J. Biochem. 122, 525-530 (1997)

Identification and Subcellular Localization of a Novel Mammalian Dynamin-Related Protein Homologous to Yeast Vps1p and Dnm1p[1]

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Received for publication, March 3, 1997

The dynamin family of GTP-binding proteins are implicated in vesicular transport. These include mammalian dynamins I, II, III, and yeast Vps1p and Dnm1p. Dynamin is involved in the formation of clathrin-coated vesicles at the plasma membrane. On the other hand, Vps1p and Dnm1p appear to be involved in transport from the late Golgi compartment to vacuoles and in an endocytic process, respectively. In this study, we identified a novel human protein, named Dnm1p/Vps1p-like protein (DVLP). It resembled more closely Dnm1p and Vps1p than dynamins not only in the primary structure but also in the domain organization. DVLP mRNA was expressed ubiquitously, suggesting that this protein plays a fundamental role in cellular function. Immunofluorescence analysis of cells expressing epitope-tagged DVLP revealed that it showed a diffused perinuclear staining pattern that was not superimposed on that of the marker protein for the Golgi apparatus, *trans*-Golgi network, lysosomes, endosomes, or endoplasmic reticulum. These data suggest that DVLP is not involved in the formation of known coated vesicles.

Key words: dynamin, epitope tagging, GTP-binding protein, vesicular transport, Vps1.

In eukaryotic cells, transport of proteins between membrane-bound compartments along the exocytic and endocytic pathways is mediated by carrier vesicles that bud from a donor compartment and are targeted to and fuse with an appropriate acceptor compartment (for review, see Refs. *1-3*). To date, four types of coated transport vesicles have been identified (*1-3*). Clathrin-coated vesicles containing the AP-1 and AP-2 adaptor complexes bud from the *trans*-Golgi network (TGN) and the plasma membrane, respectively. COP II-coated vesicles bud from the endoplasmic reticulum (ER). COP I-coated vesicle coats assemble onto cisternae of the Golgi apparatus and onto the intermediate compartment between the ER and Golgi. A variety of GTP-binding proteins have been shown to play pivotal roles in budding, targeting and fusion of these transport vesicles (for review, see Ref. *4*). These GTP-binding proteins are classified into three broad families: (i) Rab/Ypt and ARF/Sar1 subfamilies of small GTP-binding proteins (*4-6*); (ii) heterotrimeric G proteins, which also have well-established roles as signal transducers (*7*); and (iii) the dynamin family of large GTPases, which includes mammalian dynamins I, II, and III, and *Saccharomyces cerevisiae* Vps1p and Dnm1p (for review, see Refs. *8-11*).

Dynamin I was first identified as a microtubule-binding protein that is predominantly expressed in neural tissues (*12-14*). Studies using GTP-binding domain mutants of dynamin I have suggested that it is involved in receptor-mediated endocytosis *via* clathrin-coated pits (*15-17*). Recent morphological studies have shown that dynamin I assembles into rings around the necks of invaginated coated pits, thereby suggesting a model that GTP hydrolysis of dynamin I squeezes the necks until membrane fission occurs (*18, 19*). Dynamin II is expressed ubiquitously (*20, 21*), while dynamin III (originally referred to as dynamin-2) is expressed predominantly in the testis and brain (*22, 23*).

The *VPS1* gene was identified as one of more than 50 yeast genes that are required for sorting of soluble proteins into the yeast lysosome known as the vacuole (*24*). Its product, Vps1p, has been suggested to be involved in formation of vesicles from the late Golgi compartment, corresponding to the TGN in animal cells, and in Golgi membrane protein retention (*25, 26*). A more recently identified Vps1p homologue, Dnm1p, has been suggested to be involved in transport of endocytosed vesicles at some step before fusion with the late endosome (*27*). In view of

[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 Special Research Project on Circulation Biosystem in the University of Tsukuba, the Ciba-Geigy Foundation (Japan) for the Promotion of Science, the Asahi Glass Foundation, the Sumitomo Foundation, Ono Pharmaceutical Co., Ltd., and Sankyo Co., Ltd.

