J. Biochem. 125, 780-789 (1999)

Dynamin II Is Involved in Endocytosis but Not in the Formation of Transport Vesicles from the *trans*-Golgi Network[1]

Kazuo Kasai,*,[2] Hye-Won Shin,† Chisa Shinotsuka,† Kazuo Murakami,* and Kazuhisa Nakayama†,‡,[3]

*Institute of Applied Biochemistry, †Institute of Biological Sciences, and ‡Gene Experiment Center, University of Tsukuba, Tsukuba Science City, Ibaraki 305-8572

Received November 6, 1998; accepted December 28, 1998

Dynamins are a family of ~100-kDa GTPases that are thought to play a pivotal role in the formation of endocytic coated vesicles. There are three dynamin genes in mammals: dynamin I is neuron-specific, dynamin II shows ubiquitous expression, and dynamin III is expressed in testis, brain, and lung. However, most studies on the functions of dynamins to date have been restricted to dynamin I. In the present study, we show that, like dynamin I, dynamin II is involved in receptor-mediated endocytosis. While this study was in progress, Jones *et al.* [Jones, S.M., Howell, K.E., Henley, J.R., Cao, H., and McNiven, M.A. (1998) *Science* 279, 573-577] reported that dynamin II is localized in the *trans*-Golgi network (TGN) and involved in the formation of constitutive transport vesicles and clathrin-coated vesicles from this compartment. However, immunofluorescence analyses and experiments using cells transfected with dominant-negative dynamin II failed to show any evidence for localization of dynamin II in the TGN or for its involvement in vesicle formation from this compartment. Our data thus indicate that dynamin II is involved in endocytosis but not in the formation of transport vesicles from the TGN.

Key words: dynamin, endocytosis, *trans*-Golgi network, vesicular transport.

Dynamins are a family of high molecular weight GTP-binding proteins with an intrinsic GTPase activity that regulate formation of endocytic coated vesicles (for reviews, see Refs. *1-6*). Mammalian dynamin family is composed of three members, dynamins I, II, and III, with some splicing variants. Dynamin I is expressed exclusively in neural tissues, dynamin II is expressed ubiquitously, and dynamin III is enriched in brain, lung and testis (*7*). The *Drosophila shibire* gene product shows ~70% amino acid identity to mammalian dynamins. Since temperature-sensitive *shibire* mutants show a pleiotropic defect in endocytosis (*8-10*), it has been believed that mammalian dynamins are also involved in endocytosis. Studies using cells transfected with GTP-binding defective mutants of dynamin I have established a role for dynamin in receptor-mediated endocytosis in mammalian cells (*11-13*). Later morphological analysis and *in vitro* experiments have shown that dynamin self-assembles into rings and/or spirals around the necks of invaginated clathrin-coated pits, thereby suggesting a fascinating model that conformational change of dynamin induced by GTP hydrolysis leads to pinching off the budding coated vesicles (*14, 15*). Most recently, elegant cell-free studies have confirmed this model, although GTP hydrolysis appears not to be necessary for the vesicle formation (*16, 17*).

Given that there are many vesicular trafficking events within cells, it is possible that dynamins are involved not only in endocytosis from the plasma membrane but also in vesicle formation from other intracellular compartments. One candidate step is the formation of clathrin-coated vesicles from the *trans*-Golgi network (TGN), because this is thought to take place by a similar mechanism to the formation of such vesicles from the plasma membrane, although they use different adaptor protein complexes, AP-1 and AP-2, respectively (*18, 19*). The data of Henley and McNiven support this possibility (*20*). They raised antibodies against synthetic peptides corresponding to conserved regions of the dynamin family and showed by immunofluorescence microscopy that these antibodies strongly labeled the Golgi complex in cultured cells. An immunoelectron microscopic study further suggested the localization of dynamin II in the TGN, although uncertainty remained about its subcellular localization (*21*). Taking into account its ubiquitous expression, we speculated that dynamin II might play a role in vesicle formation from the

[1] This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, the University of Tsukuba Research Projects, the Asahi Glass Foundation, the Sumitomo Foundation, the Uehara Memorial Foundation, and Sankyo Co., Ltd.

[2] Recipient of a fellowship from the Japanese Society for Promotion of Science for Japanese Junior Scientists.

[3] To whom correspondence should be addressed. Tel.: +81-298-53-6005, Fax: +81-298-53-6006, E-mail: kazunaka@sakura.cc.tsukuba.ac.jp

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; NRK, normal rat kidney; TGN, *trans*-Golgi network; VSVG, vesicular stomatitis virus G-protein. For the reasons described in the text, we used HA-tagged dynamin IIaa in the present study and refer to it as dynamin II in the text for convenience, except where specifically indicated.

