Vear, a Novel Golgi-associated Protein with VHS and γ-Adaptin “Ear” Domains*

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Intracellular membrane trafficking, involving budding, transport, and fusion of transport vesicles, provides the mechanism for the movement of proteins between different membrane compartments of a cell (for a review, see Refs. 1 and 2). The specificity in the system is conferred by a selective recognition of the cargo, its specific sequestration in appropriate transport vesicles at the budding site, and discriminating docking at the target membrane. Instrumental in the initiating events of the vesicular trafficking are cytosolic proteins that assemble at the vesicle budding sites to form “coats.” The best known coats involve heterotetrameric protein complexes called adaptor complexes 1 and 2 (AP-1 and AP-2), which mediate the recruitment of clathrin to the vesicle budding site and are associated with trafficking from the trans-Golgi network to the late endocytic pathway and from the plasma membrane to the early endosomes, respectively. Other thoroughly studied coats are formed by the “coater” proteins COPI and COPII, which are responsible for vesicle formation in the endoplasmic reticulum/Golgi and in the early secretory pathway, respectively. COP proteins are not associated with clathrin (3, 4). AP-3, another adaptor complex originally thought to be associated with non-clathrin-dependent membrane trafficking in the trans-Golgi network, has also been shown to bind clathrin (5).

All the AP proteins have a similar heterotetrameric structure, being composed of two heavy chains (adaptins) and one medium (μ) and one small (σ) chain (6). The compositions of the AP proteins are as follows. AP-1: γ- and β1-adaptins/μ/σ1; AP-2: α- and β2-adaptins/μ/σ2; and AP-3: δ- and β3-adaptins/μ/σ3. In electron microscopy, AP proteins show an overall structure consisting of a bulky body that is formed by the N-terminal “head” domains of the two adaptins and by the smaller subunits embedded between them and of “ears” that protrude from the body and that correspond to the C termini of the adaptins (6–8). There is also a considerable degree of sequence similarity between the various subunits and between the subunits in different species, suggesting conserved functional features. The β-adaptins are ~85% identical, whereas the others are <50% similar. All the adaptins have a similar domain structure: a large N-terminal head domain, a proline- and glycine-rich hinge region, and a C-terminal appendage or ear (7, 9). α- and γ-adaptins have the least overall identity (only 25%). There are similarities also between μ- and σ-subunits (10).

Based on morphological and functional studies, it is suggested that there are at least 12 different sorting events in vesicular membrane trafficking that putatively employ coat proteins (11). On the other hand, not as many coat proteins are known, calling for the presence of novel adaptor or coat proteins. Indeed, several novel isoforms or homologs of known adaptor/coat proteins (see Refs. 12 and 13) and membrane-associated protein complexes that bind to AP proteins (14, 15) have recently been described in both mammals and yeast, suggesting that related but variable sets of subunits may be utilized to match the specificity requirements of the trafficking events. There is also an emerging group of proteins that are thought to be involved in the determination of the specificity of the vesicle fusion events. They are loosely called “tethering” proteins due to their extended shape and capacity to mediate interactions between different membrane compartments (16–18). One of the best studied is trh, which contains a VHS and a FYVE domain, forms coiled-coil structures, and associates with endocytic vesicles (19).

In this study, we report the molecular cloning and initial characterization of a novel Golgi-associated protein that contains a VHS domain, which is characteristic of endocytosis-associated proteins (20, 21), and a C-terminal end similar to the
ear domain of γ-adaptin. It shows a distinct membrane association and a Golgi-associated localization as determined by cell fractionation, immunofluorescence microscopy, and transfection experiments. Due to these features, we suggest that it plays a role in vesicular trafficking events in or in the immediate vicinity of the Golgi complex. Based on the sequence similarities (VHS domain and ear domain of γ-adaptin), we call this protein “Vear.”