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Abbreviations: TGN, *trans*-Golgi network; ER, endoplasmic reticulum; PCR, polymerase chain reaction; EST, expressed sequence tag; ORF, open reading frame; HA, hemagglutinin; DVLP, Dnm1p/Vps1p-like protein; DVH, dynamin/Vps1p homology; BFA, brefeldin A.

the similarity to mammalian dynamins, these yeast GTP-binding proteins may also play a role in fission of vesicles budding from some intracellular compartments. However, mammalian counterparts of these yeast gene products have not yet been identified. We here report the cloning and characterization of a human protein homologous to yeast Vps1p and Dnm1p.

EXPERIMENTAL PROCEDURES

cDNA Cloning—A hybridization probe was generated from random-primed HepG2 cell cDNA using polymerase chain reaction (PCR) and oligonucleotide primers, 5′-GGT-GCTAGAATTTGTTATATTTTCCA-3′ and 5′-CATAGT-TCTCTTCACCAAAGATGA-3′, synthesized on the basis of GenBank human expressed sequence tags (ESTs), accession numbers R10223 and T71617, respectively, and used to screen $\sim 2 \times 10^5$ phages of a HepG2 cDNA library (*28*). Six positive phages were isolated and their cDNA insert was subcloned into the *Not*I site of the pBluescript-II KS(+) vector (Stratagene, La Jolla, CA). One of the six cDNAs was presumed to cover the entire coding sequence, and its sequence was determined from both strands using a BcaBest sequencing kit (Takara Shuzo, Kyoto).

Northern Blot Analysis—Total RNAs (15 μg) isolated from mouse tissues were subjected to Northern blot analysis as described previously (*29*). The probe used was a cDNA fragment of human DVLP covering a coding region for amino acid 489 to the COOH terminus and a 206-bp 3′-untranslated region.

Plasmid Construction—An epitope sequence of influenza virus hemagglutinin (HA) was introduced into the pcDNA3 expression vector (Invitrogen, San Diego, CA) by ligation of a double-stranded oligonucleotide coding for the epitope sequence between the *Hin*dIII and *Bam*HI sites. The resultant vector was designated pcDNA3-HAN. A *Bam*HI recognition sequence was introduced upstream of the initiation codon of the human DVLP cDNA by PCR amplification of the cDNA in pBluescript-II using a primer, 5′-AT*GGATCC*ACCATGGAGGCGCTAATTCCTGTC-3′ (where the initiation codon is underlined and the *Bam*HI sequence is shown in italics) and the T7 sequencing primer. A cDNA fragment between the *Bam*HI site and a *Not*I site at the 3′-terminus was ligated into the *Bam*HI-*Not*I sites of pcDNA3-HAN. The resultant expression vector for human DVLP with an NH_2-terminal HA epitope was designated pcDNA3-HAN-DVLP.

Antibodies—Polyclonal rabbit anti-α-mannosidase II, polyclonal rabbit anti-lamp-1, and monoclonal mouse anti-TGN38 were kindly provided by Dr. K.W. Moremen (University of Georgia, GA; *30*), Dr. K. Akasaki (Fukuyama University, Fukuyama; *31*), and Dr. G. Banting (University of Bristol, Bristol, UK; *32*), respectively. Monoclonal mouse anti-protein disulfide isomerase was purchased from StressGen Biotechnologies (Victoria, Canada). Monoclonal mouse (12CA5) and polyclonal rabbit (HA.11) antibodies against the HA epitope were from Boehringer Mannheim GmbH (Mannheim, Germany) and Berkeley Antibodies (Richmond, CA), respectively. FITC-conjugated anti-mouse and anti-rabbit IgGs were from Jackson ImmunoResearch Laboratories (West Grove, PA). Texas Red-conjugated anti-mouse and anti-rabbit immunoglobulins were from Amersham International (Buckinghamshire, UK).

