Association of a Dynamin-like Protein with the Golgi Apparatus in Mammalian Cells

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Abstract. Dynamins are a family of 100-kD GTPases comprised of at least three distinct gene products and multiple alternatively spliced variants. Homologies with the shibire gene product in Drosophila melanogaster and with Vps1p and Dnmlp in Saccharomyces cerevisiae suggest that dynamins play an important role in vesicular transport. Morphological studies have localized brain dynamin to coated pits and tubular invaginations at the plasma membrane, where it is believed to facilitate the formation of endocytic vesicles. Because similar membrane-budding events occur at the Golgi apparatus and multiple dynamin isoforms exist, we have studied the distribution of dynamins in mammalian cells. To this end, we generated and characterized peptide-specific antibodies directed against conserved regions of the dynamin family. By immunoblot analysis, these antibodies reacted specifically with a 100-kD protein in fibroblasts that sedimented with membranes and microtubules in vitro in a manner similar to brain dynamin. By immunofluorescence microscopy, these antibodies strongly labeled the Golgi complex in cultured fibroblasts and melanocytes, as confirmed by double labeling with a Golgi-specific antibody. Furthermore, Western blot analysis showed significant enrichment of a 100-kD dynamin band in Golgi fractions isolated from the liver. To substantiate these findings, we used a specific antidynamin antibody to immunoisolate Golgi membranes from subcellular Golgi fractions, as determined by EM and immunoblot analysis. This study provides the first morphological and biochemical evidence that a dynamin-like protein associates with the Golgi apparatus in mammalian cells, and suggests that dynamin-related proteins may have multiple cytoplasmic distributions. The potential contributions of dynamin to the secretory and endocytic pathways are discussed.

Dynamin is a 100-kD GTP-binding protein that was originally isolated from the bovine brain because of its ability to cross-link and bundle microtubules in vitro in a nucleotide-dependent manner (Shetner and Vallee, 1989). The GTPase activity of dynamin is stimulated by microtubules (Maeda et al., 1992; Shetner and Vallee, 1992; Tuma et al., 1993), phospholipids (Tuma et al., 1993), phosphorylation (Robinson et al., 1993), and certain Src homology 3 domain-containing proteins (Gout et al., 1993; Herskovits et al., 1993b; Scaife et al., 1994). While its precise function is presently undefined, substantial evidence has implicated dynamin at an early stage in endocytosis. Dynamin is believed to be the mammalian homologue of the shibire gene product in Drosophila melanogaster (Chen et al., 1991; van der Bliek and Meyerowitz, 1991), in which a temperature-sensitive mutation renders these flies paralyzed because of an inability to recycle synaptic vesicles at nerve terminals (e.g., Poodry and Edgar, 1979; Kosaka and Ikeda, 1983a; Costello and Salkoff, 1986; Koenig and Ikeda, 1989). Ultrastructural studies of different cell types from mutant shibire flies show an accumulation of membrane invaginations and a depletion of subplasmalemmal vesicles at the cell surface, consistent with a perturbation in endocytosis (Kosaka and Ikeda, 1983b; Costello and Salkoff, 1986; Kim and Wu, 1987; Kessell et al., 1989; Koenig and Ikeda, 1990). Furthermore, mutant forms of dynamin, when overexpressed in cultured cells, cause a marked reduction in receptor-mediated endocytosis (Herskovits et al., 1993a; van der Bliek et al., 1993; Damke et al., 1994, 1995). Finally, recent ultrastructural studies have confirmed the presence of dynamin on endocytic structures (Damke et al., 1994; Takei et al., 1995).

Several past studies suggested that dynamin was restricted to neural tissues (Scaife and Margolis, 1990; Nakata et al., 1991; Chen et al., 1992). This exclusive neuronal distribution was unexpected, since mutations in the shibire gene affect a number of different cell populations, including muscle (Buzin et al., 1978; Costello and Salkoff,...
Tuma et al., 1993) or an unidentified membrane compart-
tment. Interestingly, fibroblasts that were immunopa-
lized Dynl, which is involved in protein sorting at the
Golgi apparatus (Rothman et al., 1990; Vater et al.,
1992; Wilsbach and Payne, 1993), and the recently identi-
fied Dnm1p, which is involved in endosomal protein traf-
ficking in yeast (Gammie et al., 1995).

Although dynamin associates with microtubules in vitro,
this has not been observed in vivo. Rather, immuno-
chemical studies on the distribution of dynamin in PC12
cells (Scapce and Margolis, 1990) and intact neurons (Noda
et al., 1993; Gass et al., 1995) reveal diffuse and punctate
staining patterns that spread throughout cell bodies, neu-
rites, and growth cones. A similar distribution is observed
in transfected cells overexpressing Dyn1 (Herskovits et al.,
1993a). Western blot analysis of subcellular fractions from
the brain shows dynamin immunoreactivity in both mem-
brane and cytosolic fractions. It is unclear whether dy-
namin associates with synaptic vesicles (Noda et al., 1993;
Tuma et al., 1993) or an unidentified membrane compart-
ment (Scapce and Margolis, 1990) in these preparations.
More recently, dynamin has been localized to clathrin-
coated pits at the plasma membrane in cultured cells
(Damke et al., 1994), and to the necks of membrane invag-
inations and clathrin-coated pits in an isolated synaptoso-
mal preparation (Takei et al., 1995). It is expected that
the multiple dynamin isoforms and related proteins will
have different distributions and functions that are not yet de-
defined in mammalian cells. We have therefore constructed a
library of five affinity-purified pAbs directed against three
distinct regions of rat brain Dyn1 that are highly con-
served within the dynamin family of proteins. Using mor-
phological and biochemical methods, we examined the in-
tracellular localization of dynamin in primary cultures of
human cells of two different embryonic origins: fibroblasts
(mesenchymal) and melanocytes (neural crest). By immu-
noprecipitation and Western blot analysis, these poly-
clonal antibodies (pAbs) specifically recognized purified
Dyn1, as well as dynamin from crude extracts of rat brain and cultured human fibroblasts. Indirect immu-
nofluorescence microscopy of cultured cells showed that
these antibodies consistently labeled a perinuclear
membrane network that localized with a Golgi-specific an-
tibody, but not with fluorescently labeled endocytic com-
partments. Interestingly, fibroblasts that were immuno-
labeled with a pAb to Vpslp also revealed a reticular Golgi
association identical to that seen with the multiple antidy-
namin antibodies. Consistent with these morphological
observations, Western blot analysis of subcellular organelle
fractions isolated from rat liver showed a significant en-
richment of a 100-kD dynamin protein in Golgi-enriched
fractions as compared to a total membrane fraction. Fur-
thermore, we demonstrate that a specific antidynamin an-
tibody immunoisolated Golgi membranes from two separa-
t subcellular Golgi fractions, as determined by EM and
Western blot analysis. These biochemical and morphologi-
cal observations provide the first evidence that a dynamin-
like protein is associated with the Golgi apparatus in mam-
malian cells and may be involved in vesicular trafficking
events of the secretory pathway.