**EXPERIMENTAL PROCEDURES**

*General Methods—* Standard solutions; buffers; and procedures for purification and precipitation of DNA, restriction enzyme digestion, and ligations reactions were as described by Sambrook et al. (22). Sequencing was done using an automated ABI PRISM 377XL DNA Sequencer (Perkin-Elmer). Synthetic oligonucleotides were obtained from Amersham Pharmacia Biotech. For sequence analysis and alignments, the Genetics Computer Group Wisconsin Package Program Suite 9.0 and CLUSTAL X (23) programs were used.

*Data Base Searches and Sequence Analysis—* BLAST searches (24) of EST databases were carried out on a World Wide Web server at NCBI. As a query, nucleotides 13–420 of the sequence encoding the VHS domain of EAST (GenBank accession number AY224514) (25) were used. In reiterative runs, several matching and overlapping EST clones were found.

*Northern Blot Analysis—* The cDNA corresponding to nucleotides 501–950 of the coding region of Vear was amplified by polymerase chain reaction (PCR) using primers labeled with [α-32P]dCTP (Amersham Pharmacia Biotech) using an Oligolabelling Kit (Amersham Pharmacia Biotech). Northern analysis of multiple human mRNA samples was performed on a multiple-tissue Northern blot filter (CLONTECH) following the manufacturer’s instructions. Total RNA from the cell lines was prepared using the SV Total RNA Isolation System (Promega). 20 μg of total RNA was transferred to a Hybond-N nylon filter (Amersham Pharmacia Biotech). The blots were stripped and reprobed with the control β-actin cDNA as recommended by the manufacturer. Autoradiographic images were acquired using a PhosphorImager (Molecular Dynamics, Inc.).

*Cell Culture—* COS-7 and MDBK cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and antibiotics.

*Antibodies—* Antibodies to Vear were raised as follows. The full-length sequence of Vear was amplified by polymerase chain reaction using *Pfu* polymerase (Stratagene). The product was subcloned into the *BamHI/NotI* cloning site of the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech). Expression of the fusion protein in bacterial cells was induced with isopropyl-1-thio-β-D-galactopyranoside (0.25 mM) at room temperature for 3–5 h. The cells were spun down, resuspended in PBS containing 1% Triton X-100, sonicated, and incubated on ice for 10 min. After centrifugation, supernatants were incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The beads were washed several times in PBS, and the fusion protein was eluted with 20 mM reduced glutathione in 0.1 M Tris-HCl, pH 8.0. The purifying beads was used to immunize rabbits. The antisera were collected and affinity-purified on CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech) coated with the fusion protein. The monoclonal anti-γ-adaptin antibody was from Transduction Laboratories (A36120). The polyclonal anti-HA antibody was purchased from Santa Cruz Biotechnology (sc-805).

*Immunoblotting—* For immunoblotting, cells were washed in ice-cold PBS and then solubilized in lysis buffer (50 mM Heps, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 25 mM NaF, 10 μM ZnCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were incubated on ice for 10 min and cleared by centrifugation 14,000 × *g* for 10 min. The supernatants were collected and used in the assays. Immunoblotting was carried out essentially as described previously (26).

*Transient Transfection—* The cDNAs used for the transfection experiments were produced by polymerase chain reaction using *Pfu* polymerase (Stratagene). Full-length Vear (amino acids 1–612; Vearfull), the VHS domain of Vear (amino acids 29–165; VearVHS), the C-terminal end of Vear (amino acids 170–612; VearC), and the ear domain of Vear (amino acids 483–605; Vearear) were cloned into the *BamHI/NotI* cloning site of the pRK5 vector (a gift from Dr. J. Schlessinger). The HA epitope tag was added to the C terminus of the constructs by primer design. The authenticity of the constructs was confirmed by DNA sequencing. Transient transfections were done using Fugene 6 reagent (Boehringer Mannheim Biochemicals) according to the manufacturer’s instructions.