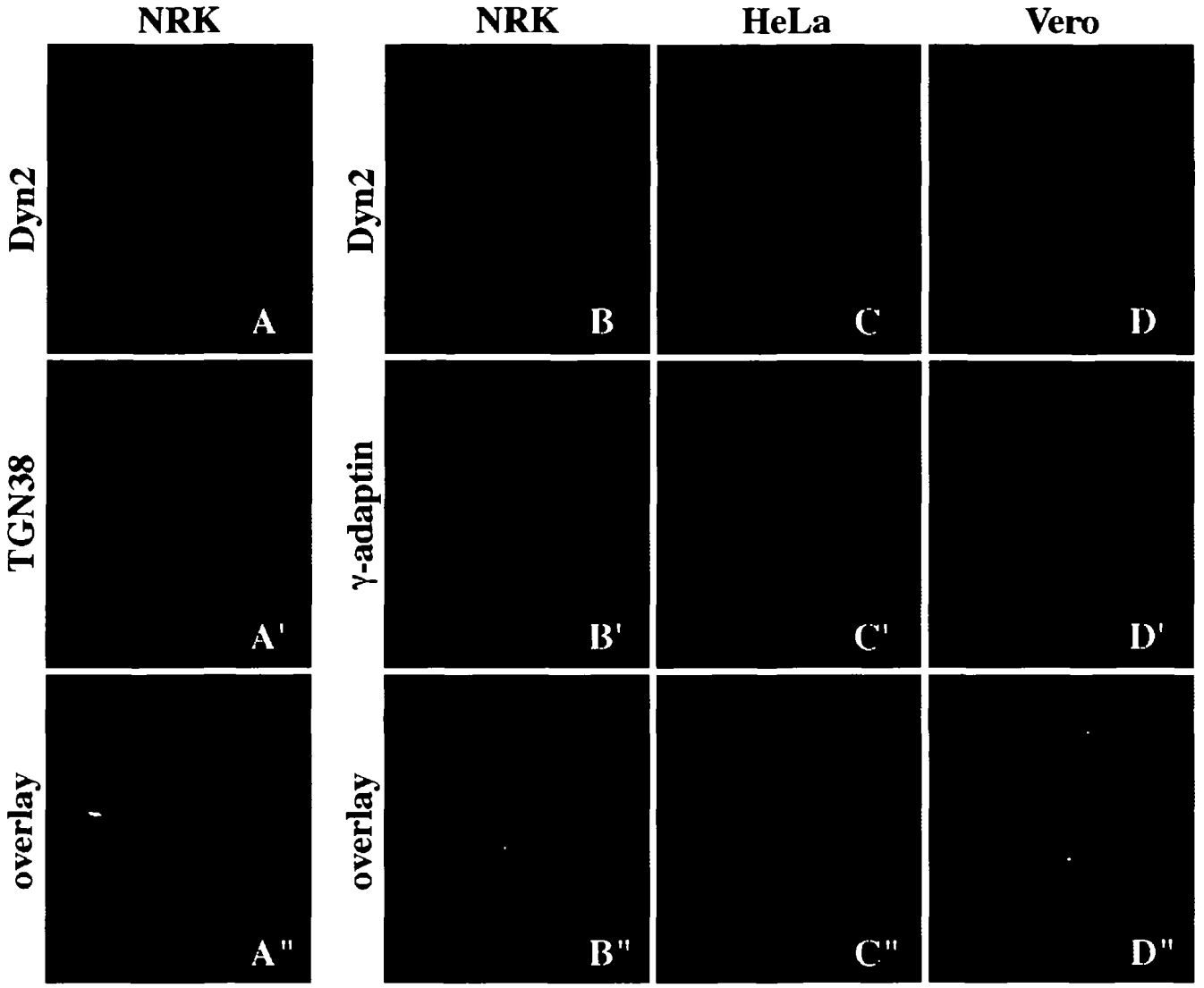


Fig. 1. **Subcellular localization of dynamin II in various cell lines.** NRK (panels A and B), HeLa (panel C), and Vero (panel D) cells transfected with pcDNA3-HA-Dyn2a were double-stained with monoclonal rat anti-HA antibody, 3F10 (top panels), and either monoclonal mouse anti-TGN38 antibody, 2F7.1 (panel A′), or monoclonal mouse anti-γ-adaptin antibody, #88 (panels B′, C′, and D′). Overlays are shown in the bottom panels.

TGN. In an attempt to support this speculation, we expressed dynamin II in various culture cell lines and examined its subcellular localization by indirect immunofluorescence microscopy. However, we could not observe typical Golgi-like staining for dynamin II. While this study was in progress, however, Jones *et al.* reported that dynamin II tagged with green fluorescent protein (GFP) was localized in the TGN and in a cell-free assay anti-dynamin antibodies inhibited the formation of constitutive exocytic vesicles and clathrin-coated vesicles from the TGN (*22*). We therefore began to address this issue in detail.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Expression vectors for dynamin Iaa (pcDNA3-HA-Dyn1a) and dynamin IIaa (pcDNA3-HA-Dyn2a) with an influenza virus hemagglutinin (HA) epitope sequence were constructed by subcloning of cDNA fragments covering the entire coding sequence of rat dynamin Iaa and dynamin IIaa (kindly provided by Dr. Thomas Südhof, University of Texas Southwestern Medical Center, Dallas, TX; Ref. *23*), respectively, into the multiple cloning site of pcDNA3-HAN, a derivative of pcDNA3 (Invitrogen, Carlsbad, CA) with the HA sequence downstream of the cytomegalovirus promoter (*24*). An expression vector for dynamin IIba (pcDNA3-HA-Dyn2b) was constructed by replacing a cDNA region of pcDNA3-HA-Dyn2a with the corresponding dynamin IIba cDNA region. An expression vector for GFP-tagged dynamin IIaa (pEGFP-Dyn2a) was constructed by subcloning of the dynamin IIaa cDNA fragment into the multiple cloning site of pEGFP-C1 or pEGFP-N1 (Clontech, Palo Alto, CA). K44A mutation was introduced into the dynamin I or II cDNA by a PCR-based strategy. An expression vector for cathepsin D (pcDNA3-CatD) was constructed by subcloning into pcDNA3 of a cDNA fragment covering the entire coding sequence of

human cathepsin D, which was obtained by PCR-amplification of a human liver cDNA library using a set of primers synthesized on the basis of the published sequence (*25*). An expression vector for vesicular stomatitis virus G-protein (VSVG) tsO45 tagged with GFP (*26*) was a kind gift from Dr. Jennifer Lippincott-Schwartz (NIH, Bethesda, MD). The DNA insert was transferred into pcDNA3 (pcDNA3-VSVG-EGFP) and used for transfection.

Antibodies—Monoclonal mouse (2F7.1) and polyclonal rabbit (AWα29) antibodies against rat TGN38 (*27*) were kindly provided by Dr. George Banting (University of Bristol, Bristol, UK). Monoclonal mouse anti-γ-adaptin antibody (clone #88) was purchased from Transduction Laboratories (Lexington, KY). Monoclonal rat (3F10) and monoclonal mouse (12CA5) antibodies against the HA epitope were from Boehringer Mannheim GmbH (Mannheim, Germany). Polyclonal rabbit anti-human cathepsin D antibodies were from Calbiochem-Novabiochem (San Diego, CA). All fluorescence-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunofluorescence Analysis—Rat hepatocyte Clone 9 cells, HeLa cells, monkey kidney Vero cells or normal rat kidney (NRK) cells grown in wells of eight-well Lab-Tek-II chamber slides (Nunc, Roskilde, Denmark) were transfected with the dynamin expression vector (1.5 μg/well) using a TransIT-LT1 polyamine transfection reagent (Pan Vera, Madison, WI), cultured at 37°C for 12 h, and fixed and permeabilized with methanol at −20°C for 5 min. The fixed and permeabilized cells were then processed for indirect immunofluorescence microscopy as described previously (*24, 28*). Briefly, the cells were incubated with anti-HA antibody in combination with either anti-TGN38 or anti-γ-adaptin antibody, then incubated with FITC- and Cy3-conjugated secondary antibodies. The stained cells were observed with a laser-scanning confocal microscope (TCS-NT, Leica Lasertechnik GmbH, Heidelberg, Germany). To examine the effects of dynamin expression on transferrin uptake, HeLa cells were transfected with the dynamin vector, then sequentially incubated with normal medium for 12 h, with serum-free medium containing 0.1% bovine serum albumin for 1 h to deplete transferrin, and with serum-free medium containing 0.1% bovine serum albumin and 40 μg/ml BODIPY-transferrin (Molecular Probes, Eugene, OR). The cells were then fixed with phosphate buffered saline containing 4% paraformaldehyde and 3% sucrose and permeabilized with Tris-buffered saline containing 50 μg/ml digitonin. The fixed and permeabilized cells were stained with anti-HA antibody and Cy3-conjugated secondary antibody. To examine the effects of dynamin expression on VSVG-GFP transport, Clone 9 cells were co-transfected with the dynamin vector (0.5 μg/well) and pcDNA3-VSVG-EGFP (1.0 μg/well), and cultured at 39.5°C for 12 h. Prior to fixation and permeabilization with cold methanol, some wells of cells were further incubated at 32°C for 1 h or 3 h. The fixed and permeabilized cells were stained with anti-HA antibody and Cy3-conjugated secondary antibody.

