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CLATHRIN-COATED VESICLES FROM HUMAN PLACENTA CONTAIN GTP-BINDING PROTEINS

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Biochemical and morphological techniques were used to investigate the GTP-binding proteins of clathrin-coated vesicles. Binding of [^{3}H]GTP to clathrin coats was demonstrated by electron microscopic autoradiography. Purified coated vesicles bound 5.2 pmol [^{35}S]GTPγS/mg protein. Addition of GTP or GTPγS, but not ATP nor GMP, inhibited binding of [^{35}S]GTPγS to intact coated vesicles and Triton X-100-extracted coats. A series of 23-24 kDa GTP-binding proteins with isoelectric points between pH 5-8 were detected in coated vesicles. We suggest that the low molecular weight ras-like GTP-binding protein(s) play a role in regulating vesicle-mediated protein transport or signal transduction within intracellular organelles.

A number of steps in the vesicular transport of proteins within eukaryotic cells have been shown to be linked to GTP-binding proteins. In yeast, SAR1, YPT1, and SEC4 genes code for low molecular weight ras-like GTP-binding proteins that are believed to play a role during different stages of secretion (1-3). Recent observations from our laboratory indicate that GTP-binding proteins regulate *in vitro* fusion of macrophage endosomes (4, 5).

Selective transport of receptor-ligand complexes from the plasma membrane and the *trans*-Golgi network is facilitated by clathrin-coated vesicles (6). Since GTP-binding proteins have been identified as playing a role in both inward and outward membrane traffic, it seemed likely that these proteins would be associated with the vesicles that mediate protein transport. With this in mind, we assayed purified clathrin-coated vesicles for associated GTP-binding proteins. Coated vesicles were found to contain several low molecular weight GTP-binding proteins.

METHODS: *Coated Vesicle Isolation*. All procedures were carried out at 4°C. Human placenta was resuspended in three volumes of 250 mM sucrose, 1 mM $MgCl_2$, 0.5 mM EGTA, 20 mM MES, pH 6.5 (homogenization buffer) and homogenized with a Waring blender. The homogenate was centrifuged at 11,000 x g for 30 minutes and the supernatant was decanted and centrifuged at 29,000 x g for 4.0 hours. The resulting pellet was resuspended in homogenization buffer, layered onto a 30% sucrose cushion, and centrifuged at 80,000 x g for 5 min. The 30% sucrose

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layer and the pellet were discarded. The supernatant was again layered onto 30% sucrose, centrifuged at 80,000 x g for 5 min, and the sucrose cushion and pellet were discarded. The supernatant was layered onto 10% Ficoll, 45% D_2O, 1 mM $MgCl_2$, 20 mM MES, pH 6.5 and centrifuged at 100,000 x g for 120 min. The pellet was resuspended in homogenization buffer and centrifuged at 20,000 x g for 10 min. The resulting supernatant was layered onto 15% Ficoll in 75% D_2O, 140 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 20 mM MES, pH 6.5 and centrifuged at 80,000 x g for 14 hours. The pellet containing coated vesicles was resuspended in homogenization buffer to 5 mg/ml final protein concentration. Clathrin coats were prepared by diluting the vesicles with 9 volumes of 1% Triton X-100 in homogenization buffer and incubating the sample at 22°C for 30 min. Clathrin coats were collected by centrifugation at 100,000 x g for 60 min. The phospholipid content was measured by following the methods described by Rouser et al., (7). Proteins were measured according to Bradford (8).

GTP-binding assays. Binding of [^{35}S]GTPγS to clathrin-coated vesicles was determined by incubating the vesicles (10 µg total protein/100 µl) at 22°C for 60 min in 2 µCi [^{35}S]GTPγS (120 Ci/mmol), 250 mM sucrose, 1 mM $MgCl_2$, 0.5 mM EGTA, and 20 mM HEPES, pH 7.0 (binding buffer). The vesicles were collected by centrifugation at 200,000 x g for 30 minutes and washed twice with binding buffer. Competition for [^{35}S]GTPγS binding was determined by adding various concentrations of GTP, GTPγS, ATP, and GMP to the vesicles prior to addition of [^{35}S]GTPγS. The association of GTP-binding proteins with clathrin coats was determined by extracting [^{35}S]GTPγS bound to coated vesicles with 1% TX-100 for various periods of time. Clathrin coats were collected by centrifugation at 200,000 x g for 30 minutes, washed with binding buffer, and coat-associated radioactivity was determined by liquid scintillation counting.