Materials and Methods

Materials

Tissues used for this study were harvested from 150–250 g male Sprague
Dawley rats (Harlan, Madison, WI). Taxol was a gift from the National
Cancer Institute (Bethesda, MD). Other reagents were from Aldrich
Chemical Company, Inc. (Milwaukee, WI), Bio-Rad Laboratories (Her-
cules, CA), Calbiochem Corp. (La Jolla, CA), Electron Microscopy Sci-
ences (Fort Washington, PA), Pharmacia Fine Chemicals (Uppsala, Swe-
den), or Sigma Chemical Co. (St. Louis, MO), unless specified otherwise.
A pAb to rab 6 was purchased from Santa Cruz Biotechnology, Inc.
(Santa Cruz, CA), and an mAb against β-COP was obtained from Sigma
Chemical Co. Secondary antibodies were FITC- or TRITC-conjugated
anti-rabbit and goat anti-mouse (Kirkegaard & Perry Laboratories,
Gaithersburg, MD) for immunocytochemical staining; alkaline phos-
phatase– or HRP-conjugated goat anti-rabbit and goat anti-mouse (Bio-
source International, Camarillo, CA) were used for Western blot analysis.
The following antibodies were obtained as gifts: the Golgi-specific mAb
10E6 (Dr. W. Brown, Cornell University, Ithaca, NY), a pAb to the cata-
ytic domain of rat liver Golgi α-mannosidase II (Dr. M. Gist Farquhar,
University of California, San Diego, CA), a rabbit anti-rab 6 (Dr. B.
Goud, Institut Pasteur, Paris, France), mAb 3D1 against RER-associated
ribophorin II (Dr. D. Meyer, University of California, Los Angeles), and
pAbs and mAbs to Vpslp (Dr. T. Stevens, University of Oregon, Eugene).
Partially purified brain dynamin was a gift from Dr. G. Waters (Princeton
University, Princeton, NJ).

Cells and Culture Conditions

Primary cultures of human foreskin melanocytes and fibroblasts were pro-
vided by the laboratory of Dr. M. Pittelkow (Mayo Clinic, Rochester,
MN). Cells were grown at 37°C with 5% CO2 in a humid environment us-
ing standard tissue culture procedures. Melanocytes were cultured in
MCDB 153 media supplemented with 0.1 mM ethanolamine, 0.1 M
phosphorylethanolamine, and 0.5 μM hydrocortisone (Pittelkow and Shipley,
1989), while fibroblasts were cultured in MEM-a medium with l-gluta-
mic acid, ribonucleosides, and deoxyribonucleosides (GIBCO BRL, Grand
Island, NY) containing 10% FBS. Cells were grown in Falcon petri dishes
(Fisher Scientific, Pittsburgh, PA) and on 12-mm round cover glasses for
protein extraction and immunocytochemistry, respectively.

Production and Affinity Purification of pAbs

Peptides against the deduced amino acid sequence of rat brain Dyn1 (Obar
et al., 1990) were synthesized and conjugated to keyhole limpet hemocya-
in by the Mayo Protein Core Facility at the Mayo Clinic. These peptides
were suspended in PBS and Freund’s adjuvant, injected into New Zealand
white rabbits, and the antisera were collected after subsequent boost in-
jections. The peptide sequences (single-letter amino acid notation) and
the corresponding antibody names are LTLVLPLGMTK (amino acids 132–
143; MC12), LAYVGGQGSKSVLFE (amino acids 34–48; MC13 and
MC14), ARDVLKENLPLRGGYGVNRSQKD (amino acids 216–

1. Abbreviations used in this paper: CM, crude membrane fraction; D-PBS,
Dulbecco’s PBS; Dyn1, dynamin I; Dyn2, dynamin II; pAb, polyclonal ant-

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Protein concentrations were determined with bichoninic acid according to the manufacturer (Pierce), using BSA as a standard. Cellular proteins were separated by discontinuous SDS-PAGE under reducing conditions (Laemmli, 1970) on 5-10% and 5-12% polyacrylamide gradient gels or 7.5-9% linear gels, using a bisacrylamide/acrylamide ratio of 1:37.5. Protein bands were visualized with Coomassie brilliant blue or by silver staining (ICN Radiochemicals, Irvine, CA). Alternatively, proteins were electrophoretically transferred to polyvinylidifluoride membranes (Millipore, Bedford, MA) and then analyzed using Western blotting (Towbin et al., 1979). Membranes containing transferred proteins were blocked in TBS (20 mM Tris, pH 7.5, 150 mM NaCl) containing 5% BSA and 0.05% Tween 20 before incubation with primary antibodies. Immunoreactive bands were detected with an appropriate secondary antibody conjugated either to alkaline phosphatase or HRP. After each step, the membranes were washed with TBS containing 0.05% Tween 20. Membranes were developed with either bromochloroindolyl phosphate and nitro blue tetrazolium to visualize alkaline phosphatase or for enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) and were exposed to autoradiographic film (Eastman Kodak Co., Rochester, NY) to detect HRP. To quantitate the relative density of immunoreactive bands, exposed autoradiographic films were scanned with a densitometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) or with a Umax Technologies, Inc. (Freemont, CA) scanner and analyzed with Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA) and Image 1 (National Institutes of Health, Bethesda, MD) software. To estimate molecular weights, bands were referred to a high molecular weight standard mixture (SDS-6H, Sigma Chemical Co.) or to the following prestained standards: lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, β-galactosidase, and myosin (Bio-Rad).

