*). This difference was more apparent in differentiated cells in areas in the vicinity of the TGN (Fig. 4, *bottom*). These results suggest the existence of membrane structures that at steady state are coated with adaptors, but not clathrin. In addition, staining in the vicinity of the plasma membrane was more pronounced with anti-clathrin than with anti-β adaptin antibody, this difference being particularly apparent in nondifferentiated fibroblasts (Fig. 4, *top*). A number of possible explanations for this result exist: for example, flat clathrin lattices, which occur at the initial stages of coated pit formation, may hinder the binding of anti-adaptin antibody. Alternatively, the ratio of adaptins to clathrin may actually be lower at early stages of coat formation. More experiments are required to distinguish among these possibilities.

Biochemical Analysis of Isolated Coated Vesicles

The morphological results shown above suggest that the process of adipocytic differentiation is accompanied by an in-

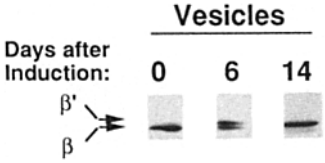

Figure 5. Analysis of clathrin-coated vesicles. Clathrin-coated vesicles were prepared from cells harvested after 0, 6, or 14 d of differentiation. Approximately 5 μg of coated vesicle protein was analyzed by electrophoresis on 7.5% polyacrylamide gels and immunoblotting with mAb 100.1. The primary antibody was detected using HRP-coupled anti-mouse antibodies and enhanced chemiluminescence.

crease in coated membrane formation at the TGN. We analyzed isolated clathrin-coated vesicles from 3T3-L1 cells to determine whether the biochemical composition of such vesicles would reflect the apparent distribution of clathrin lattices in intact cells. Immunoblotting with mAb 100.1 reveals two closely spaced bands migrating at 100–110 kD (Fig. 5). Based on the reported mobilities of the β adaptins, and on further experiments described below, we have denoted the upper band as β' and the lower band as β. The signal obtained with the antibody was reasonably linear, and could detect two- to tenfold differences in the amount of adaptors present in the gels (not illustrated). In coated vesicles from nondifferentiated fibroblasts, the lower band was the predominant species stained by the antibody (Fig. 5, *Day 0*). Interestingly, after 6 d of differentiation, both bands displayed approximately equal staining intensity (Fig. 5, *Day 6*), and after 14 d the upper band was clearly the predominant species (Fig. 5, *Day 14*). These results indicate that the composition of β adaptins in coated vesicles changes dramatically during differentiation of 3T3-L1 cells. The increase in the proportion of β' over β adaptin suggests that in adipocytes a larger proportion of the purified CVs are derived from the TGN. These results are consistent with the observed distribution of clathrin and β adaptins by immunofluorescence.

Biochemical Analysis of Subcellular Fractions

To begin to understand the biochemical basis for the increase in the proportion of TGN-derived coated vesicles, we analyzed the abundance and cellular distribution of clathrin and the adaptins after subcellular fractionation. For these experiments, the cytosolic (unassembled) fraction was separated from a particulate fraction (assembled) which comprises all structures which sediment by high speed centrifugation as described in Materials and Methods (>90 S). Serial dilutions of each fraction were analyzed by PAGE and immunoblotting with mAbs 100.1 and Chc5.9. Shown in Fig. 6 *A* are lanes which were selected from the serial dilutions based on their equivalent amount of staining with mAb 100.1. When the immunoblots are analyzed by densitometry (Fig. 6 *B*), and the results are normalized for the total yield of each fraction (Fig. 6, see legend) it is apparent that whereas only 30–40% of the total cellular clathrin heavy chain is in the cytosol, almost 70% of the β adaptins are detected in this fraction. Although the different isotypes of antibodies used preclude drawing quantitative conclusions about the absolute amounts of clathrin and β adaptins, the relative ratios of these components in the cytosol compared to coated vesicles suggest that coat formation may be more limited by clathrin availability than by adaptor availability Surprisingly, neither the concentration (relative to clathrin), nor the relative ratios of β to β' adaptins in the unassembled, cytosolic fraction changed with differentiation (Fig. 6 *A*, *U*, compare *day 0* and *13*; and Fig. 6 *B*, *U*, *shaded bars*, *right* and *left*). Thus, the marked increase in β' adaptin observed in purified clathrin-coated vesicles with differentiation (Fig. 5) was not reflected by significant changes in the major cellular pool of adaptors.