*Cell Fractionation—* COS-7 cells transfected with HA-Vearfull in the pRK5 vector were washed twice in ice-cold PBS and pelleted by centrifugation at 1000 × *g* for 5 min at 4 °C. The pellets were then resuspended in ice-cold homogenization buffer (0.25 M sucrose and 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitors) and sonicated. The nuclei and cell debris were removed by centrifugation at 1000 × *g* for 15 min at 4 °C. The post-nuclear supernatant was then centrifuged for 60 min at 100,000 × *g* to obtain cytosolic and total membrane fractions. The pelleted membranes were resuspended in Laemmli sample buffer. Equal amounts of cytosolic and membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting.

*Immunofluorescence Microscopy—* For immunofluorescence microscopy, the cells were grown on glass coverslips. They were washed in Hank’s salt solution and then fixed either with 4% paraformaldehyde in 100 mM Pipes, pH 6.8, 5 mM EGTA, 2 mM MgCl2, and 0.2% Triton X-100 for 10 min or with −20 °C methanol for 10 min. After washing in PBS, the cells were incubated with 10% fetal bovine serum in PBS/glycine for 30 min. They were then overlaid with the primary antibody for 30 min; washed; and incubated with the appropriate Texas Red-conjugated (Jackson ImmunoResearch Laboratories, Inc.), fluorescein isothiocyanate-conjugated (Caltag Laboratories), or Oregon Green-conjugated (Molecular Probes, Inc.) secondary antibody for another 30 min. The cells were viewed under an Olympus BH2 fluorescence microscope equipped with appropriate filters.

The expression of brefeldin A (BFA) on the distribution of Vear was studied by adding BFA (Alexis Corp.) to a culture medium of MDCK cells at a final concentration of 5 μg/ml. After incubation for 2, 5, and 30 min at 37 °C, the cells were fixed with −20 °C methanol for 10 min and handled for the immunofluorescence detection of Vear and γ-adaptin as described above.

**RESULTS**

*Molecular Cloning and Sequence Analysis of Vear—* EST data bases were utilized to identify novel proteins that could qualify as proteins involved in vesicular trafficking by virtue of their domain structure. We were especially interested in proteins carrying a VHS domain, a recently discovered domain present in several endocytic proteins (20). A search using the BLAST algorithm and the nucleotide sequence encoding the VHS domain of EAST (nucleotides 13–420) as a query yielded six matching and overlapping ESTs: AA671009, AA279107, AA235896, AA307157, AA070902, and AI017433. Aligning the sequences suggested the presence of an open reading frame encoding a protein of 613 amino acids with a calculated molecular mass of 67,149 Da.

EST clone AA070902 was purchased from the Human Genome Mapping Project Research Center (Hinxton, Cambridge, United Kingdom). Sequencing of it yielded a nucleotide sequence almost identical to that derived from the aligned sequences of AA671009, AA279107, AA235896, AA307157, AA070902 and AI017433. The sequence consists of a 5′-untranslated region of 21 nucleotides, an open reading frame of 1842 nucleotides, and a 3′-untranslated region of 134 nucleotides. The deduced amino acid sequence is shown in Fig. 1A. Based on the structural features of the protein, we call it Vear (see below). A schematic representation of the structural features and the domain structure of the protein is shown in Fig. 1B. Later on, we noticed that a human chromosome 16 bacte...
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**Fig. 1.** The predicted amino acid sequence and the domain structure of Vear. A, the deduced amino acid sequence of Vear. The VHS domain is shown in boldface, and the ear domain is underlined. B, schematic representation of the domain structure of Vear. αα, α-amino acids.