**Purification of Dynamic and GTPase Assay**

Dynamin was purified using a modification of the method by Scaife and Margolis (1990). Fresh rat brains were homogenized in a glass tissue grinder (Wheaton Scientific Products) or to the following prestained standards: lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, β-galactosidase, and myosin (Bio-Rad).

**Immunochemistry and EM**

For immunochemistry microscopy, cells were grown on coverslips for 2-7 d. Cells were rinsed with D-PBS at 37°C and fixed for 15-20 min with 2-3% formaldehyde in Pipes buffer (0.1 M Pipes, pH 6.95, 3 mM MgSO4, 1 mM EGTA), which had been prewarmed to 37°C. After rinsing with D-PBS, cells were permeabilized with 0.1% digitonin in D-PBS for 1 h at 37°C. Cells were then washed extensively with D-PBS, rinsed briefly with distilled water, and mounted on a glass slide in mounting reagent (SlowFade or SlowFade Light; Molecular Probes, Inc., Eugene, OR). For double-labeling experiments, cells were incubated simultaneously with both primary antibodies, rinsed with D-PBS, and incubated with two appropriate fluorescently labeled secondary antibodies. Cells were viewed with an Axiosvert 35 epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 50- or 100-W Mercury lamp using either a 63× or 100× objective lens (Zeiss Plan-Neofluar, NA 1.25 and 1.30, respectively). Images were recorded on Tmax p3200 or Ektachrome 400 film (Eastman Kodak Co.) with a 35-mm camera.

To label late endosomes and lysosomes, human fibroblasts were incubated with media containing 0.5 mg/ml lysine-fixable, FITC-conjugated dextran (3,000 mol wt; Molecular Probes) for 3.5 h at 37°C, rinsed with four changes of HBSS (Sigma Chemical Co.), and incubated for an additional 35 min at 37°C in media alone. Cells were then processed for indirect immunofluorescence microscopy as described above. In some cases, cells were photographed before immunochemical processing to compare the effect of processing on the dextran label.

For analysis by EM, suspended membrane (5-10 μl) and immunolabeled (0.2-1 mg beads) fractions were fixed in 2.5% glutaraldehyde (in 50 mM NaH2PO4/Na2HPO4, pH 7.4, 5 mM MgCl2, 1 mM EDTA) for 0.5-1 h at 4°C. Membrane fractions were then embedded in 0.6% agarose and fixed an additional 1-6 h at 4°C in fresh 2.5% glutaraldehyde fixative. After rinsing in PBS, the fractions were postfixed in 2% osmium tetroxide.
for 1.5 h, rinsed extensively in PBS, and stained overnight with 0.5% uranyl acetate. Fractions were then rinsed with two changes of PBS, dehydrated in a graded series of 50–100% ethanol, embedded in Quetol 651 (Ted Pella, Inc., Redding, CA), and sectioned with a diamond knife. Thin sections were poststained with lead citrate and viewed with a transmission electron microscope (JEOL 1200; JEOL USA, Peabody, MA) at 60 kV.

Subcellular Fractionation and Immunolocalization

For microtubule-binding experiments, fibroblasts and brains were homogenized as described above in PEM/G buffer containing 10 μg/ml soybean trypsin inhibitor and leupeptin. Homogenates (H) were centrifuged at 4°C for 20 min at 50,000 g and pellets collected as the heavy membrane fraction (P1). Supernatants were centrifuged at 170,000 g for 45 min and pellets collected as the microsomal fraction (P2), while supernatants were saved as the cytosolic fraction (S2). Microtubules were assembled from purified bovine brain tubulin as described above, stabilized with 20 μM taxol, and added to the S2 fraction. After a 30-min incubation at 37°C to allow dynamin to bind to the microtubules, this fraction was centrifuged at 170,000 g for 45 min at 20°C to yield a microtubule pellet (MTP) and supernatant (S3).

Enriched organelle fractions were isolated from rat liver according to the separate methods of Hamilton and co-workers (1991) and Howell and Palade (1982), as described in Marks et al. (1994). In addition, a modification of the method of Leelavathi et al. (1970) was used essentially as previously described (Fath et al., 1994). Briefly, a rat was anesthetized with pentobarbital (0.1 cc/100 g), and the liver was removed after perfusing with 40 ml of ice-cold PKM buffer (100 mM KH2PO4/KHPO4, pH 6.5; 5 mM MgCl2; 1 mM PMSF; 10 μg/ml leupeptin, soybean trypsin inhibitor, and p-Toluenesulfonyl-L-arginine methyl ester; 5 μg/ml pepstatin; 50 μM EDTA) containing 0.5 M sucrose. All the following steps were done at 4°C, and sucrose concentrations were maintained in PKM buffer with the aid of Millipore filters. The liver was finely minced and homogenized in 5 vol of 0.5 M sucrose with a polytron (Brinkmann Instruments, Inc., Westbury, NY), and the homogenate was centrifuged at 600,000 g for 10 min to obtain a postnuclear supernatant (S1). The postnuclear supernatant (10 ml) was layered over a 10-mL cushion of 1.3 M sucrose and was centrifuged at 50,000 g for 60 min. The supernatant was aspirated and the interface collected as the cytosolic fraction (CM). The CM fraction was adjusted to 1.1 M sucrose in 6.5 ml and layered onto a second discontinuous sucrose gradient containing 6.5-ml steps with sucrose concentrations as follows: 1.4, 1.3, 1.25, 1.1 (CM), and 0.5 M. This discontinuous sucrose gradient was centrifuged at 90,000 g for 1.5–2 h, and the flocculent material at the 0.5/1.1 M interface collected (3–5 ml) as the mixed Golgi fraction (G). This mixed Golgi fraction was adjusted to 0.5 M sucrose in a volume of 30 ml and was centrifuged at 10,000 g for 15 min. The pellet was gently resuspended in 5 ml of 0.5 M sucrose (0.25 ml) as stacked Golgi fractions (Gs).