Whereas the ratio of the β and β' adaptins in the cytosolic fraction did not change with differentiation, the ratio of adaptins in total particulate fractions did reflect the ratio observed in purified coated vesicles (compare Fig. 6 *A*, lanes *A*, days *0* and *13* with Fig. 5, days *0* and *14*). However, the differences observed in the assembled fraction were less pronounced than those observed in purified coated vesicles. To corroborate these apparent differences, we simultaneously analyzed the unassembled, assembled, and coated vesicle fractions derived from the same preparation of cells. Serial dilutions of each fraction were analyzed by PAGE and immunoblotting. Shown in Fig. 7 are lanes from each fraction

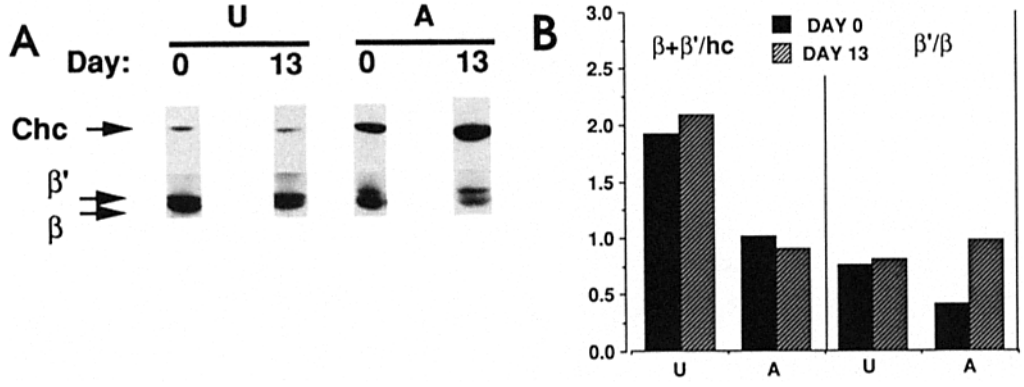

Figure 6. Analysis of assembled and unassembled fractions. (*A*) Cells were grown to confluence in 60 mm dishes, and harvested after 0 or 13 d of differentiation. Assembled (*A*) or cytosolic, unassembled (*U*) fractions were separated by high speed centrifugation. The supernatant (unassembled) and the pellet (assembled) were brought to equal volumes (500 μl). Serial dilutions of the assembled and unassembled fractions were analyzed by electrophoresis on 7.5% polyacrylamide gels, and immunoblotting with a mixture of anti-clathrin heavy chain mAb Chc5.9 and anti-β and β' adaptin mAb 100.1. The lanes shown in the figure were selected based on their similar content of adaptins, and represented the amount present in 50 μl of unassembled and 120 μl of assembled fractions. The position of the clathrin heavy chain (*Chc*) and of the β and β' adaptins are indicated. (*B*) Densitometric scanning. The relative ratios of adaptins to clathrin (*left*) and of β' to β adaptins (*right*) in the assembled (*A*) and unassembled (*U*) fractions of cells after 0 (■) or 13 (▨) d of differentiation were calculated from the intensity of each band, assessed by scanning with a laser densitometer.