Electron microscopy. Coated vesicles were processed for electron microscopy to monitor purity. The vesicles were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate pH 7.4, post-fixed in 1.5% osmium tetroxide, incubated with 4% uranyl acetate, dehydrated with a graded series of ethanol, infiltrated with propylene oxide, and embedded in Polybed 812 (Polysciences Inc., Washington, Pa.). Tissue was sectioned with a diamond knife, post-stained with uranyl acetate and lead citrate, and examined in a Zeiss 10A electron microscope. Electron microscopic autoradiography was performed by incubating clathrin coats (60 µg total protein) in 240 µl binding buffer containing 10 µCi [^{3}H]GTP (7 Ci/mmol) and 10 µM GTPγS or 10 µCi [^{3}H]GTP and 100 µM ATP. The clathrin coats were washed with binding buffer and processed for electron microscopy as described above. Light gold thin sections were mounted on Formvar carbon-coated nickel grids, coated with Ilford L-4 emulsion using a wire loop, and developed using the gold latensification procedure (9). Developed grids were post-stained in uranyl acetate and lead citrate and viewed in a Zeiss 10A electron microscope.

Molecular weight determination. Protein (100 µgs) from purified clathrin-coated vesicles and clathrin coats was separated by isoelectric focusing (10) and SDS-PAGE (11). The proteins were transferred to nitrocellulose (12), incubated with 25 µCi [α-^{32}P]GTP, washed, and autoradiographed (13).

RESULTS: Electron micrographs of purified clathrin-coated vesicles illustrate that >99% of the vesicles were clathrin-coated (fig 1). To test whether coated vesicles contained GTP-binding proteins, the vesicles were layered onto a linear gradient made of 2% Ficoll 9% D_2O to 20% Ficoll 90% D_2O and centrifuged for 4 hours at 80,000 x g. Frctaion from the gradient were analyzed by SDS-PAGE and the gel was stained for protein. The amount of clathrin heavy chain in each fraction was determined by densitometer tracings of the gel. Low molecular weight GTP-binding proteins were detected by probing Western-blots of proteins separated by SDS-PAGE with [α-^{32}P]GTP and exposing the blots to X-ray film. The amount of GTP-binding protein in each

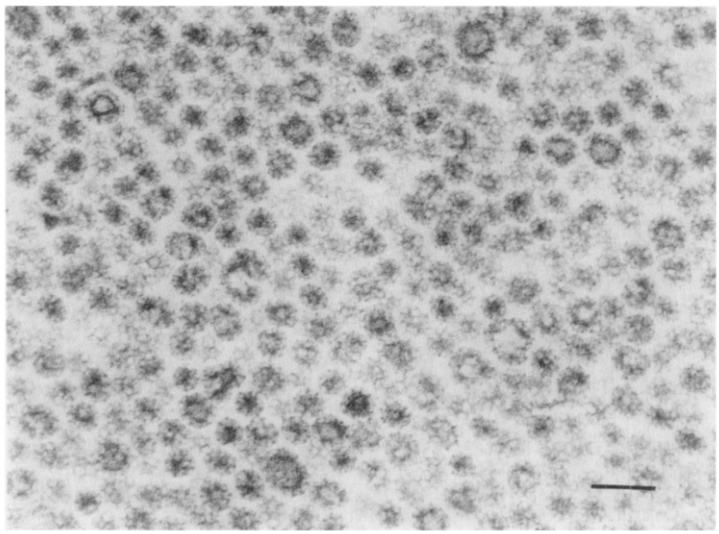

Figure 1. Electron microscopy of purified clathrin-coated vesicles and clathrin coats. Clathrin-coated vesicles were isolated and analyzed for purity by electron microscopy as described in the text (bar = 0.1 micron).

fraction was quantitated by densitometry. The densitometric tracings revealed that clathrin and low molecular weight GTP-binding proteins co-fractionate in the gradient (Fig 2).