The supernatant was centrifuged at 266,000 g for 30 min, and the pellet resuspended in 0.5 M sucrose (0.4 ml) as the Golgi vesicle fraction (Gv). A small fraction of the homogenate was centrifuged at 266,000 g for 45 min to obtain a total membrane pellet and a supernatant that was saved as the cytosolic fraction. Aliquots of all membrane fractions were diluted 5–10-fold in 0.5 M sucrose as a wash step, centrifuged at 266,000 g for 30 min, and pellets were resuspended in 2% RIPA buffer containing 1% SDS. Fractions were quick-frozen in liquid N2 and stored at −80°C before subsequent analysis by Western blotting.

Immunolocalizations were done as reviewed by Howell et al. (1989). Briefly, tosylated superparamagnetic beads (Dynabeads M-500 Subcellular; Dynal Inc., Lake Success, NY) were incubated overnight at room temperature on a rotator with a goat anti-rabbit linker antibody (Biodesign International, Kennebunkport, ME) at a concentration of 10 μg PAb/mg beads in borate buffer (100 mM H3BO3, pH 9.5). For this and all subsequent steps, beads were collected with a magnetic device (MPC, Dynal, Inc.) and washed with a magnetic device (MPC, Dynal, Inc.) and saved as the immunosolated fractions. Supernatants that remained after separation from the immunosolated fractions were centrifuged at 16,000 g for 10 min, and the pellets were resuspended in 0.25 M sucrose as the unbound fractions. Fractions were subsequently analyzed by standard EM and Western blotting techniques.

Results

pAbs Recognize Dynamin In Cell Homogenates

pAbs were generated against three different peptides representing regions of the amino-terminal domain of rat brain Dyn1 (Fig. 1) that are conserved among the dynamin family of proteins (Obar et al., 1990; Chen et al., 1991; van der Bliek and Meyerowitz, 1991; Nakata et al., 1993; Cook et al., 1994; Sontag et al., 1994). Two of these peptides spanned the first (MC13 and MC14) and second (MC12) regions of the dynamin tripartite GTP-binding motif (Obar et al., 1990), and they are conserved among the various dynamin-related proteins. A third peptide sequence (MC62 and MC63) was chosen based on its high degree of conservation within the dynamin family and lack of homology with related GTP-binding proteins. Crude rabbit antisera were collected and affinity purified, and the specificity of each antibody was determined by Western blot analysis. As shown in Fig. 2, each antidynamin antibody recognized a prominent protein in the brain and fibroblast extracts that migrated with purified brain dynamin. A similar profile was observed with pAbs MC14 and MC62 (data not shown).
Fibroblasts were grown to extreme confluency for this study, and were harvested using a prolonged incubation in 0.25% trypsin. Under these conditions, multiple 80-90-kD bands were detected by SDS-PAGE of the immunoprecipitate obtained with MC63 (Fig. 3 A), as well as by Western blot analysis of the initial protein extract using either MC63 or an antibody that is specific for the internal region of Dyn2 (data not shown). These bands probably correspond to proteolytic fragments of dynamin since they were recognized by the Dyn2-specific antibody by immunoprecipitation and Western blot analysis (data not shown). To further characterize the proteins in these samples, the immunoprecipitates from the brain were transferred to polyvinylidifluoride membranes and processed for Western blot analysis using antibodies to Dyn1, kinesin, β-COP, and Vps1p. Fig. 3 B shows that the prominent 100-kD protein immunoprecipitated by each of the antidynamin pAbs (MC12, MC13, and MC63) was indeed dynamin, as determined by a Dyn1-specific tail antibody. Kinesin was not detected in the antidynamin antibody immunoprecipitates, but was present only in the antikinesin antibody immunoprecipitate and in the crude brain homogenate (Fig. 3 B). Likewise, the Golgi-associated coat protein β-COP was not recognized in any of the immunoprecipitates (data not shown). In contrast, mAbs or pAbs to Vps1p did not detect an 80-kD Vps1p band in the immunoprecipitates that was seen in crude yeast extracts. Interestingly, however, the pAb against Vps1p recognized both purified and immunoprecipitated brain dynamin, suggesting a cross-reactivity of this antibody.

To characterize the 100-kD immunoreactive dynamin-like protein that was detected in crude homogenates of both brain and cultured fibroblasts, we tested whether this protein sedimented with membranes and microtubules, as has been demonstrated previously for brain dynamin (Scaife and Margolis, 1990). Brain and fibroblast homogenates were centrifuged at 30,000 g to pellet the heavy membrane fractions and the supernatants centrifuged at 170,000 g to obtain microsomal pellets and cytosolic fractions. Microtubules that had been assembled from purified bovine brain tubulin were incubated with the cytosolic fractions for 30 min at 37°C and then collected by centrifuging at 170,000 g. Fig. 4 shows that substantial amounts of the dynamin-like protein associated with heavy membrane and microsomal fractions from both brain and fibroblasts, while less was found in cytosolic fractions. In addition, the soluble protein pelletted with exogenously added microtubules in the absence of nucleotide. These results indicate that the 100-kD proteins that are recognized by our antidynamin antibodies in both neuronal and nonneuronal cell types are biochemically similar to brain dynamin.

**Distribution of Dynamin Immunoreactivity in Cultured Cells**

To define the cellular localization of dynamin, we used each of the affinity-purified antidynamin antibodies for indirect immunofluorescence analysis of fixed cells. As shown in Fig. 5, three different antidynamin antibodies (MC12, MC13, and MC63) labeled an elaborate perinuclear membrane network and a population of punctate vesicular structures in cultured human fibroblasts. Similar results were obtained using pAbs MC14 and MC62 (data not shown). Additionally, these localizations were susceptible to harsh detergent extractions, indicating an association...
Peptide antibodies to dynamin recognize a 100-kD protein in brain and fibroblast homogenates that sediments with crude membranes and microtubules. Western blot analysis of purified rat brain dynamin (D) and subcellular fractions obtained from rat brain and human fibroblasts stained with antidynamin antibody MC63. Lanes were loaded with four times more fibroblast protein than that of brain. Fractions include crude homogenates (H), 30,000 g (P1) and 170,000 g (P2) membrane pellets, 170,000 g supernatants (S2), microtubule pellets (MtP), and resulting supernatants (S3). Note that while there is considerably more dynamin in the brain as compared to fibroblast homogenates, both dynamin proteins behave similarly. Dynamin from both cell types sediments with membranes (P1 and P2) and exogenously added microtubules (MtP).