Immunofluorescence Analysis—pcDNA3-HAN-DVLP was transfected into mammalian culture cell lines using CellPhect (Pharmacia Biotech, Uppsala, Sweden), Clonfectin (Clontech, Palo Alto, CA), or Lipofectamine (Life Technologies, Gaithersburg, MD). The cells were cultured for 24 h and then trypsinized, plated onto wells of eight-well Lab-Tek chamber slides (Nunc, Roskilde, Denmark), and cultured for a further 24 h. The cells were then processed for indirect immunofluorescence analysis as described previously (*33, 34*). Briefly, the cells were fixed and permeabilized, then incubated sequentially with either a combination of monoclonal mouse anti-HA antibody and polyclonal rabbit antibody against an endogenous protein or one of polyclonal anti-HA antibody and monoclonal antibody against an endogenous protein, and with either a combination of FITC-conjugated anti-mouse and Texas Red-conjugated anti-rabbit IgGs or a combination of FITC-conjugated anti-rabbit and Texas Red-conjugated anti-mouse IgGs, respectively. The stained cells were observed with a laser-scanning confocal microscope (TCS4D, Leica Lasertechnik GmbH, Heidelberg, Germany).

RESULTS AND DISCUSSION

Genetic studies in yeast have identified a panel of genes encoding proteins involved in vesicular transport. Vps1p and Dnm1p, both of which are homologous to mammalian dynamins, have been identified by such studies and suggested to play some role in sorting of proteins from the late Golgi compartment (corresponding to the TGN in animal cells) into vacuoles (corresponding to mammalian lysosomes) and in an endocytic process, respectively. However, mammalian counterparts of the yeast GTP-binding proteins have not been identified so far. We exploited the availability of large data bases of ESTs to identify the putative mammalian proteins. A search of the GenBank EST data base using the TBLASTN algorithm revealed that several human ESTs encoded sequences homologous to *S. cerevisiae* Vps1p and Dnm1p but not significantly to mammalian dynamins. By PCR amplification of human hepatoma HepG2 cell cDNA using a set of primers specific to these ESTs, we obtained a partial cDNA fragment and used it as a hybridization probe to screen a HepG2 cDNA library. Sequence analysis of hybridizing cDNA clones revealed that a clone had a 2.2-kb open reading frame (ORF) coding for a 736-amino acid protein; we hereafter refer to this putative protein as DVLP (for Dnm1p/Vps1p-like protein).

Figure 1 shows the cDNA sequence and the deduced amino acid sequence of human DVLP. Like other dynamin family members, DVLP has a well-conserved NH_2-terminal GTP-binding domain containing a tripartite GTPase/GTP-binding consensus site. Data base search revealed that DVLP is most similar to an ORF in a cosmid clone (T12E12) identified by the *Caenorhabditis elegans* genome project with a 60% overall amino acid identity. In the amino acid sequence and the domain organization, DVLP resembles not only the putative *C. elegans* protein but also a putative dynamin-like protein (DymA) of *Dictyostelium discoideum* (42% overall identity), *S. cerevisiae* Dnm1p (45% overall identity), and Vps1p (42% overall identity), and a putative

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                  M  E  A  L  I  P  V  I  N  K  L  Q  D  V  F  N  T  V  G  A  D  I  I  Q  L  P  Q  I  V  V  V  G  T  Q  S  S  G  K  S  39

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CAGAACACATCACACATTTAATCCAAATAATAAATGGCTGTTTCTAAAG                                                                                                                2449
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Fig. 1. **Nucleotide and deduced amino acid sequences of human DVLP.** GTPase and GTP-binding motifs are shown in dark boxes. Regions that show significant homology to other dynamin family members (GTP-binding, DVH1, and DVH2 domains) are underlined.