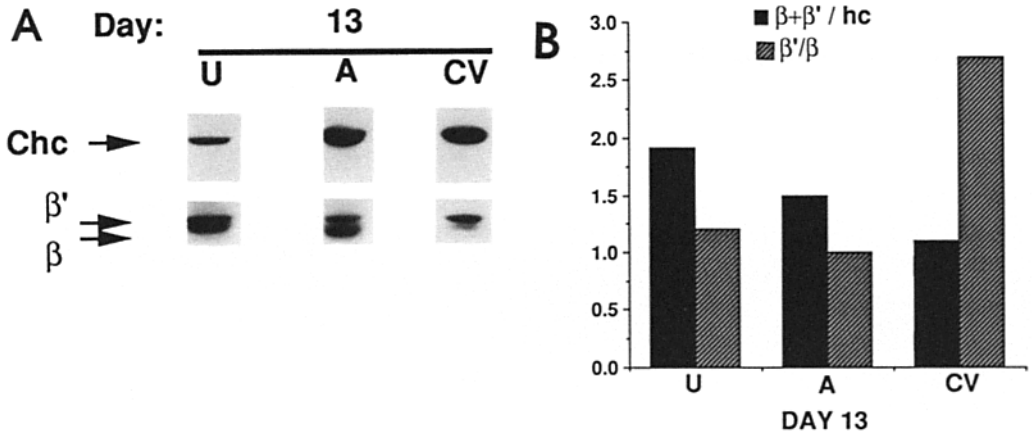

Figure 7. Comparison of assembled, unassembled and coated vesicle fractions. (*A*) Four 150 mm dishes of cells differentiated for 13 d were harvested and homogenized in 8 ml of MES buffer. A 500-μl aliquot was taken after homogenization for the preparation of assembled and unassembled fractions, and the rest was used to prepare 150 μl of purified clathrin-coated vesicles as described in Materials and Methods. Serial dilutions of the fractions obtained were analyzed by electrophoresis on 7.5% polyacrylamide gels, and immunoblotting with a mixture of anti-clathrin heavy chain mAb Chc5.9 and anti-β and β′ adaptin mAb 100.1. The lanes shown in the figure were selected based on their similar content of adaptins, and represented the amount present in 50 μl of unassembled (~50 μg of protein), 100 μl of assembled (~50 μg of protein) and 6.2 μl of the purified coated vesicle (~3 μg of protein) fractions. The position of the clathrin heavy chain (*Chc*) and of the β and β′ adaptins are indicated. (*B*) Densitometric scanning. The relative ratios of adaptins to clathrin (■) and of β′ to β adaptin (▨) in the assembled (*A*), unassembled (*U*), or coated vesicle (*CV*) fractions of cells after 13 d of differentiation were calculated from the intensity of each band, assessed by scanning with a laser densitometer.

selected based on their approximately equivalent staining for adaptins. This experiment shows that the ratio of adaptins to clathrin is higher in the assembled fraction compared to coated vesicles, and highest in the cytosolic fraction (Fig. 7, *A* and *B*). Furthermore, the ratio of β′ to β adaptins is also different in each fraction, being highest in pure, coated vesicles and lowest in the cytosolic fraction (Fig. 7, *A* and *B*). These biochemical results are consistent with the hypothesis that cells contain a population of membranes that at steady state are coated with adaptins but not clathrin. These results are consistent with the partial lack of colocalization of clathrin and adaptins observed by immunofluorescence.

Sedimentation Analysis of Cytosolic Adaptors

The interpretation of the results shown above is contingent on the notion that the concentration of cytosolic adaptins reflects the concentration of functional adaptors. However, the presence of β′ in the cytosolic fraction of fibroblasts may not reflect the presence of AP-1 complexes, but rather the presence of free β′ adaptin chains. To rule out this possibility we took advantage of the different sedimentation coefficients of the free β adaptins (Ahle and Ungewickell, 1989) and the complete adaptors (Virshup and Vann Bennett, 1988), which allow their separation on sucrose gradients. Aliquots of the