Figure 4. Peptide antibodies to dynamin recognize a 100-kD protein in brain and fibroblast homogenates that sediments with crude membranes and microtubules. Western blot analysis of purified rat brain dynamin (D) and subcellular fractions obtained from rat brain and human fibroblasts stained with antidynamin antibody MC63. Lanes were loaded with four times more fibroblast protein than that of brain. Fractions include crude homogenates (H), 30,000 g (P1) and 170,000 g (P2) membrane pellets, 170,000 g supernatants (S2), microtubule pellets (MtP), and resulting supernatants (S3). Note that while there is considerably more dynamin in the brain as compared to fibroblast homogenates, both dynamin proteins behave similarly. Dynamin from both cell types sediments with membranes (P1 and P2) and exogenously added microtubules (MtP).

Figure 3. Multiple antibodies specifically immunoprecipitate dynamin. (A) A silver-stained gel showing the protein profile of immunoprecipitates obtained from a crude brain extract (Brain) with MC12 (12), MC13 (13), and MC63 (63) reveals comigration of a major protein band detected at 100 kD and purified brain dynamin (D, arrow). This band represents the only protein consistently recognized by the multiple antibodies to dynamin that was not also immunoprecipitated with a pAb to kinesin (44) or normal rabbit serum (S). Only MC12 immunoprecipitated a protein detected as a minor band at ~80 kD. Proteins were also immunoprecipitated from a fibroblast total protein extract (Fibro) using the MC63 and kinesin antibodies. The corresponding silver-stained gel indicates that a protein doublet specifically recognized by MC63 migrates with purified brain dynamin. The less intense bands at 80–90 kD most likely represent proteolytic fragments of dynamin that have been described previously (Hinshaw and Schmid, 1995; Tuma et al., 1993), since these are detected with an antibody that is specific for Dyn2 by Western blot analysis (data not shown). Immunoglobulin heavy (hc) and light (lc) chains are denoted by arrowheads. (B) Proteins immunoprecipitated from the brain were probed with multiple antibodies for Western blot analysis. The Dyn1-specific carboxyl-terminal tail antibody (Dyn1) detected purified brain dynamin (D) and the major 100-kD protein that was recognized by each of the multiple antibodies to dynamin (12, 13, and 63). Kinesin was detected only in the lanes containing crude brain extract (Br) or an immunoprecipitate obtained with an antibody to kinesin (44), and not in the lanes containing immunoprecipitates obtained with the antibodies to dynamin. Interestingly, a pAb to Vpslp not only recognized Vpslp in the crude yeast extract (Y), but also detected both purified and immunoprecipitated brain dynamin. A mAb to Vpslp, however, reacted only with the crude yeast extract and not with any of the immunoprecipitated proteins. The corresponding molecular weights are listed to the right of each panel.
Figure 5. Multiple antibodies to dynamin label the Golgi apparatus in fibroblasts. Cultured human fibroblasts were processed for double-label immunofluorescence microscopy using three different affinity-purified antibodies to dynamin: MC12 (a), MC13 (b), and MC63 (c). (a’–c’) The Golgi-specific mAb 10E6. Note the strong colocalization of dynamin immunoreactivity (green) and the Golgi apparatus (red). Identical staining patterns are seen with two other antidynamin antibodies (MC14 and MC62) not shown. In addition to the Golgi stain, punctate vesicular structures are also seen. No immunofluorescence staining was detected when preimmune sera or void volumes from the antibody affinity purification step were used (data not shown). (a’”–c’”) Corresponding phase contrast images. Bars, 20 μm.

Golgi structure, while late endosomes and lysosomes appeared as punctate vesicles that had a wider distribution than and shared little overlap with the dynamin label. Because early recycling endosomal compartments may have a perinuclear distribution, cells were also fixed immediately after incubation with fluorescently labeled dextran or transferrin before dynamin staining. In these preparations, we observed little, if any, overlap between dynamin and the labeled markers, indicating that they localize to distinct compartments.

Membrane-associated Dynamin Is Enriched in Subcellular Golgi Fractions

To support the morphological observations indicating that a dynamin-like protein associated with the Golgi apparatus, we used Western blot analysis to test for dynamin immunoreactivity on Golgi membrane fractions isolated from rat liver. We used three separate protocols, including those of Hamilton and co-workers (1991), Leelavathi et al. (1970), as modified by Fath et al. (1994), and Howell and Palade (1982), as described in Marks et al. (1994), to obtain preparations of Golgi membranes enriched significantly over those present in whole tissue homogenates. Because the results obtained by these methods were similar, only those from the modified Leelavathi preparation are shown here. With this method, we obtained a postnuclear supernatant, a total membrane pellet, a crude initial membrane fraction, a highly enriched mixed Golgi fraction, and fractions containing either Golgi vesicles or Golgi stacks. These subcellular fractions were immunoblotted (Fig. 9 A) for dynamin and several organelle marker proteins including α-mannosidase II (Moremen et al., 1991), β-COP (Allen and Kreis, 1986), and rab 6 (Goud et al., 1990) for the Golgi apparatus, and ribophorin II for the RER (Hortsch et al., 1986). As demonstrated in Fig. 9 A and Table I, which are representative of multiple experiments, both the dynamin immunoreactivity and the immunoreactivity for the different Golgi-associated proteins increased significantly in the stacked Golgi fraction compared to the total membrane fraction. In contrast, the RER-associated ribophorin II was significantly deenriched in this fraction. As shown in the corresponding electron micrographs (Fig. 9, B and C), this stacked Golgi...
Figure 6. Dynamin immunoreactivity on the Golgi apparatus in melanocytes. Cultured human melanocytes were processed for indirect immunofluorescence microscopy as was done for the fibroblasts in Fig. 5. Antidynamin antibodies MC12 (a), MC13 (b), and MC63 (c) all label the Golgi apparatus. No staining was observed when preimmune sera were used (data not shown). (a'–c') Corresponding phase-contrast images. Bars, 10 μm.