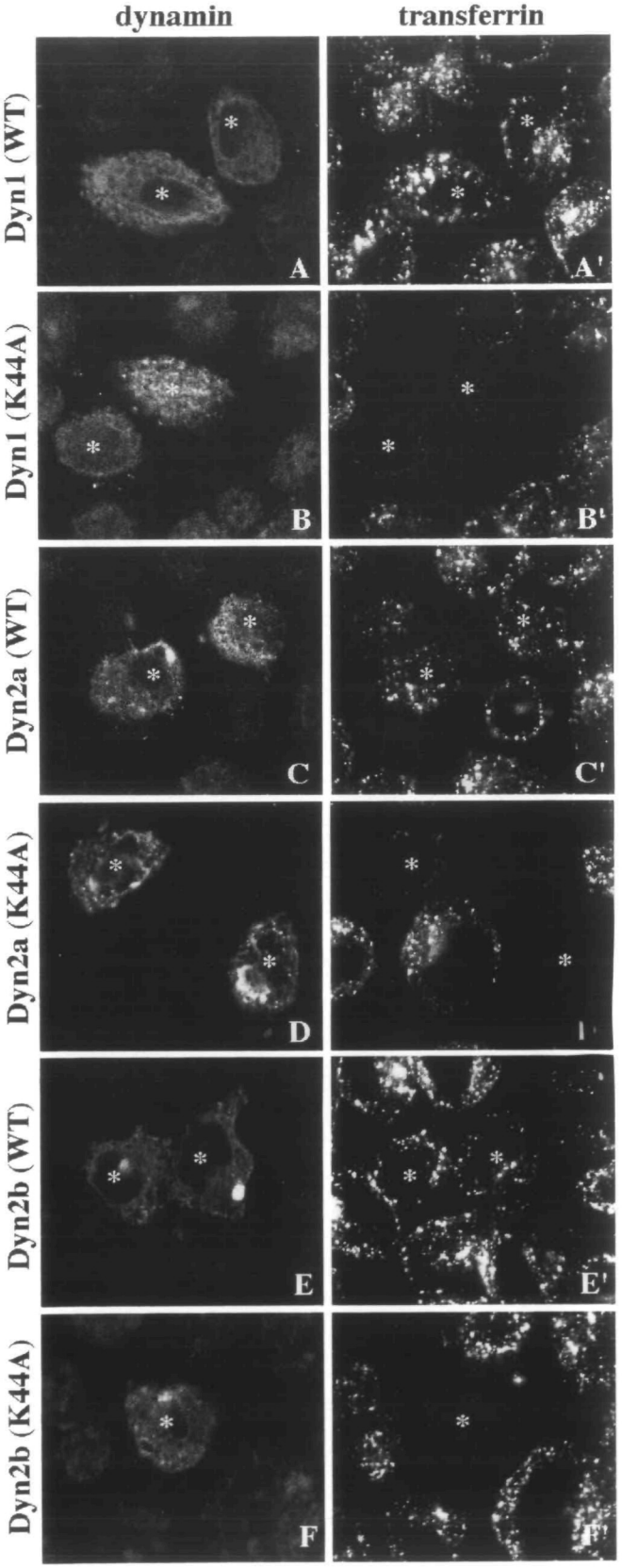
Metabolic Labeling and Immunoprecipitation—Mouse pituitary AtT-20 cells grown in wells of six-well cluster plates were co-transfected with the dynamin expression vector (0.7 μg/well) and pcDNA3-CatD (0.3 μg/well) with a FuGENE 6 transfection reagent (Boehringer Mannheim GmbH, Mannheim, Germany). After the transfection, the cells were incubated with methionine-free medium for 1 h, then with methionine-free medium containing 150 μCi/ml of EXPRESS Methionine/Cysteine protein labeling mix (NEN Life Science Products, Boston, MA) for 7 h. The cells and media were separately collected. The cells were then lysed in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.1% SDS). The cell lysates and the media were subjected to immunoprecipitation with anti-human cathepsin D antibody and protein A-Sepharose (Sigma, St. Louis, MO), electrophoresed on a 12.5% SDS-polyacrylamide gel, and analyzed using a BAS5000Mac bioimaging analyzer (Fuji Photo Film, Tokyo).