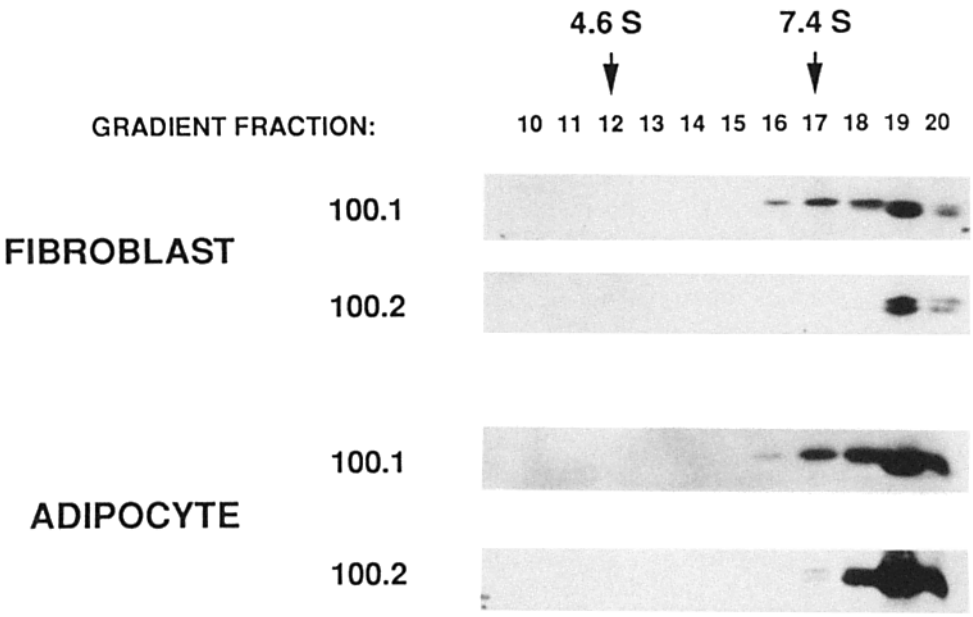

Figure 8. Analysis of the cytosolic fraction from 3T3-L1 cells after sucrose gradient centrifugation. Cells were harvested before (*FIBROBLAST*) or after 10 d of differentiation (*ADIPOCYTE*). Cytosolic fractions were prepared by high speed centrifugation, and 500 μg of protein separated on a calibrated linear 5–20% sucrose gradient as described in Materials and Methods. Gradient fractions were removed from the top of the tube, and analyzed by electrophoresis on 7.5% polyacrylamide gels and immunoblotting with anti-β and β′ adaptin mAb 100.1. The blot was subsequently re-probed with anti-α adaptin mAb 100.2. Primary antibodies were detected using HRP-coupled anti-mouse antibodies and enhanced chemiluminescence. The positions of bovine serum albumin (*4.6S*) and aldolase (*7.4S*) used to calibrate the gradient, are indicated. No specific signal was detected in the lighter fractions (1–10) of the gradient.

cytosolic fraction from nondifferentiated fibroblasts, or from adipocytes harvested after 10 d of differentiation were fractionated by centrifugation on calibrated linear 5–20% sucrose gradients. Fractions were analyzed by PAGE and immunoblotting with mAb 100.1 All the detectable β adaptin signal sediments at the region above 8 S, where AP-1 and AP-2 (8.6 S) would be expected to sediment (Fig. 8). No detectable signal is detected in the region of 4.6 S, which is the reported sedimentation coefficient for the monomeric β adaptins (Ahle and Ungewickell, 1989). Thus, this experiment suggests that essentially all the cytosolic β adaptins are complexed into adaptors, and are not present as free subunits.

More detailed analysis of the blot shown in Fig. 8 indicates that the upper β′ adaptin band is present in a complex of slightly lower sedimentation coefficient (peak in fraction 17) than the one containing the lower, β adaptin band (peak in fraction 19). The lower β adaptin completely cosediments with AP-2, as suggested by the results obtained after reprobing the blot with an antibody (mAb 100.2), which recognizes specifically the α adaptin of the AP-2 complex. These results are relevant in that they provide strong support for our initial assumption that the upper band detected by mAb 100.1 corresponds to β′, whereas the lower band corresponds to β. It is of interest that in 3T3-L1 cells mAb 100.2 recognizes two α adaptins, which may correspond to the two isoform αa and αc (Robinson, 1989). Most importantly, the relative abundance and sedimentation properties of the β, β′, or α adaptins were similar between the cytosolic fractions obtained from fibroblasts or from adipocytes, indicating that the differentiation process is not accompanied by significant changes in the concentration or gross biochemical properties of these components.

Discussion

We report in this study that the process of adipocytic differentiation of 3T3-L1 cells is accompanied by a pronounced change in the composition of clathrin-coated vesicles in these cells. Whereas in nondifferentiated fibroblasts a large proportion of coated vesicles are plasma membrane derived, in differentiated cells clathrin-coated vesicles appear to be principally derived from the TGN. This finding is supported both by biochemical analysis, which indicates the predominance of β over β′ adaptin in coated vesicles purified from fibroblasts, and the reversal of this ratio in coated vesicles purified from adipocytes, and by immunofluorescence microscopy, which indicates a larger concentration of both clathrin and adaptins in the perinuclear region of adipocytes compared to fibroblasts. The change in the relative abundance of TGN- vs. PM-derived vesicles raises questions on the mechanisms that may potentially be involved in the assembly of these structures, and questions on the physiological significance of this phenomenon.