**Vear Antibody**—Northern blot analysis of human tissue revealed two major messages of 3.5 and 3.9 kb that were most abundant in kidney, skeletal muscle, and cardiac muscle (Fig. 3A). They were also seen at a lower level in placenta, lung, thymus, and spleen. In kidney, an abundant message of 5.2 kb was also seen. It was expressed at a lower level also in the other tissues mentioned above. A low level of expression of these species was seen in brain, colon, and small intestine. The nature of the multiple RNA species seen in some tissues is currently unknown, but could correspond to alternatively spliced forms of the Vear mRNA. We also carried out Northern hybridization of the total RNAs isolated from HeLa, COS-7, MDBK, and MDCK cells. A distinct band of 3.5 kb, corresponding to a similar-sized band in tissues, was seen in all cell lines. In HeLa and COS-7 cells, messages of 3.9 and 5.2 kb, respectively, were also seen (Fig. 3B).

Western blot analysis of the cell solubilizes with anti-Vear antibody detected a single band of 75 kDa in COS-7, HeLa, MDBK, and MDCK cells. A distinct band of 3.5 kb, corresponding to a similar-sized band in tissues, was seen in all cell lines. In HeLa and COS-7 cells, messages of 3.9 and 5.2 kb, respectively, were also seen (Fig. 3B).

**Tissue Distribution of Vear and Characterization of Anti-Vear Antibody**—Northern blot analysis of human tissue revealed two major messages of 3.5 and 3.9 kb that were most abundant in kidney, skeletal muscle, and cardiac muscle (Fig. 3A). They were also seen at a lower level in placenta, lung, thymus, and spleen. In kidney, an abundant message of 5.2 kb was also seen. It was expressed at a lower level also in the other tissues mentioned above. A low level of expression of these species was seen in brain, colon, and small intestine. The nature of the multiple RNA species seen in some tissues is currently unknown, but could correspond to alternatively spliced forms of the Vear mRNA. We also carried out Northern hybridization of the total RNAs isolated from HeLa, COS-7, MDBK, and MDCK cells. A distinct band of 3.5 kb, corresponding to a similar-sized band in tissues, was seen in all cell lines. In HeLa and COS-7 cells, messages of 3.9 and 5.2 kb, respectively, were also seen (Fig. 3B).

Western blot analysis of the cell solubilizes with anti-Vear antibody detected a single band of 75 kDa in COS-7, HeLa, MDBK, and MDCK cells (Fig. 4A, lanes 5–8). No bands were detected using a preimmune serum, attesting to the specificity of the reaction (lanes 1–4). The detection of a single band in all cell lines suggests a correspondence with the 3.5-kb mRNA species, which was also shared by all the cells tested (Fig. 3B). The results from Western blotting of the COS-7 cells transfected with the truncated parts of Vear with anti-Vear and anti-HA antibodies are shown in Fig. 4B. A distinct binding of anti-Vear antibody to the C-terminal part (lane 3), but not to the VHS or ear domain (lanes 1 and 2), of Vear is shown. The positive reaction with anti-HA antibody of all the HA-tagged proteins (lanes 4–6) indicates that the peptides were appropriately expressed and transferred to the filter. These results show that the epitope recognized by anti-Vear antibody lies between amino acids 170 and 482, an area that shows no significant homology to any other protein in the data banks.

**Immunofluorescence Microscopy**—The subcellular distribution of Vear in MDCK, COS-7, HeLa, and human embryonic skin cells (Fig. 5, a, c, e, and g) was studied by immunofluorescence microscopy using anti-Vear antibody. In all cells tested,
Vear was localized to a mostly tubular perinuclear structure closely resembling the Golgi complex both in localization and appearance. No staining was seen when preimmune serum was substituted for anti-Vear antibody. Moreover, the staining was completely inhibited by preincubating the antibodies with the recombinant glutathione S-transferase-Vear fusion protein (Fig. 5h, inset) but not with glutathione S-transferase alone (data not shown). This shows that anti-Vear antibody detects...
endogenous Vear, that the staining reaction is specific, and that Vear is probably associated with the Golgi in different types of cells.