dynamin-related protein (C12C2.08) identified by the *Schizosaccharomyces pombe* genome project (42% overall identity) (Fig. 2). The NH_2-terminal GTP-binding domain of DVLP also shows an extensive homology to mammalian dynamins (Fig. 2; the overall identity to rat dynamin Ia was 37%). However, the dynamin-related proteins including DVLP do not resemble dynamins in the COOH-terminal half for lack of the important functional domains; namely, the pleckstrin homology domain that has been proposed to be important for protein-protein interaction and for protein-phospholipid interaction (reviewed in Refs. *35* and *36*), and the proline-rich domain that has been shown to be responsible for binding to proteins containing the SH3 domain and to microtubules (reviewed in Ref. *9*). Another structural feature that distinguishes the dynamin-related proteins from dynamins is that only the former has a relatively longer spacer sequence (18-48 amino acids) that interrupts the NH_2-terminal conserved region containing the GTP-binding motifs, GXXXXGKS and DXXG (data not shown). However, a central region that we designated as the DVH1 domain (DVH for dynamin/Vps1p homology) and a region designated as the DVH2 domain, which is flanked by the pleckstrin homology and proline-rich domains of dynamins, show a significant homology to the corresponding regions of dynamin-related proteins (Fig. 2). Vallee and Okamoto (*9*) has proposed that the DVH2 domain of dynamin forms a coiled-coil structure. By using the COILS program, Ver. 2.2 (*37, 38*), we confirmed that the DVH2 domain of dynamin-related proteins including DVLP also has a potential to form a coiled-coil structure (data not shown). Since the coiled-coil structure is known to

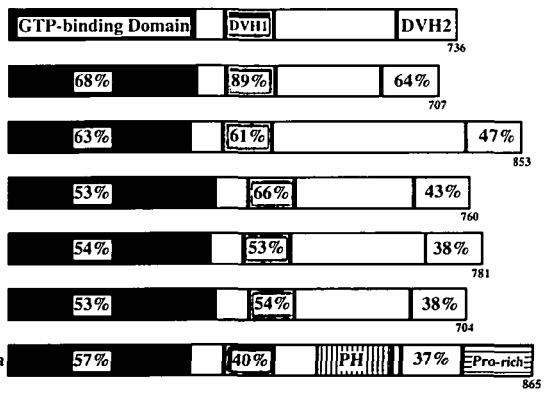

Fig. 2. **Schematic representation of the structures of DVLP and other dynamin family members.** The percentage amino acid identity in each domain of each member with DVLP is shown. PH, pleckstrin homology domain; Pro-rich, Pro-rich domain; h, human; ce, *Caenorhabditis elegans*; dd, *Dictyostelium discoideum*; sc, *Saccharomyces cerevisiae*; sp, *Schizosaccharomyces pombe*; r, rat.

be involved in homo- and hetero-dimerization and oligomerization of proteins (*37, 38*), the dynamin-related proteins might form a homodimer or homo-oligomer, and/or interact with other proteins *via* the DVH2 domain. Collectively, the structural features suggest that DVLP plays a role similar to that of yeast Vps1p and Dnm1p rather than that of dynamins.

To determine whether DVLP performs a general vesicle trafficking function in all tissues or is restricted to a subset of tissues, we performed Northern blot analysis. As shown

in Fig. 3, a predominant transcript of 3.0 kb was found in all examined tissues, with a relatively higher expression level in the brain, heart, and testis. A minor transcript of 2.7 kb was also detected. It is unclear whether these transcripts represent products *via* alternative splicing of the same primary transcript or derive from a distinct, but related, gene. We favor the former possibility, since the DVLP cDNA probe used for the Northern blot analysis covered a region relatively unconserved among the dynamin family members. In any case, the ubiquitous expression suggests that DVLP plays a fundamental role in cellular function.