The fraction is replete with Golgi cisternae as well as coated buds and lipoprotein-containing vesicles that are characteristic of Golgi membranes. Despite the substantial enrichment of Golgi membranes in this fraction, it was important to define the amount of endocytic components that were present, since dynamin has been observed to associate with plasmalemmal coated pits and membrane invaginations. To this end, we immunoblotted all Golgi frac-
Figure 7. The Golgi apparatus in fibroblasts is labeled with a pAb to Vpslp. Cultured human fibroblasts were processed for indirect immunofluorescence microscopy using a pAb to Vpslp that cross-reacts with dynamin (Fig. 3 B). (a) This antibody strongly labels the Golgi apparatus. No staining was detected using an mAb to Vpslp (data not shown). (b) Corresponding phase-contrast image. Bar, 20 μm.

The protein has been used as a marker to study the maturation and posttranslational processing of proteins in the hepatic secretory and endocytic pathways (e.g., Sztul et al., 1985; Larkin et al., 1986). The 105-, 116-, and 120-kD forms of the receptor represent its passage through the RER and early Golgi, the Golgi, and plasma membrane and endocytic compartments, respectively. While the endocytic 120-kD form of the polymeric IgA receptor was present in all the Golgi fractions, it was not enriched in the stacked Golgi fraction as compared to the crude initial membrane fraction. There was a significant enrichment (more than sixfold), however, of the immature RER/Golgi form of the receptor in this stacked Golgi fraction. Thus, while putative endosomal compartments may contaminate all fractions to some extent, dynamin-enrichment parallels an increase in the secretory form of the polymeric IgA receptor.

To confirm the association of a dynamin-like protein with Golgi membranes, we isolated dynamin-immunoreactive membranes from subcellular Golgi fractions obtained from liver. Fractions enriched in Golgi membranes were prepared by two different methods, including that used to obtain the stacked Golgi fraction as discussed above and one used by Howell and Palade (1982) to obtain a sample enriched in Golgi heavy and Golgi light fractions, as described previously (Marks et al., 1994). These fractions were incubated with superparamagnetic beads that had been precoated with either antidynamin antibody MC63

Figure 8. Dynamin immunoreactivity in fibroblasts does not localize with late endosomes and lysosomes. Cultured human fibroblasts were incubated with FITC-conjugated dextran to label late endosomes and lysosomes (see Materials and Methods) before fixing and processing for indirect immunocytochemistry with the antidynamin antibody MC63. (a and c) Dynamin immunoreactivity detected with a TRITC-conjugated secondary antibody labels the Golgi apparatus (red). (b and c) Internalized dextran in late endosomes and lysosomes (green). Note that there is little overlap between the two signals (c, yellow). No qualitative differences in the dextran labeling were observed as a result of immunocytochemical processing (data not shown). Bar, 15 μm.
Methods). Fractions were characterized using antibodies against three different Golgi marker proteins, α-mannosidase II (Mann II), β-COP, and rab 6; the RER-associated protein, ribophorin II (Ribo II); and the polymeric IgA receptor (IgA rec). A dynamin-immunoreactive band that is detected in the postnuclear supernatant migrates with purified brain dynamin (D, 1 µg/lane). This dynamin-immunoreactive protein is desorbed in the total membrane pellet, indicating that it does not preferentially associate with membranes. Upon enriching for Golgi membranes, as determined by the increased immunoreactivity for α-mannosidase II, β-COP, rab 6, and the 105-kD form of the polymeric IgA receptor (IgA rec, lower band), there is a concomitant increase in dynamin immunoreactivity, with the greatest enrichment in the stacked Golgi fraction. This stacked Golgi fraction has little ribophorin II immunoreactivity, indicating that the RER is not enriched. Also, note that there is no increase in the immunoreactivity for the 120-kD form of the polymeric IgA receptor (IgA rec, upper band), indicating that endocytic compartments, likewise, are not enriched in this stacked Golgi fraction. Lines to the lower right of the panel indicate the positions, from top to bottom, of the 120-, 116-, and 105-kD forms of the polymeric IgA receptor, respectively. An equal amount of protein was loaded for each liver fraction (50 µg/lane). (B and C) Electron micrographs demonstrating numerous Golgi profiles in the isolated stacked Golgi fraction, including stacked Golgi elements (asterisks) and lipoprotein-containing vesicles (arrowheads). Coated buds and vesicles can also be seen (C, small arrows). Bars, 0.3 µm.

| Protein | TM | CM | G  | Gs |
|---------|----|----|----|----|
| Dymanin | 1.0| 1.7| 1.8| 5.1|
| Man II  | 1.0| 1.4| 3.1| 3.8|
| β-COP   | 1.0| 2.6| 2.8| 5.3|
| Rab 6   | 1.0| 1.8| 7.9| 15.6|
| Ribo II | 1.0| 1.2| 0.4| 0  |
| IgA rec 120 | 1.0| 3.3| 1.0| 1.0|
| IgA rec 116*| 1.0| 3.6| 2.2|    |
| IgA rec 105*| 1.0| 2.9| 6.5|    |

Subcellular fractions isolated from rat liver were analyzed by Western blotting as in Fig. 9 and for densitometry as described in Materials and Methods. TM, total membrane fraction; CM, crude initial membrane fraction; G, mixed Golgi fraction; Gs, stacked Golgi fraction; Mann II, α-mannosidase II; Rab 6, ribophorin II; and IgA rec 120, 116, and 105 represent the 120-kD (plasma membrane/endosomal), 116-kD (late Golgi), and 105-kD (RER/early Golgi) forms of the polymeric IgA receptor, respectively. Relative densitometric values for each fraction were normalized to the total membrane fraction and are represented as fold increase.