Receptors for IgG, transferrin, and ferritin are insoluble in Triton X-100-extracted clathrin coats (14). When coated vesicles were extracted with 1% Triton X-100, smooth vesicle contamination and membranes within coated vesicles were removed (compare fig 1 with fig 3; 14). SDS-PAGE of clathrin-coats extracted with Triton X-100 revealed no qualitative nor quantitative

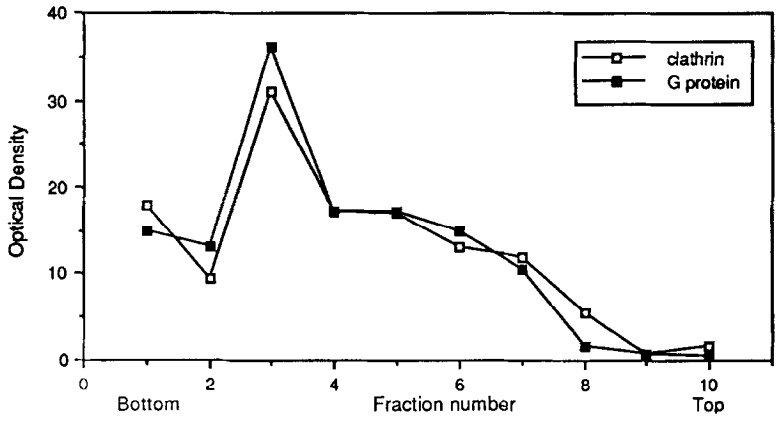

Figure 2. Co-fractionation of GTP-binding proteins and clathrin in Ficoll density gradients. Purified clathrin-coated vesicles (200 µg protein) were centrifuged through 10 ml Ficoll gradients. The distribution of clathrin heavy chain and total low molecular weight GTP-binding proteins in the gradient was determined by densitometer tracings of protein stained gels and autoradiographs of Western blots probed with [α-^{32}P]GTP, respectively.

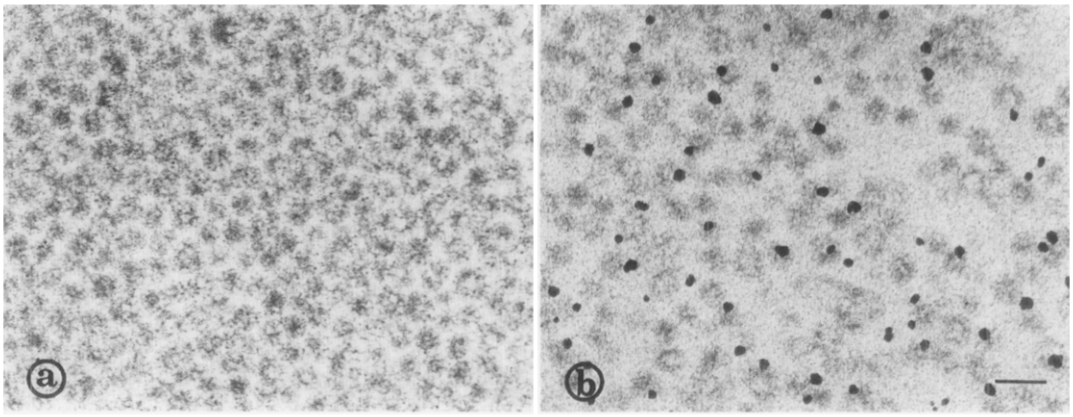

Figure 3. Electron microscopic autoradiography of [^{3}H]GTP-binding proteins in clathrin coats. Purified clathrin coats were incubated in the presence of GTPγS (panel a) or ATP (panel b) and [^{3}H]GTP, washed, and processed for electron microscopic autoradiography (bar = 0.1 micron).

difference in the protein content when compared to normal coated vesicles (data not shown; 15). This indicates that the detergent-extractable proteins of coated vesicles constitutes a small percentage of the total protein. To test whether clathrin coats retained GTP-binding proteins, [^{35}S]GTPγS was bound to clathrin-coated vesicles and the vesicles were washed and extracted with 1% Triton X-100. After 4 and 16 hours of extraction with 1% Triton X-100, 60% and 30% of the binding activity was preserved, respectively.