To determine the subcellular localization of DVLP, we performed indirect immunofluorescence analysis. To this end, an expression vector for DVLP tagged with an influenza virus HA epitope sequence (pcDNA3-HAN-DVLP) was constructed and transfected into various culture cell lines. We introduced the HA epitope at the NH_2-terminus of DVLP, since the NH_2-terminal tag has been shown not to alter the intracellular localization of dynamin I (*17*); we have also confirmed that the HA tag did not affect the dynamin localization (data not shown). As shown in Fig. 4, staining of transfected rat Clone 9 hepatocytes with an antibody against the HA epitope showed a diffuse perinuclear pattern for DVLP with an NH_2-terminal HA tag (HA-DVLP) (panels A, C, E, and G). Expression of COOH-terminally HA-tagged DVLP (DVLP-HA) gave rise to a similar staining pattern (data not shown). Furthermore, a similar perinuclear staining for HA-DVLP was observed with other cell lines, such as monkey kidney Vero cells and Chinese hamster ovary cells (data not shown). These make it unlikely, but not impossible, that such a tag could have affected the subcellular localization of DVLP.

Taken together with its similarity to yeast Vps1p and

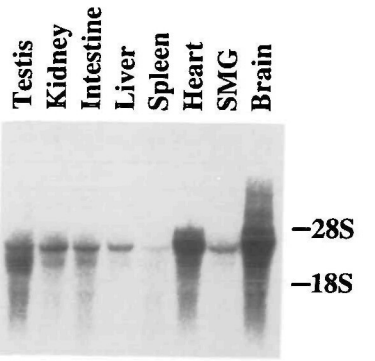

Fig. 3. **Northern blot analysis of DVLP mRNA.** Total RNAs (15 μg) isolated from mouse tissues were electrophoresed, blotted onto a membrane, and hybridized with a cDNA probe covering a region of human DVLP relatively unconserved among the dynamin family members, as described under "EXPERIMENTAL PROCEDURES." The positions of 28S and 18S ribosomal RNAs are indicated. SMG, submandibular gland.