RESULTS

Because most studies on the role of dynamins to date have focused upon dynamin I, little information is available on dynamin II. A morphological study revealed that dynamin II was localized in clathrin-coated pits on the plasma membrane (*29*). Another study suggested localization of dynamin II in the TGN, although uncertainty remained about its subcellular localization (*21*). In a first attempt to address our speculation that dynamin II may play a role in vesicle formation from the TGN, we transiently transfected various cell lines with the expression vector for HA-tagged dynamin II (pcDNA3-HA-Dyn2a), and compared its subcellular localization with that of TGN38, a TGN marker protein, or that of γ-adaptin, a subunit of the TGN-associated clathrin adaptor AP-1 (Fig. 1). In NRK cells transiently transfected with the HA-dynamin II vector and stained with anti-HA antibody, the dynamin II staining was distributed throughout the cytoplasm, as observed for dynamin I by other researchers (*12, 13*; see also Fig. 3), although some vesicular structures were often observed (panels A and B). A similar staining pattern was observed using monoclonal anti-dynamin antibody, hudy-1 (data not shown). By contrast, TGN38 and γ-adaptin showed a typical Golgi-like paranuclear localization in these cells (panels A′ and B′). As a result, little overlap between dynamin II and the TGN markers (pseudocolored yellow region) was observed (panels A″ and B″). Although we used cells fixed with cold methanol for the immunostaining, because anti-TGN38 antibody was less reactive in cells fixed with paraformaldehyde, a similar dynamin II staining pattern was observed in cells fixed with 4% paraformaldehyde (data not shown). Furthermore, essentially the same results were obtained using HeLa (panels C–C″) and Vero (panels D–D″) cells transfected with the HA-dynamin II construct, excluding the possibility that the observations using NRK cells were not specific for the cell line used as the host. Thus, dynamin II appeared not to be specifically localized in the TGN.

The marked structural similarity of dynamin II to dynamin I makes it likely that dynamin II is also involved in receptor-mediated endocytosis. However, no such involvement has yet been reported, although immunoelectron microscopic study has shown that dynamin II is distributed on coated pits on the plasma membrane (*29*). To address this issue, we constructed an expression vector for a K44A mutant of dynamin II [pcDNA3-HA-Dyn2a (K44A)], transfected it into HeLa cells, and examined its effect on the uptake of fluorescence-labeled transferrin,

because a K44A mutant of dynamin I is defective in GTP binding and hydrolysis by virtue of the point mutation in the first GTP-binding motif, and its expression in cells has been shown to dominantly inhibit internalization of transferrin (*11-13*). The results are shown in Fig. 2. Cells overexpressing wild-type dynamin I (panels A and A′) and wild-type dynamin II (panels C and C′) both showed efficient internalization of BODIPY-transferrin, as in untransfected cells. In contrast, cells overexpressing the dominant-negative mutant of dynamin I (panels B and B′) and dynamin II (panels D and D′) both failed to internalize the fluorescent transferrin: the cells expressing the dynamin mutant (marked by asterisks) showed no intracellular accumulation of the fluorescent transferrin. From these data, we conclude that dynamin II, like dynamin I, is involved in receptor-mediated endocytosis.



Because our data thus far presented show no evidence for the localization of dynamin II in the TGN, we had discontinued working on the possible involvement of dynamin II in vesicle formation from the TGN. However, Jones *et al.* (*22*) recently reported that dynamin II is localized in the TGN and involved in the formation of transport vesicles from this compartment. They showed that the labeling of GFP-tagged dynamin II expressed in a rat hepatocyte cell line, Clone 9, is superimposed on the staining for TGN38 and for the clathrin heavy chain in the paranuclear region by immunofluorescence microscopy, and that antibodies to dynamins (an antibody specific for dynamin II and pan-dynamin antibodies) are capable of inhibiting the formation of constitutive transport vesicles and clathrin-coated vesicles from the TGN in a reconstituted cell-free assay. We therefore resumed our study with intent to clarify the reason for the discrepancy between our and their data.

We first addressed whether the difference in the cell line used as the host might result in the discrepancy. We therefore transiently transfected the HA-dynamin constructs into Clone 9 rat hepatocytes, the same as those used by Jones *et al.* (*22*), and compared the localization of HA-dynamin II with that of either TGN38 or γ-adaptin by immunofluorescence microscopy (Fig. 3). However, the staining pattern for dynamin II (panel Ac) was similar to that observed in other cell lines (Fig. 1); we failed to observe a paranuclear Golgi-like staining for dynamin II, in contrast to the observation of Jones *et al.* (*22*). We examined a number of transfected cells with varying staining intensities but could not observe paranuclear staining for dynamin II, excluding the possibility that overexpression of dynamin II might have resulted in its relatively uniform cytoplasmic distribution. Furthermore, the dynamin II staining was not significantly superimposed on that for TGN38 (panels A, c–c″) or γ-adaptin (panels B, c–c″), as was the case for dynamin I (panels A and B, a–a″). Similar TGN staining patterns were observed with polyclonal anti-TGN38 antibody, AWα29 (data not shown), in place of monoclonal anti-TGN38 antibody, 2F7.1. It is interesting to note, however, that some tendency was

Fig. 2. **Effect of wild-type and K44A mutant of dynamins I and II on transferrin uptake.** HeLa cells transfected with pcDNA3-HA-Dyn1a (panel A), pcDNA3-HA-Dyn1a(K44A) (panel B), pcDNA3-HA-Dyn2a (panel C), pcDNA3-HA-Dyn2a(K44A) (panel D), pcDNA3-HA-Dyn2b (panel E), or pcDNA3-HA-Dyn2b(K44A) (panel F) were incubated with BODIPY-transferrin (right panels) followed by staining with anti-HA antibody (left panels) as described under "EXPERIMENTAL PROCEDURES."