*Since little polymeric IgA receptor immunoreactivity was detected in the total membrane fraction, relative values were normalized to the crude initial membrane fraction.
Golgi membranes are immunoisolated using an antibody to dynamin. (A) Starting and immunoisolated fractions were analyzed by immunoblotting with an antibody to the Golgi protein α-mannosidase II. Golgi fractions (Golgi) were isolated from liver using two different established methods, and consisted of either a stacked Golgi fraction (Prep I), or a mixture of Golgi heavy and Golgi light membrane fractions (Prep II; see Materials and Methods). These fractions were incubated with beads that were coated with affinity-purified antibody MC63, and the material that bound (Beads) or did not bind (Unbnd) to the beads was collected. Protein loads were equivalent to the starting Golgi fraction for each lane. There was a significant accumulation of the Golgi marker protein in the immunoisolated bead fractions, while very little remained in the unbound fractions. (B–D) Electron micrographs representative of the immunoisolated fraction obtained from the stacked Golgi fraction with antibody MC63-coated beads, as described above. Multiple Golgi profiles (asterisks), lipoprotein-containing vesicles (arrowheads), and fenestrated membranes associated with Golgi elements (C and D, open arrows) are clearly visible. (E) Electron micrograph representative of a control fraction obtained after incubating the stacked Golgi fraction with beads that were coated with an anti-rabbit IgG linker antibody alone. Dark granules are noncellular debris. Few, if any, membranes are associated with the beads. Bars, 0.6 μm (B and E) and 0.15 μm (C and D).

Discussion

A Dynamin-related Protein on the Golgi Apparatus

In this study, we have used multiple pAbs made to distinct sites of dynamin to determine its cellular distribution by indirect immunofluorescence microscopy of cultured cells. In addition, we have performed Western blot and EM analysis of both Golgi-enriched and dynamin-immunore-

branes indiscriminately, but recognized a specific dynamin antigen associated with the Golgi cisternae. As shown in the corresponding electron micrographs, the morphology of the immunoisolated membranes is consistent with that of Golgi (Fig. 10, B–D) including numerous cisternae, fenestrations, and lipoprotein-containing vesicles. In contrast, isolations obtained with control beads were devoid of membranes (Fig. 10 E). These results confirm that a dynamin-like protein is present on Golgi membranes.

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active membrane fractions isolated from the liver. These pAbs were directed against synthetic peptides representing three different regions conserved within the dynamin family of proteins (Fig. 1) and should recognize all of the known dynamin isoforms. By Western blot analysis (Fig. 2) and immunoprecipitation studies (Fig. 3), we show that these pAbs are both sensitive and specific for dynamin. These pAbs react with a 100-kD protein in fibroblasts that has similar biochemical properties to brain dynamin, such as binding and pelleting with membrane fractions and assembled microtubules in vitro (Fig. 4). We assume that this immunoreactive protein is Dyn2, which is found in all tissues (Cook et al., 1994; Sontag et al., 1994), since the other dynamin isoforms are restricted to neural tissues and testis (Nakata et al., 1991, 1993; Cook et al., 1994; Sontag et al., 1994).

The immunocytochemical studies described here indicate that dynamin has two prominent localizations in the cell populations examined. These include a ubiquitous, punctate staining of the cytoplasm that is generally consistent with the observations of others (Scaife and Margolis, 1990; Herskovits et al., 1993a; Noda et al., 1993; Gass et al., 1995) and unexpectedly, a marked staining of the Golgi apparatus, as confirmed by double-labeling studies with a Golgi-specific mAb (Fig. 5). This Golgi localization is striking and observed consistently in all the cell types examined using several different affinity-purified antidynamin antibodies (Figs. 5 and 6). Most often, both of these staining patterns are seen concomitantly in the same cell, indicating that the recognized antigen may have a differential distribution. These morphological observations are reinforced further through Western blot analysis of subcellular fractions isolated from the liver (Fig. 9). Although prevalent in a cytosolic fraction, the amount of a 100-kD dynamim-immunoreactive protein in a highly enriched Golgi membrane fraction is increased more than fivefold when compared to a total membrane fraction (Table 1). This result argues that the association of this dynamin-like protein with the Golgi fraction is physiological and does not represent random sticking to membranes. We find it significant that this enrichment of membrane-associated dynamin parallels the enrichment of several different Golgi-associated proteins in this same fraction. Furthermore, only dynamin is recognized in this enriched Golgi fraction, which is replete with other associated GTP-binding proteins. A concern with any subcellular fractionation procedure is that an isolated fraction may be comprised of vesiculated membranes from multiple types of organelles. It is for this reason that we have used three separate methods to obtain fractions enriched in Golgi marker proteins and show that dynamin is coenriched in each of these fractions over a total membrane fraction. Furthermore, we have used an antibody to the polymeric IgA receptor to show that the 120-kD plasmalemmal/endosomal form of this receptor is not enriched in these Golgi membrane preparations. To confirm that it is Golgi and not other membranes that are responsible for the dynamin enrichment in these fractions, we immunosolated membranes using beads coated with the sensitive and specific antidynamin antibody MC63. Substantially, these MC63-coated beads bound to Golgi elements, as determined both biochemically and morphologically (Fig. 10). Since MC63 specifically immunoprecipitated dynamin from total protein extracts of brain, fibroblasts, and liver (Fig. 3 and data not shown), and since Western blot analysis of subcellular Golgi fractions indicated this antibody reacted with only a single 100-kD protein that migrated with purified dynamin (Fig. 9), we are convinced that a dynamin-like protein is localized to the Golgi apparatus.