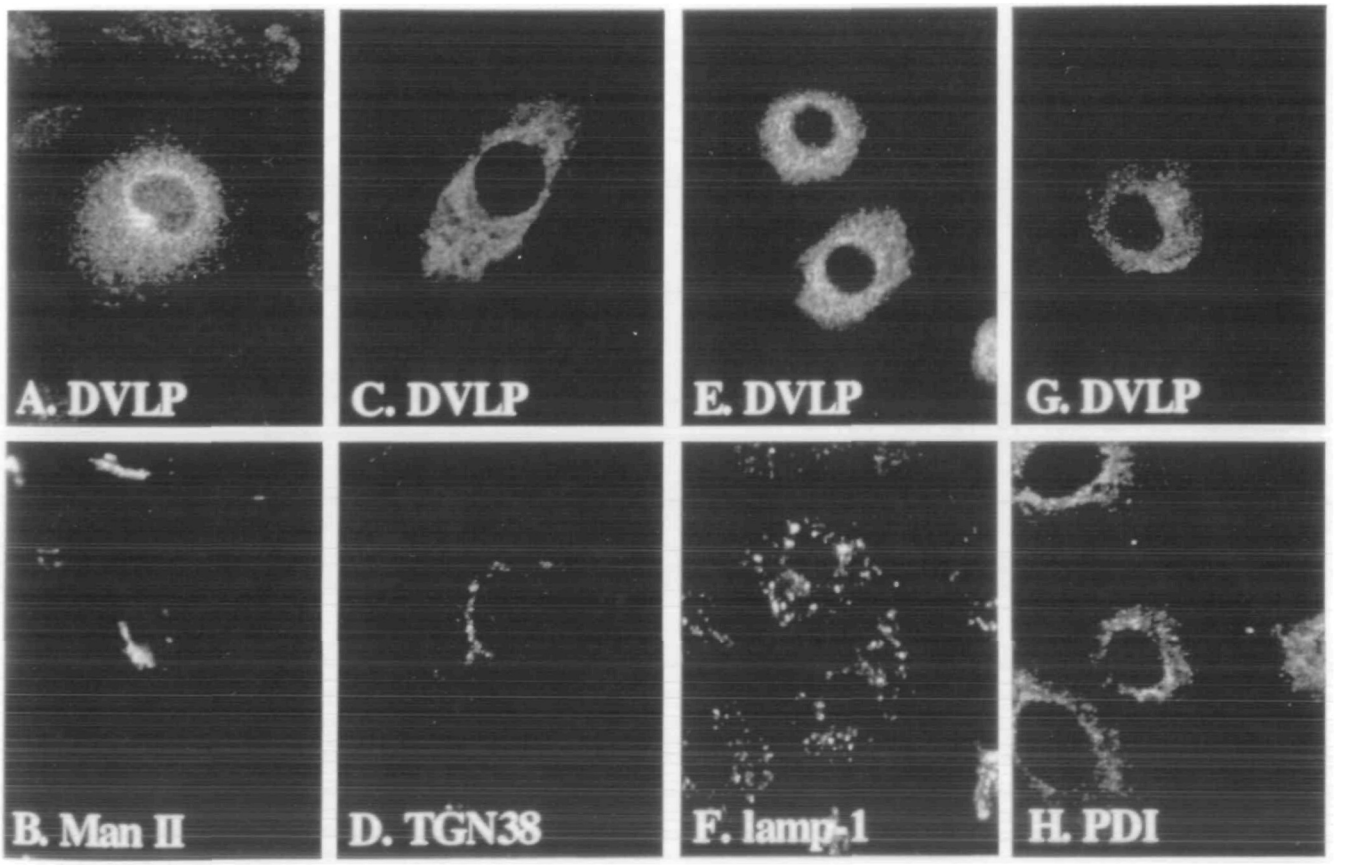

Fig. 4. **Indirect immunofluorescence analysis of cells expressing epitope-tagged DVLP.** Clone 9 cells transiently transfected with the expression vector for NH_2-terminally HA-tagged human DVLP (pcDNA3-HAN-DVLP) were stained with a combination of anti-HA antibody (panels A, C, E, and G) and antibody against either α-mannosidase II (Man II; panel B), TGN38 (panel D), lamp-1 (panel F), or protein disulfide isomerase (PDI; panel H) as described under "EXPERIMENTAL PROCEDURES" and observed with a confocal microscope.

Dnm1p in the primary structure and domain organization, these findings suggested that DVLP might localize to the TGN and/or lysosomal/endosomal compartments, for the following reasons. First, Vps1p is responsible for sorting of proteins from a late Golgi compartment into vacuoles and for membrane protein retention in the late Golgi (*24-26*). Second, Dnm1p participates at some step before fusion of endocytosed vesicles with late endosomes (*27*). Third, it has recently been reported that the Golgi complex in mammalian cells is strongly labeled not only with peptide-specific antibodies directed against conserved regions of the dynamin family but also with those against yeast Vps1p (*39*). However, the diffused perinuclear staining of HA-DVLP was not superimposed on a relatively compact perinuclear staining of a resident Golgi membrane enzyme, α-mannosidase II (compare panels A and B), or a TGN membrane protein, TGN38 (panels C and D). Furthermore, the DVLP staining was not superimposed on a punctate staining of a lysosomal membrane protein, lamp-1 (panels E and F), or transferrin receptor (data not shown), which is known to recycle between endosomal compartments and the cell surface. Since the relatively broad perinuclear staining pattern for DVLP appeared to represent an ER structure, transfected cells were then double-stained for DVLP and protein disulfide isomerase, a resident ER protein (panels G and H). However, the staining for DVLP was not identical, albeit rather similar, to that for protein disulfide isomerase.