One possible mechanism whereby the assembly of clathrin cages at the PM or TGN could be regulated would be by changes in the cellular concentration of specific adaptors. However, the results presented here do not suggest that the concentration of cellular AP-1 adaptors is the limiting factor for assembly of TGN-derived clathrin coats in fibroblasts. A large proportion (60–70%) of cellular adaptors were found in the cytosolic fraction of both fibroblasts and adipocytes, and the ratio of adaptins to clathrin was significantly greater in the cytosol than in membranes or purified clathrin-coated vesicles. Moreover, the concentration of cytosolic AP-1 did not increase (relative to AP-2 or clathrin) during differentiation, whereas the proportion of clathrin-coated vesicles containing AP-1 increased dramatically. Thus, whereas a small increase in the synthesis of AP-1 which is not reflected by its cytosolic concentration may occur to accommodate the increase in TGN–clathrin lattice formation during differentiation, our results suggest that mechanisms additional to the synthesis of specific adaptors operate to regulate the assembly of clathrin lattices at the PM or TGN. Examples of rapid redistribution of cytsolic clathrin to the plasma membrane in response to stimulation of cells by growth factors (Corvera, 1990; Connoly et al., 1984), and of a redistribution of clathrin from the Golgi to the plasma membrane during endocytosis in macrophages (Takemura et al., 1986), have been previously documented. The results shown here illustrate an example of a stable redistribution of clathrin to the TGN during the process of cellular differentiation. The binding of γ adaptin to the TGN has been shown to be enhanced by AlF, a trimeric G protein activator, and by GTPγS (Robinson and Kreis, 1992; Wong and Brodsky, 1992). Mechanisms involving the induction of specific GTP binding proteins could potentially be involved in producing a stable redistribution of clathrin lattices to the TGN during adipocytic differentiation.

The increased formation of TGN-derived clathrin lattices in adipocytes raises questions about the physiological significance of this phenomenon. One of the key features of the differentiation process is the development of mechanisms that insure the intracellular sequestration of specific molecules, among which are the glucose transporter isoforms GLUT-1 and GLUT-4 (Yang et al., 1992). Little is known about the cellular basis of this sequestration process, but it is likely to result from the efficient retrieval of proteins from the plasma membrane, their efficient retention in intracellular stores, or a combination of both processes. Recent results in yeast cell mutants deficient in the clathrin heavy chain have led to the hypothesis that intracellular clathrin coats play an important role in intracellular retention of certain proteins (Seeger and Payne, 1992*a*,*b*). Our results showing that the development of intracellular retention mechanisms for glucose transporters correlates with an increase in intracellular clathrin lattices support the hypothesis that these structures may play a role in facilitating the intracellular retention of transporter proteins.

This study also suggests the presence in cells of a significant population of membranes coated with adaptors, but not with clathrin. Double staining with anti-clathrin and anti-β adaptin antibodies revealed the presence of structures that stain with anti-adaptin antibody, but not with anti-clathrin. In addition, biochemical analysis of cellular fractions indicated that the ratio of β adaptins to clathrin is significantly higher in a total assembled fraction that contains all cellular elements with sedimentation coefficients higher than 90 S, than in purified clathrin-coated vesicles. It is possible that this difference in the ratio of adaptins to clathrin may result from artificial aggregation of adaptors during the fractionation procedure; however, aggregation only seems to occur to AP-2 when purified to near homogeneity (Beck and Keen, 1991), and has not been found

to occur to AP-1. Moreover, repeated re-centrifugation of the cytosolic fractions after separation of the particulate pellet did not yield aggregates containing APs (results not shown). Thus, this possibility appears unlikely. Rather, the combination of morphological and biochemical results suggest that adaptors can be found assembled onto membranes in the absence of clathrin coats. Interestingly, endocytic membranes coated with adaptors but not clathrin have been detected in other cells (Guagliardi et al., 1990), and a specific role for adaptors in the fusion of early endosomes has been proposed (Beck et al., 1992). Experiments involving the fungal toxin BFA suggest that the TGN behaves functionally as an organelle independent of the Golgi (Reaves and Banting, 1992). In this compartment, adaptors may play a role in preserving the structural identity of the TGN and its functional separation from the endocytic and secretory pathways in normal cells.

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