The specificity of [^{35}S]GTPγS binding to purified clathrin-coats was determined by measuring binding in the presence of competing GTP, GTPγS, ATP, and GMP. Both 1 μM GTP or GTPγS completely inhibited binding of 0.1 μM [^{35}S]GTPγS; however, 100 μM ATP or GMP inhibited binding by less than 40%. We also compared the specific activity of GTP-binding proteins associated with purified clathrin-coated vesicles to total membranes prepared from placenta. The coated vesicles bound 5.2 picomol GTPγS/mg protein (13.0 picomol GTPγS/μmol lipid-associated phosphate) and the total membranes bound 8.1 picomol GTPγS/mg protein (10.4 picomol GTPγS/μmol lipid-associated phosphate).

In order to confirm that the clathrin-coated vesicles contained GTP-binding proteins, electron microscopic autoradiography using [^{3}H]GTP bound to clathrin coats was performed. Binding of [^{3}H]GTP to clathrin coats was detected by the formation of silver grains. This process was inhibited by addition of GTPγS (fig 3a), but not ATP (fig 3b). Less than 10% of the silver grains generated were present in areas lacking clathrin coats.

The GTP-binding proteins in coated vesicles were further characterized by separating the proteins using two dimensional gel electrophoresis and transferring the proteins to nitrocellulose. The blot was incubated with [α-^{32}P]GTP and analyzed by autoradiography. Partial renaturation of some ras-like GTP-binding proteins occurs under these conditions (13). Figure 4

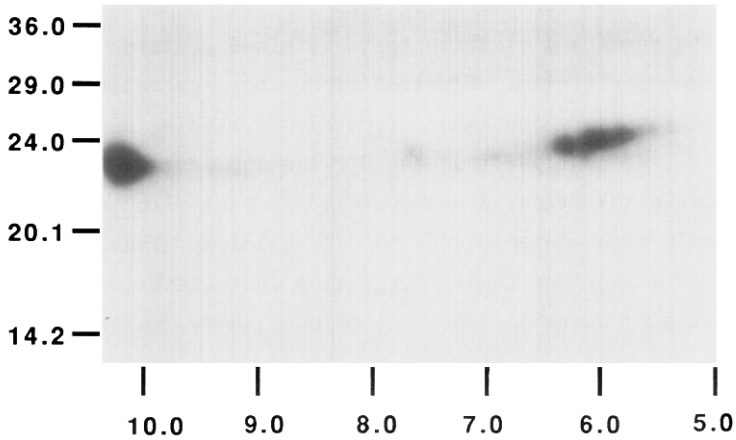

Figure 4. Two dimensional gels blotted for [α-^{32}P]GTP-binding proteins. Proteins of purified clathrin-coated vesicles (100 µg) were separated by isoelectric focusing followed by SDS-PAGE and subsequently transferred to nitrocellulose. The blot was incubated in [α-^{32}P]GTP, washed, and exposed to X-ray film. The numbers on the left side of the gel represent molecular mass markers and those below the gel represent the pI of the proteins.

illustrates that GTP-binding proteins with molecular weights from 23-24 kDa form a spot at the origin of the first dimension and a series of spots between pH 5-8. The same GTP-binding proteins were also identified in TX-100-extracted clathrin coats (data not shown).