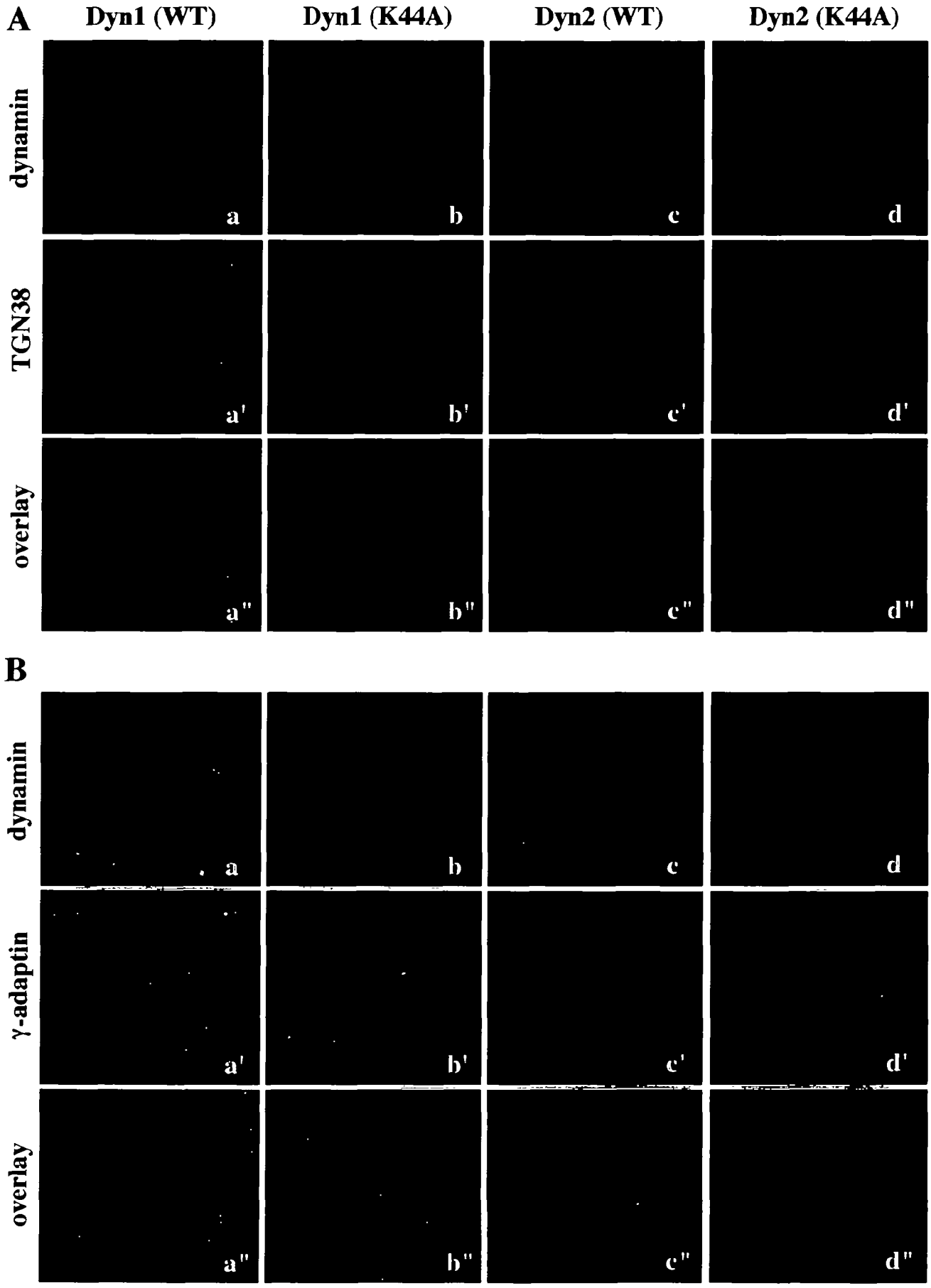


Fig. 3. **Subcellular localization of wild-type and K44A mutant of dynamins I and II in Clone 9 cells.** Clone 9 cells transfected with pcDNA3-HA-Dyn1a (panels Aa and Ba), pcDNA3-HA-Dyn1a(K44A) (panels Ab and Bb), pcDNA3-HA-Dyn2a (panels Ac and Bc), or pcDNA3-HA-Dyn2a(K44A) (panels Ad and Bd) were double-stained with anti-HA antibody (A and B, top panels) and either anti-TGN38 (A, middle panels) or anti-γ-adaptin (B, middle panels) antibody. Overlays are shown in the bottom panels.

observed when the K44A mutant of dynamin II was expressed in Clone 9 cells: namely, the dynamin II mutant was often localized in large vesicular structures throughout the cytoplasm (panel Ad) as compared with the relatively uniform distribution of wild-type dynamin II (panel Ac); and in cells expressing the dynamin II mutant the staining patterns for TGN38 (panel Ad″) and γ-adaptin (panel Bd″) were more compact than those in untransfected and wild-type dynamin II expressing cells (panels A and B, c″), although such compact TGN structures were also observed in cells expressing the K44A dynamin I mutant (panels A and B, b″). Because TGN38 and other TGN-membrane proteins, such as furin, cycle between the TGN and plasma membrane, and because retrieval of these proteins from the plasma membrane is mediated by clathrin-coated vesicles, we speculate that the reduced intensity of the TGN38-staining at the TGN may be due to the reduced retrieval of this membrane protein from the plasma membrane caused by expression of the dominant-negative mutant of dynamin II or dynamin I.

Another difference between our experimental system and that of Jones *et al.* (*22*) is that we used HA-tagged dynamin II, while they used the GFP-tagged form. We therefore constructed an expression vector for dynamin II tagged with GFP at the NH_2- or COOH-terminus, and transfected it into Clone 9 cells. As shown in Fig. 4, panel A, NH_2-terminally GFP-tagged dynamin II was distributed throughout the cytoplasm in a pattern not significantly different from that of HA-dynamin II (Fig. 2A, panel c). A similar labeling pattern was observed for the COOH-terminally tagged form (data not shown). Again, no significant overlap between the labeling of GFP-dynamin II and the staining for TGN38 was observed (compare panels A and A′). Like cells expressing the HA-tagged K44A dynamin II mutant, the cells expressing the GFP-tagged mutant showed vesicular distribution of dynamin II (panel B) and rather compact staining for TGN38 (panel B′).

A third difference between the experimental systems of us and Jones *et al.* is that they used Clone 9 cell lines stably expressing GFP-dynamin II, whereas we used cells transiently expressing dynamin II. It was possible that overexpression of dynamin II might have resulted in its aberrant subcellular localization, although we examined a number of individual transfected cells with varying levels of dynamin II expression but could not observe its TGN-like localization in any of them. We therefore established Clone 9 cell lines stably expressing GFP-dynamin II and examined its subcellular localization. As shown in Fig. 5, the labeling of GFP-dynamin II was not significantly superimposed on the staining for TGN38 (panels A and A′) or γ-adaptin (panels B and B′).