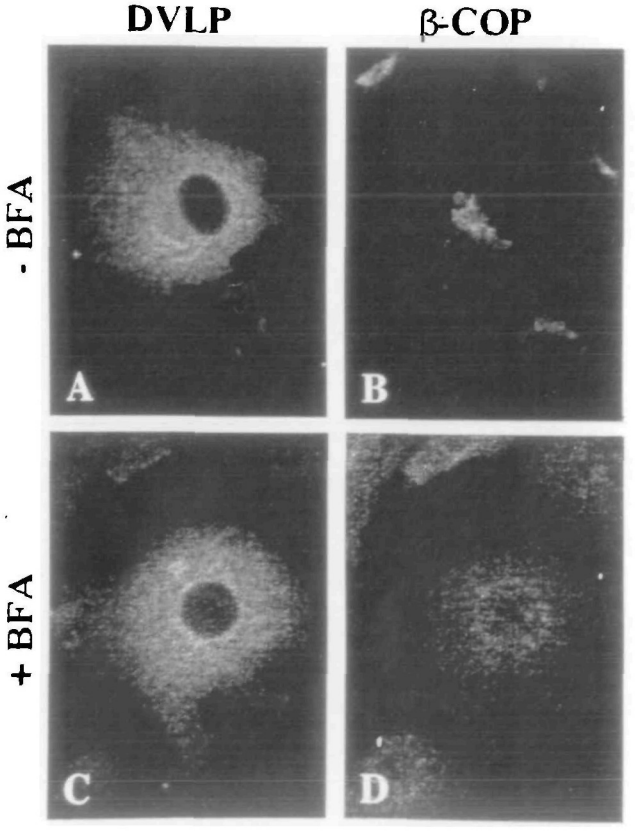

Fig. 5. **Effect of BFA on DVLP localization.** Clone 9 cells transiently transfected with pcDNA3-HAN-DVLP were incubated in the presence (panels C and D) or absence (panels A and B) of 5 μg/ml BFA for 30 min, then processed for staining with a combination of anti-HA antibody (panels A and C) and antibody against β-COP (panels B and D), as described under "EXPERIMENTAL PROCEDURES," and observed with a confocal microscope.

We then examined effects of a fungal antibiotic, brefeldin A (BFA), on the DVLP localization, since this drug affects the organization of various intracellular organelles. Upon treatment of cells with this drug, components of the COP I coat and the AP-1 adaptor were first dissociated from membranes, then the Golgi apparatus was tubulated and fused with the ER, while tubules extending from the TGN, lysosomes, and endosomes were concentrated around the microtubule organizing center (for review, see Ref. *40*). As shown in Fig. 5, when Clone 9 cells transfected with HA-DVLP were treated with BFA (5 μg/ml) for 30 min, the DVLP staining pattern was not altered as compared with untreated control (compare panels A and C), whereas β-COP, a component of the COP I coat was dissociated from membranes by the BFA treatment (panels B and D). This supports the observations that DVLP was co-localized with neither of the marker protein for the Golgi apparatus, TGN, lysosomes, nor endosomes.

BFA is known to block the formation of COP I-coated vesicles from the Golgi and AP-1 clathrin-coated vesicles from the TGN. On the other hand, the formation of COP II-coated vesicles from the ER or AP-2 clathrin-coated vesicles from the plasma membrane is not affected by this drug (*1, 40*). In view of the finding that DVLP was co-localized with neither of the examined marker proteins, it is unlikely that DVLP is involved in the formation of these known coated vesicles. Homologues of components of the AP-1 and AP-2 adaptor complexes have recently been identified and shown to form a novel adaptor complex (*41*). Furthermore, by searching the EST data base, we have found that several ESTs encode parts of proteins homologous but not identical to known clathrin adaptor components (data not shown). Taken together, these findings suggest that DVLP may be involved in the formation of unidentified coated vesicles. Although morphological studies have revealed that dynamin associates with the necks of invaginated AP-2 clathrin-coated pits to form a helical structure at the plasma membrane (*18*), there has been no report on such a structure at other intracellular compartments. It is therefore also possible that the function of DVLP is not similar to that of dynamins. In any case, intracellular vesicular transport must be more complicated than was thought previously. To gain further insight into the role of DVLP, we are now working to identify proteins that interact with this novel GTP-binding protein.

We would like to thank Drs. Kelley W. Moremen, Kenji Akasaki, and George Banting for providing antibodies.

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