To explore more closely the putative Golgi association of Vear, we compared the distribution of Vear with the localization of \( \alpha \)-adaptin, a well established marker of the Golgi complex (33, 34). Using double staining immunofluorescence microscopy for Vear (Fig. 5, a, c, e, and g) and for \( \gamma \)-adaptin (Fig. 5, b, d, f, and h), an extensive codistribution was seen between Vear and \( \gamma \)-adaptin in MDCK, COS-7, HeLa, and human embryonic skin cells. However, within the Golgi-like structure, the staining patterns were nonidentical; Vear was always seen in coarser, slightly tubular structures, whereas \( \gamma \)-adaptin was seen associated with more delicate vesicular or dot-like structures.

BFA is a fungal metabolite that inhibits coat formation in Golgi membranes, thereby causing reversible apparent dissolution of the Golgi complex in many cells (35). Due to this property, treatment with BFA can be used to explore the putative association of proteins with the Golgi apparatus and also their functional features (36–38). \( \gamma \)-Adaptin and \( \beta \)-COP are examples of Golgi proteins that are “sensitive” to BFA, i.e. they show an altered subcellular distribution upon BFA treatment of cells (34).

Fig. 6 shows the distribution of Vear and \( \gamma \)-adaptin in MDCK cells treated with BFA for 0, 5, and 30 min. No distinct changes were seen in cells treated for up to 2 min. In cells exposed to BFA for 5 min (Fig. 6 c) or 30 min (Fig. 6 e), on the other hand, a distinct disassembly of the tubular Vear-positive structure to numerous and more widely distributed vesicles was seen. A similar but not identical change was seen in the distribution of \( \gamma \)-adaptin (Fig. 6, d and f). These results strongly suggest that Vear is associated with a BFA-sensitive subcompartment of the Golgi.

Cell Fractionation Studies—The subcellular distribution of Vear was also studied by subjecting Vear-transfected COS-7 cells to fractionation by ultracentrifugation, followed by Western blotting of the post-nuclear membrane and cytosolic fractions using anti-Vear antibody. Vear was seen to be present almost exclusively in the membrane fraction (Fig. 7); only a faint band corresponding to Vear was seen in the cytosolic fraction after a longer exposure (data not shown). This indicates that most of Vear is associated with cellular membranes and is well in line with the immunofluorescence microscopy observations that showed a Golgi-associated localization without any major cytosolic distribution.

Overexpression of Vear in Cultured Cells—To further investigate the localization and function of Vear in cultured cells, we tagged recombinant full-length Vear and its distinct domain regions with the HA epitope (HA-Vear) and transiently overexpressed these in cultured COS-7 cells (Fig. 8) and MDCK cells (data not shown). In most transfected cells, HA-Vear\textsuperscript{full} was seen confined to the Golgi-like perinuclear structure. Double staining with anti-\( \gamma \)-adaptin antibodies showed a distinct codistribution verifying the Golgi-associated localization (Fig.
In most HA-Vear full-transfected cells, a distinctly dense, sharply delineated, “compacted” staining pattern was seen with anti-HA antibody. This was always associated with a correspondingly condensed \(\gamma\)-adaptin distribution, which clearly differed from the more “non-compacted” distribution seen in nontransfected cells (Fig. 8b). A similar distribution was seen with HA-Vear C. In most cells, HA-Vear C showed a perinuclear Golgi-like distribution (Fig. 8c), which was similar to the compacted distribution of HA-Vear full and completely coincided with the distribution of \(\gamma\)-adaptin (Fig. 8d). In nontransfected cells, a less compact and wider Golgi-associated distribution of \(\gamma\)-adaptin was seen (Fig. 8b, d, and e, asterisks). In HA-Vear full-transfected cells (Fig. 8e), a Golgi-associated staining was also seen. However, there was no distinct change in the morphology of the Golgi as judged by staining for \(\gamma\)-adaptin in transfected and nontransfected cells (Fig. 8f). In marked contrast to the above, HA-VearVHS showed a widespread membrane- and vesicle-associated distribution in the cytoplasm without any preferential localization to the Golgi complex (Fig. 8g). Its pattern was clearly different from that of the Golgi and was not associated with any change in the Golgi morphology as judged by staining with anti-\(\gamma\)-adaptin antibody (Fig. 8h).