Dynamin II exists as four alternative splicing variants, which differ in two regions from each other (*23*). The first differentially spliced region is composed of 46 amino acids with 13 amino acid substitutions in the middle of the dynamin II polypeptide, and the second is a 4-amino-acid sequence, which is either present in or absent from different variants, 70 amino acids downstream of the first region (reviewed in Ref. *4*). In the above experiments, we used the dynamin IIaa variant. Although the variant was the same as that used by Jones *et al.* (*22*), we nonetheless constructed an expression vector for dynamin IIba (pcDNA3-HA-Dyn2b) and examined the possibility that the splicing variants of dynamin II showed different subcellular distributions. As shown in Fig. 4C, the localization of wild-type dynamin IIba was not significantly different from that of wild-type dynamin IIaa. In addition, cells expressing the

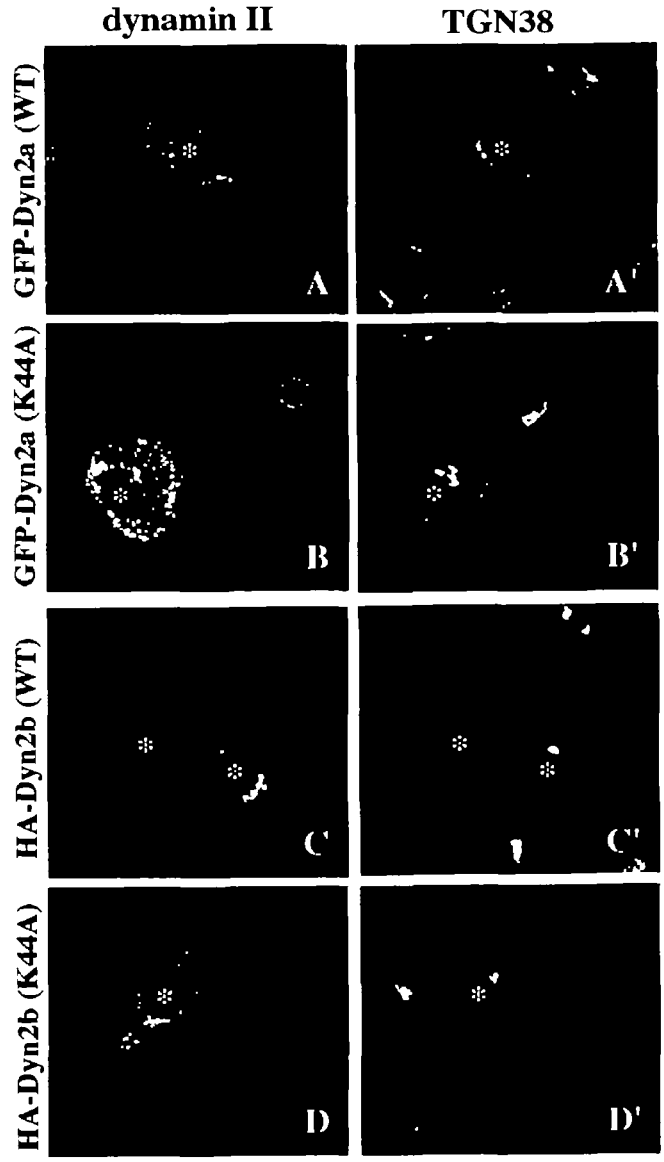


Fig. 4. **Subcellular localization of wild-type and K44A mutant of GFP-tagged dynamin IIaa and HA-tagged dynamin IIba.** A and B, Clone 9 cells transfected with pEGFP-Dyn2a (panel A) or pEGFP-Dyn2a(K44A) (panel B) were stained with anti-TGN38 antibody (panels A′ and B′). C and D, Clone 9 cells transfected with pcDNA3-HA-Dyn2b (panel C) or pcDNA3-HA-Dyn2b(K44A) (panel D) were double stained with anti-HA (panels C and D) and anti-TGN38 (panels C′ and D′) antibodies.

K44A mutant of dynamin IIba showed vesicular staining, as was observed for the dynamin IIaa mutant. We also examined the effect of the dynamin IIba mutant on uptake of BODIPY-transferrin and found that it inhibited the receptor-mediated endocytosis (Fig. 2, panels F and F′), as did the mutants of dynamin I (panels B and B′) and dynamin IIaa (panels D and D′). Thus, we failed to show any difference between dynamins IIaa and IIba in their subcellular localization or involvement in the receptor-mediated endocytosis. We therefore used HA-tagged dynamin IIaa in the following experiments.

Because Jones *et al.* (*22*) reported that the formation of constitutive transport vesicles and clathrin-coated vesicles from the TGN in a cell-free system could be inhibited by anti-dynamin antibodies, we examined the effect of expression of the K44A dynamin II mutant on the formation of constitutive exocytic vesicles and clathrin-coated vesicles from the TGN. To examine the effect of the dynamin II mutant on the constitutive vesicle formation, Clone 9 hepatocytes were co-transfected with the expression vectors for the HA-tagged dynamin mutant and for GFP-tagged VSVG tsO45. The transfected cells were incubated at non-permissive temperature (39.5°C) to accumulate the viral glycoprotein in the endoplasmic reticulum (ER), then at permissive temperature (32°C) to allow the transport of the viral protein from the ER to the plasma membrane through the Golgi apparatus. If dynamin II is involved in the formation of constitutive transport vesicles from the TGN, its dominant-negative mutant is expected to cause an arrest of the VSVG at this compartment after exit from the ER upon shift to the permissive temperature. As shown in Fig. 6, when the cells transfected with either wild-type or mutant dynamin II were incubated at 39.5°C, VSVG-GFP was localized in reticular structures resembling the ER (panels G and G′, and J and J′, respectively). When the temperature was shifted to 32°C for 1 h, irrespective of the expression of wild type or mutant dynamin II (panels H and H′, and K and K′, respectively), the labeling of the viral glycoprotein was observed in the Golgi region and on the plasma membrane. When the cells were incubated at 32°C for 3 h (panels I, I′, L, and L′), the strongest labeling was observed on the plasma membrane. These observations indicate that the transport of VSVG-GFP to the plasma membrane *via* the Golgi apparatus is normal even in the presence of wild type or mutant dynamin II. Similar results were obtained with cells expressing wild-type or mutant dynamin I (panels A–F and A′–F′). Thus, neither the dominant-negative mutant of dynamin II nor that of dynamin I appeared to affect the transport of VSVG from the ER to the plasma membrane through the Golgi apparatus.