It has previously been suggested that high molecular weight GTP-binding proteins (eg. G_i or G_s) may be susceptible to irreversible denaturation after SDS-PAGE (16). Cholate is an effective detergent for extracting clathrin trimers from coated vesicles (17) and active high molecular weight GTP-binding proteins from placental membranes (18). This detergent was therefore used to solubilize coated vesicles and the proteins were separated by gel permeation chromatography using a Superose 12 HR 10/30 column (Pharmacia). This technique is commonly used to separate the higher mobility (115 kDa) trimeric GTP-binding proteins from the lower mobility (50 kDa) ras-like GTP-binding proteins (19). The GTP-binding proteins apparently have a higher molecular weight when analyzed by gel filtration chromatography compared to SDS-PAGE due to their association with cholate. The [^{35}S]GTPγS binding activity of fractions eluted from the column exhibited a major peak near a molecular mass of 50 kDa (data not shown). A second peak of binding activity at higher molecular weights was not observed. We also failed to detect the trimeric GTP-binding proteins by using antibodies (serum code A-569, 20) that recognize the α subunit (data not shown).

DISCUSSION: The results presented in this paper demonstrate that clathrin-coated vesicles contain multiple low molecular weight GTP-binding proteins. Since preparations of clathrin-coated vesicles are commonly contaminated with

smooth vesicles, these contaminating vesicles were removed by extraction with detergent. The remaining clathrin coats retained proteins that bind GTP with high nucleotide specificity. Electron microscopy autoradiography was used to conclusively demonstrate the association of GTP-binding proteins with pure clathrin coats. We conclude that the GTP-binding proteins present in the coated vesicles preparations are associated in part with coated-vesicles rather than only with contaminating smooth vesicles. These results are consistent with a previous report suggesting that clathrin-coated vesicles may contain GTP-binding proteins (21). More importantly, we provide definitive evidence that low molecular weight GTP-binding proteins are present in these vesicles and initially characterize these proteins.

Detergent extraction of coated vesicles for 16 hours resulted in a 70% decrease in GTP-binding activity. Under these same conditions we found that less than 5% of the total protein was extracted. Since the lipid bilayer was extracted with detergent and the coat proteins remained intact, it is likely that the GTP-binding proteins are retained in coated vesicles by their association with the lipid bilayer. This is consistent with the finding that ras proteins are anchored to membranes by lipid moieties attached to the COOH-terminus of the protein (22). Since a significant amount of the GTP-binding proteins were retained in detergent extracted coats, it is also possible that some of the GTP-binding proteins are associated with the coat proteins.

It is difficult to access the source of the size and charge heterogeneity observed in the low molecular weight GTP-binding protein pool. One explanation is that these proteins represent multiple gene products. Alternatively, some of these proteins may be coded for by the same gene and these variations could arise from differences in post-translational modifications (eg., proteolytic processing, polyisoprenylation, or phosphorylation). Whether the same GTP-binding proteins are present in Golgi-derived or plasma membrane-derived coated vesicles also remains to be determined. Heterogeneity in GTP-binding proteins identified in this study may reflect the heterogeneity in the origin of coated vesicles.

The low molecular weight GTP-binding proteins include approximately 30 known ras-like proteins whose cellular functions are largely unknown, however their intracellular distribution suggests that they play roles in organellar traffic and other intracellular interactions (23). These proteins have been implicated as playing a role during secretion by chromaffin granules (24) and fusion between endoplasmic reticulum-derived vesicles (25). Recently, the ras-related rab gene family has been implicated in vesicular transport through the secretory and endocytic pathways (26, 27). Our lab has developed an *in vitro* assay to show that plasma membrane-derived vesicles, which largely consist of clathrin-coated vesicles, can fuse with early endosomes (28). This

fusion event is inhibited by GTPγS (4). These observations support the possibility that the GTP-binding proteins associated with clathrin-coated vesicles may function during vesicular transport by mediating vesicle fusion. Alternatively, these proteins may function in other cellular roles such as signal transduction. Thus, signals generated by low molecular weight GTP-binding proteins may not be confined to the plasma membrane and may be disseminated by the vesicles that regulate receptor-mediated endocytosis.

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