**DISCUSSION**

In this study, we have identified and characterized Vear, a novel Golgi-associated protein with a unique domain structure. Vear is expressed at low levels in most human tissues and at a high level in kidney and skeletal and cardiac muscle. Both endogenous and exogenously expressed Vear proteins are confined to the Golgi complex, in which they colocalize with the Golgi marker \(\gamma\)-adaptin.

The Golgi association of Vear was demonstrated by both immunofluorescence microscopy and cell fractionation studies. Moreover, HA-tagged Vear assembled on the Golgi upon cell transfection. No significant cytosolic pool of either endogenous or exogenously expressed Vear was identified, attesting to the predominantly membrane-associated localization. Comparison with \(\gamma\)-adaptin showed a close codistribution in cultured cells, suggesting a Golgi-associated localization, which was further supported by a similar response of both Vear and \(\gamma\)-adaptin to treatment with BFA. It is noteworthy, however, that, in closer
Double staining was carried out with anti-HA antibody (d7182 VearC, HA-Vear ear, and HA-Vear VHS in cultured COS-7 cells. Subcompartment marked by the presence of g showed a codistribution with g distribution pattern of exogenously expressed Vear; it also more accurate assignment of Vear as to the substructure of the interesting differences that could be indicative of the function proline-rich middle regions and the ear or the ear alone showed from the distribution of endogenous Vear and g accompanied by an occurrence of a compacted Golgi. This differed in many transfected cells, overexpression of Vear was accompanied by some other determinants (39). Clearly, this, together with the distinct localization of the exogenously expressed ear domain of Vear in the Golgi, strongly speaks in favor of it critically determining the Golgi-based localization of Vear.

The presence of Vear in the ear domain is of special interest, first, because in adaptins, it has been suggested to have a targeting function (32, 39), and, second, because it has not been found outside the realm of adaptins before. In adaptins, ear domains have a lower degree of similarity than, for example, the head domains, which have been shown to account, at least partially, for the specific membrane targeting and for the interaction with s-subunits in a- and g-adaptins (32, 39). On the other hand, studies employing chimeric and mutant a- and g-adaptins have shown that the ear domains also contribute to membrane binding possibly by stabilizing an association initiated by some other determinants (39). Clearly, this, together with the distinct localization of the exogenously expressed ear domain of Vear in the Golgi, strongly speaks in favor of it critically determining the Golgi-based localization of Vear.

The distinct difference seen in the Golgi organization of cells transfected with VearC and Vearear (compacted versus non-compacted Golgi) points to a potential importance of the middle portion of Vear. Interestingly, it encompasses a region with a propensity to coiled-coil arrangement and a proline-rich region. Supposedly, the presence of the coiled-coil region could lead to formation of homo- and heterodimer formation with some other proteins. This could potentially lead to not only targeting of Vear, but also to forming a closer apposition on the target organelles associated with the ends of Vear and/or its putative dimerization partner. In this sense, Vear could be a novel member of a newly described group of tethering proteins that have been described in the vesicular transport systems such as the Golgi and that typically form bridges between different membrane elements (41, 42). Further studies are needed to determine whether, analogously to the Golgi-associated members of this class of proteins, Vear could be involved in the docking of the vesicles to the membranes or in the stacking of the cisternae in the Golgi.

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FIG. 8. Immunolocalization of overexpressed HA-Vear, HA-VearC, HA-Vearear, and HA-VearVHS in cultured COS-7 cells. Shown are the results from transfection with HA-Vearfull (a and b), HA-VearC (c and d), HA-Vearear (e and f), and HA-VearVHS (g and h). Double staining was carried out with anti-HA antibody (a, c, e, and g) and anti-g-adaptin antibody (b, d, f, and h). Nontransfected cells stained with anti-g-adaptin antibody are marked with asterisks (b and d). The scale bar represents 5 μm.