We then addressed whether dominant-negative dynamin II could affect the clathrin-coated vesicle formation from the TGN. To this end, we transfected AtT-20 cells with an expression vector for human cathepsin D in combination with that for either wild-type or mutant dynamin II, and carried out a metabolic labeling and immunoprecipitation experiment to monitor the processing and secretion of pro-cathepsin D. Cathepsin D is a lysosomal hydrolase that represents the most extensively studied example of lysosomal enzyme targeting mediated by mannose 6-phosphate receptor that is known to be exported from the TGN by clathrin-coated vesicles. It is synthesized as a 53-kDa proenzyme, exported from the TGN and delivered to endosomal compartments, where the propeptide is removed to form a 47-kDa intermediate, which is subsequently converted in lysosomes to yield the mature enzyme composed of non-covalently associated 31-kDa heavy and 14-kDa light chains (see Fig. 7, lane 3). A fraction of pro-cathepsin D molecules, which do not enter the lysosomal pathway, is secreted into the medium, probably *via* the constitutive secretory pathway (see Fig. 7, lane 4). Thus, the processing of pro-cathepsin D is thought to be a good index of export from the TGN *via* clathrin-coated vesicles. As shown in Fig. 7, there was no significant difference in the ratio of the intermediate and mature forms (I + M) to total intracellular cathepsin D molecules (P+I+M) between cells expressing cathepsin D alone and those expressing cathepsin D in combination with either wild-type or mutant dynamin II (panel A, lanes 9 and 11, and panel B, filled bars), although a parallel transfection and immunostaining experiment confirmed that more than 90% of the cells expressing cathepsin D also expressed dynamin (data not shown). Furthermore, the ratio of intracellular cathepsin D molecules (C) to intracellular plus secreted molecules (C+S) was not altered by the expression of wild-type or mutant dynamin II (panel B, open bars). Similar results were obtained with cells expressing the dominant-negative mutant of dynamin I. Thus, neither the dominant negative mutant of dynamin II nor that of dynamin I appeared to affect the trafficking of cathepsin D

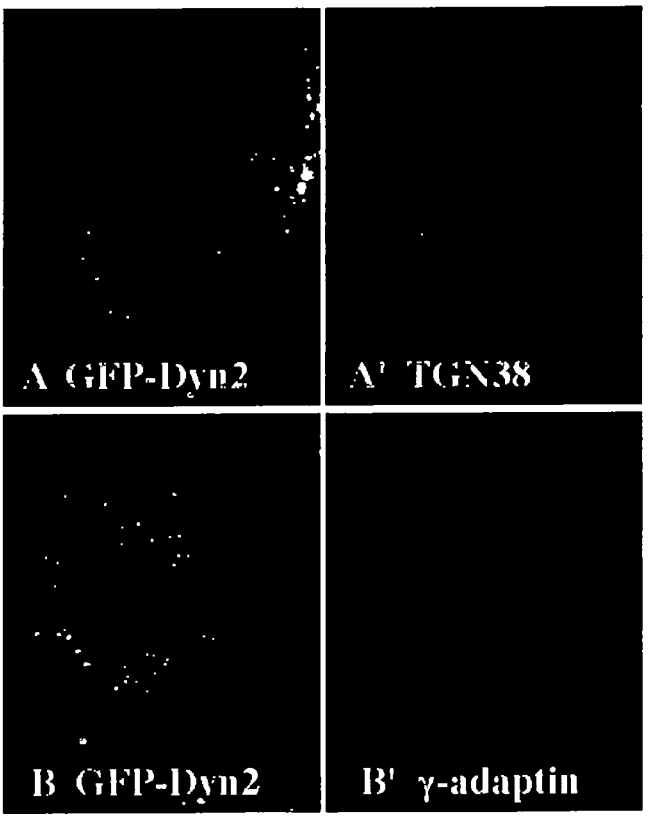


Fig. 5. **Subcellular localization of GFP-dynamin II in stable transfectants.** Clone 9 cells transfected with pEGFP-Dyn2a were selected in the presence of G418. A pool of cells stably expressing GFP-dynamin II were immunostained with anti-TGN38 antibody (panels A and A′) or with anti-γ-adaptin antibody (panels B and B′).

Fig. 6. **Effect of wild-type and K44A mutant of dynamins I and II on VSVG transport.** Clone 9 cells transfected with pcDNA3-VSVG-EGFP in combination with either pcDNA3-HA-Dyn1a (panels A, B, and C), pcDNA3-HA-Dyn1a(K44A) (panels D, E, and F), pcDNA3-HA-Dyn2a (panels G, H, and I), or pcDNA3-HA-Dyn2a(K44A) (panels J, K, and L) were incubated at 39.5°C for 12 h (panels A, D, G, and J), then at 32°C for 1 h (panels B, E, H, and K) or 3 h (panels C, F, I, and L). The cells were stained with anti-HA antibody (panels A–L). Panels A′–L′ show labeling of VSVG-GFP.