Although this is the first description of a dynamin-related protein associated with the Golgi apparatus in mammalian cells, Vps1p localizes to the Golgi in yeast, where it performs an essential function in sorting proteins destined for the vacuole (Rothman et al., 1990; Vater et al., 1992; Wilsbach and Payne, 1993). Because the amino-terminal domains of dynamin and Vps1p share 45% identity (Obar et al., 1990), it is possible that the antibodies to dynamin used in this study also recognize a mammalian Vps1p homologue. To address this, we immunoprecipitated proteins from brain protein extracts using each antidynamin antibody (MC12, MC13, and MC63) and analyzed the immunoprecipitates by SDS-PAGE and by immunoblotting with mAbs and pAbs to Vps1p. Each of the antidynamin antibodies immunoprecipitated a 100-kD protein that was recognized by a Dyn1 tail-specific antibody, indicating that this protein is indeed dynamin. In addition, the pAb to Vps1p recognized both purified brain dynamin and the immunoprecipitated dynamin (Fig. 3). In contrast, the mAb to Vps1p did not react with any immunoprecipitated protein. Remarkably, the anti-Vps1p pAb also strongly labeled the Golgi apparatus in cultured human fibroblasts (Fig. 7), whereas the anti-Vps1p mAb did not (data not shown). This provides a provocative correlation between antibodies that react with dynamin by Western blot analysis and those that label the Golgi apparatus in cultured cells by indirect immunofluorescence. The extensive characterization studies detailed here indicate that the antidynamin antibodies used in this study are specific for the dynamins, although we cannot exclude the possibility that other closely related members of the dynamin family are also recognized. Nonetheless, these results provide novel and important evidence that a dynamin-like protein is present on the Golgi apparatus in mammalian cells.

Because of the predicted endocytic function of dynamin, we were surprised to find dynamin localized to the Golgi (Figs. 5 and 6), and not endosomes and lysosomes (Fig. 8), or with clathrin at the plasma membrane (data not shown). Furthermore, immunofluorescence studies of cultured cells by others have reported a diffusible granular stain (Scaife and Margolis, 1990; Herskovits et al., 1993a), and a punctate, vesicular localization (Scaife and Margolis, 1990; Herskovits et al., 1993a; Faire and Bonder, 1993; Noda et al., 1993; Damke et al., 1994, 1995). We believe there are at least two explanations for these contrasting results. First, it is likely that the many antidynamin antibodies currently available differ in their ability to recognize endogenous dynamin. For example, we are aware of only one antibody to dynamin that localizes with clathrin and labels membrane invagination at the cell surface (Damke et al., 1994; Takei et al., 1995). A similar situation exists for the numerous antibodies generated against the microtubule-associated motor enzyme kinesin, in which only a few have been shown to consistently stain vesicular compartments (e.g., Pfister et al., 1989; Henson et al., 1992; Marks et al.,
Correlating Dynamin Localization with Function

Along with a predicted endocytic function, the shibire gene product in D. melanogaster has been proposed to play a role in other cell processes including cell adhesion, growth cone formation, neurite extension and elongation, as well as cell differentiation (Buzin et al., 1978; Kim and Wu, 1987; Poodry, 1990). It is unknown whether dynamin participates in this wide variety of cellular processes directly or via endocytic mechanisms. The morphological and biochemical observations presented here provide support for additional functions of the dynamin family through interactions with membranous organelles distinct from the endocytic pathway. Although unanticipated, the association of dynamin with the secretory pathway is supported by several previous observations made by others. For example, Western blot analysis of membrane fractions from the brain detected dynamin in fractions containing crude microsomes and synaptic vesicles (Noda et al., 1993; Tuma et al., 1993). Interestingly, EM of dynamin-enriched membrane fractions from rat brain showed a population of smooth, vesiculated membranes devoid of clathrin coats (Scaife and Margolis, 1990). In addition, a study examining the distribution of dynamin in ligated nerves showed an accumulation of dynamin on the proximal side of the ligation, where outward moving secretory vesicles collect (Noda et al., 1993). Antidynamin antibodies also strongly stained the cortical secretory granules in sea urchin eggs (Faire and Bonder, 1993). It is not yet known whether dynamin actively participates in the secretory pathways of these cells or simply acts as “passive cargo” that is moved toward the plasma membrane to aid in membrane retrieval and endocytosis.

The observations reported here, together with the results of others, provide provocative evidence that dynamins may interact with multiple membrane systems and vesicular pathways. It is attractive to speculate that, as proposed by Kelly (1995), different dynamin-like proteins may perform similar vesicle-budding or protein-sorting functions at both the plasma membrane and Golgi complex. Such a prediction is consistent with many other proteins such as ADP ribosylation factors (Peters et al., 1995; for reviews see Donaldson and Klausner, 1994; Welsh et al., 1994), clathrin (for reviews see Riezman, 1993; Robinson, 1994), adaptins (for reviews see Schmid, 1992; Robinson, 1994), and caveolins (Dupree et al., 1993; Smart et al., 1994; for review see Kurzchalia et al., 1994), which are believed to mediate vesicle budding and fusion events at the plasma membrane and the Golgi apparatus. It is certainly attractive to compare the dynamins with the adaptins that are involved in the formation of clathrin-coated vesicles, both at the cell surface during endocytosis (α and β adaptins), and at the Golgi apparatus during secretion (γ and β’ adaptins). Thus, like adaptins, the dynamins could participate in clathrin-mediated events at two cellular locations. How the different isoforms of dynamin may interact with these membrane systems needs to be defined. Future studies testing the function of dynamins in cell-free Golgi transport assays will certainly prove to be both interesting and insightful.

Primary cultures of human melanocytes and fibroblasts were kindly provided by the laboratory of Dr. M. Pittelkow. Antibodies were obtained as gracious gifts from Drs. F. Brodsky, W. Brown, E. Conibear, M. Gist Farquhar, B. Goud, D. Meyer, and T. Stevens, and purified brain dynamin was a gift from Dr. G. Waters. Special thanks to Ms. T. Cook, K. Kotz, Drs. J. Larkin and D. Marks, and Mr. D. Zacharias for critical reading of the manuscript and for providing helpful comments and discussions. Dr. J. Larkin was also instrumental in establishing the immunolocalization procedure used for this study. We thank Mr. K. Hsiao, E. Krueger, and Mrs. B. Oswald for providing expert technical assistance. This study was funded by National Institutes of Health grant DK44650 to M.A. McNiven.

Received for publication 30 June 1995 and in revised form 22 February 1996.

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