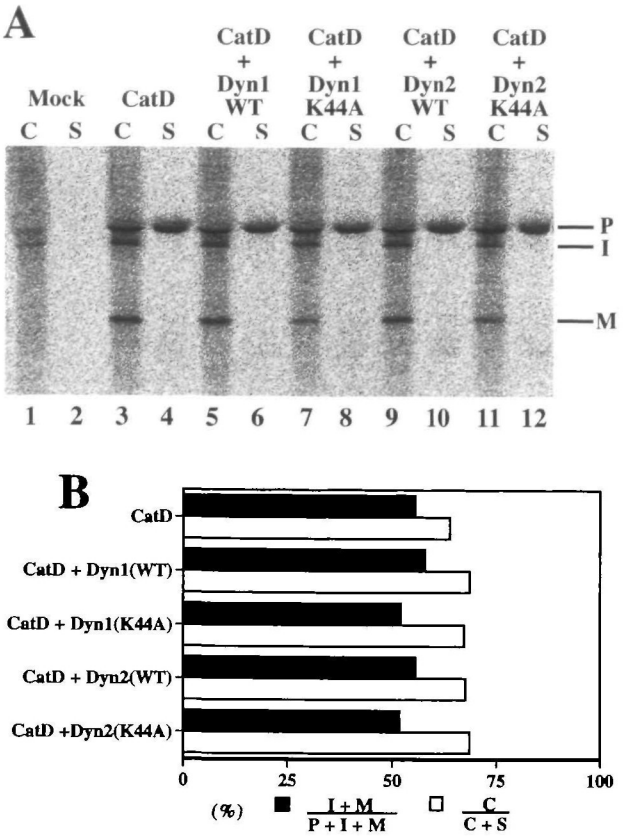


Fig. 7. **Effect of wild-type and K44A mutant of dynamins I and II on cathepsin D processing.** A: AtT-20 cells mock-transfected (lanes 1 and 2), or transfected with pcDNA3-CatD alone (lanes 3 and 4) or in combination with either pcDNA3-HA-Dyn1a (lanes 5 and 6), pcDNA3-HA-Dyn1a(K44A) (lanes 7 and 8), pcDNA3-HA-Dyn2a (lanes 9 and 10), or pcDNA3-HA-Dyn2a(K44A) (lanes 11 and 12) were metabolically labeled with ^{35}S-methionine, and the cathepsin D molecules in the cells (C) and secreted into the medium (S) were immunoprecipitated with anti-human cathepsin D antibody and subjected to SDS-PAGE as described under "EXPERIMENTAL PROCEDURES." P, I, and M indicate the positions of pro-, intermediate, and mature forms of cathepsin D, respectively. In mock-transfected cells, the bands corresponding to the positions of pro- and intermediate forms may represent the endogenous cathepsin D molecules in AtT-20 cells that cross-reacted with the anti-human cathepsin D antibody. B: The ratio of intermediate and mature forms (I+M) to total intracellular cathepsin D molecules (P+I+M) (filled bars) and the ratio of intracellular cathepsin D molecules (C) to intracellular plus secreted molecules (C+S) (open bars). The density of each band was estimated using a bioimaging analyzer and corrected by subtracting the density of the corresponding area in lanes 1 and 2.

from the Golgi through endosomes to lysosomes, indicating, although indirectly, that neither dynamin II nor dynamin I is involved in clathrin-coated vesicle formation from the TGN.

DISCUSSION

In the present study, we have shown that, like dynamin I, dynamin II is involved in receptor-mediated endocytosis (Fig. 2). This was expected to some extent on the basis of the marked structural similarity of dynamin II to dynamin I. However, we obtained no evidence for localization of dynamin II in the TGN or its involvement in vesicle formation from this compartment. The data are in quite contrast to those of Jones *et al.* (*22*): they reported that the labeling of GFP-tagged dynamin II was superimposed on the staining for TGN38 and clathrin heavy chain in the paranuclear region and that anti-dynamin antibodies were capable of inhibiting vesicle formation from the TGN in a cell-free assay. Our attempts to support their data were unsuccessful. First, HA-tagged and GFP-tagged dynamin II expressed in various cell lines, including Clone 9, the same cell line as that used by Jones *et al.* (*22*), showed a relatively uniform cytoplasmic distribution (Figs. 1, 3, 4, and 5), in contrast to the paranuclear distribution of GFP-dynamin II observed by Jones *et al.* Second, double-immunolabeling analyses (Figs. 1, 3, 4 and 5) failed to show significant colocalization of HA-tagged and GFP-tagged dynamin II with TGN38 and γ-adaptin in various cell lines, including Clone 9. Third, overexpression of the dominant-negative K44A mutant of dynamin II produced no observable effect on intracellular transport of VSVG or cathepsin D (Figs. 6 and 7), indicating that dynamin II is not involved in formation of constitutive transport vesicles or clathrin-coated vesicles from the TGN. However, we cannot exclude the slight possibility that the dynamin II mutant could not inhibit dominantly the TGN vesicle formation, even though it did inhibit receptor-mediated endocytosis from the plasma membrane.

While this manuscript was in preparation, Cao *et al.* (*30*), who belong to the same research group as Jones *et al.* (*22*), reported that GFP-tagged dynamin IIaa but not dynamin IIab showed a Golgi-like labeling and concluded that the second alternatively spliced region is a determinant of the Golgi localization of the dynamin II variant. We mainly used dynamin IIaa in the present study but failed to show its Golgi-like localization. We are unable to give a definite explanation for the discrepancy between our data and theirs on the subcellular localization of dynamin II. On the other hand, we can explain to some extent the discrepancy between our data and theirs on the involvement of dynamin II in the vesicle formation from the TGN. In their cell-free assay for vesicle formation, Jones *et al.* (*22*) used Golgi-enriched fractions prepared from rat liver by the method that they described previously (*31*). However, the Golgi-rich fractions may have also contained the plasma membrane, because the authors detected a significant amount of plasma membrane calcium ATPase in the fractions (*31*). Furthermore, as markers for formation of constitutive and clathrin-coated vesicles in their cell-free assay, Jones *et al.* (*22*) used polymeric IgA receptor and clathrin, respectively, which are also present on the plasma membrane. One point that complicates the interpretation of their data is that they used clathrin but not the TGN-specific adaptor AP-1 as a marker for clathrin-coated vesicle formation. Taken together with our observation that dynamin II as well as dynamin I is involved in receptor-mediated endocytosis, it is possible that the inhibition of the vesicle formation by anti-dynamin antibodies observed in their cell-free assay may have reflected the inhibition of clathrin-mediated endocytosis from the plasma membrane.

In the case of cells expressing the K44A mutant of dynamin I, Damke *et al.* (*13*) have reported that several membrane trafficking events, including the transport of newly synthesized transferrin receptor from the ER

through the Golgi to the plasma membrane, its recycling from endosomes, and cathepsin D trafficking to lysosomes, are unaffected, supporting our data with the dynamin I mutant. Our data on the subcellular localization of dynamin II and on the effects of its dominant-negative mutant cannot show a significant difference between dynamins I and II. Is dynamin II indeed involved in the formation of transport vesicles from the TGN, such as clathrin-coated vesicles and constitutive exocytic vesicles? On the basis of the structural and functional similarities between the AP-1 and AP-2 adaptors involved in clathrin-coated vesicle formation from the TGN and the plasma membrane, respectively, it has been supposed that these two events occur by a similar mechanism. However, there are some differences between the clathrin-coated vesicle formation from the TGN and the plasma membrane. First, although recruitment of the AP-1 adaptor onto membranes is mediated by a small GTP-binding protein, ADP-ribosylation factor, there is no evidence for its involvement in the AP-2 recruitment (for review, see Ref. *19*). Second, although several proteins that regulate clathrin-mediated endocytosis have been reported to interact with dynamin and/or AP-2 (for review, see Ref. *6*), there has been no report of the involvement of such regulatory proteins in clathrin-coated vesicle formation from the TGN. In the case of constitutive vesicle formation from the TGN, the identity of the coat itself is currently unclear. Furthermore, it is unlikely that dynamin is involved in the formation of well characterized COP I- and COP II-coated vesicles. Thus, it appears that dynamin is not always required for pinching off of budding coated vesicles.

We thank Dr. George Banting for providing anti-TGN38 antibodies, Dr. Thomas Südhof for the dynamin cDNAs, and Dr. Jennifer Lippincott-Schwartz for the VSVG-GFP vector.

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