CLONING AND CHARACTERIZATION OF VEAR, A NOVEL GOLGI-ASSOCIATED PROTEIN INVOLVED IN VESICLE TRAFFICKING

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Poussu, Anssi, Cloning and characterization of Vear, a novel Golgi-associated protein involved in vesicle trafficking

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Abstract

The control and maintenance of the character, number and protein, carbohydrates and lipid composition of intracellular compartments in a changing environment is one of the fundamental features of a living cell. It is effected, to a large measure, by vesicular traffic which connects the various cellular compartments and handles the transportation of cargo between them. Movement of cargo occurs through a transport system in membrane-bounded containers called vesicles. Vesicles originate at the donor membrane from which they are transported to target organelles where they fuse with the acceptor membrane and deliver their cargo. At the donor site, cytosolic coat proteins or 'coats' bind to the donor membrane together with GTP (guanosine 5'-triphosphate)-binding regulatory proteins first to deform a bud, which is then pinched off as a coated vesicle. During budding and targeting events, a number of regulatory proteins interact with the coat components. Currently, several different coat proteins and their adaptor proteins are known.

The purpose of this study was to characterize novel components participitating in intracellular vesicle transport. By using computer analysis and EST (expressed sequence tag) database searches, a previously unknown protein was found. Sequencing revealed the presence of a novel protein of 613 amino acids with a calculated molecular mass of 67,149 Da. Based on its structural features, possessing both a VHS domain and an "ear" domain, we named the protein Vear.

With its VHS domain in its NH_2 terminus, Vear shows similarity to several endocytosis-associated proteins. With the "ear" domain in its C-terminus, it resembles γ-adaptin, a heavy subunit of the AP-1 complex. Vear mRNA showed a widespread distribution in tissues, with high amounts of mRNA in the kidney, skeletal muscle, and cardiac muscle. At the subcellular level, Vear was localized to the Golgi complex in which it colocalized with the *trans*-Golgi marker γ-adaptin. The preferential membrane-association was demonstrated by subcellular fractionation in which Vear partitioned with the total membrane fraction. Golgi-associated subcellular localization for Vear was sensitive to a treatment with the fungal metabolite brefeldin A, suggesting an ARF (ADP-ribosylation factor)-dependent recruitment onto membranes. In transfection studies, the full-length Vear assembled on and caused structural "compaction" of the Golgi complex, while overexpression of the "ear" domain alone showed diffuse Golgi-localization without "compaction". The VHS domain, on the other hand, was mainly vesicle- and plasma membrane associated and did not show any association with Golgi. In skeletal muscle, Vear was detected preferentially in type I cells by immunohistochemistry and immunofluorescence microscopy. In normal kidney, Vear was exclusively present in glomerular epithelial cells (podocytes) and Vear protein was expressed in a developmentally regulated manner during glomerulogenesis. By immunolabeling electron microscopy, Vear was seen in vesicular and membrane structures adjacent to the Golgi complex. Vear was also abundant in the gastrointestinal tract in cells active in secretion.

The results indicate that Vear is a novel vesicle transport-associated protein, detected mainly in the Golgi complex and localized in tissues in a highly cell-type specific manner.

Keywords: Golgi, membrane, trans-Golgi network, transport

To my dear wife Hanna

Se on onnellinen, joka ei sure sitä mitä häneltä puuttuu,
vaan iloitsee siitä mitä hänellä on.
            Demokritos

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Abbreviations

aa	amino acid
AP	adaptor protein complex
ARF	ADP-ribosylation factor
ARL	ARF-like protein
ATPase	adenosine triphosphatase
BFA	brefeldin A
bp	base pair
BSA	bovine serum albumin
CCV	clathrin-coated vesicle
cDNA	DNA complementary to RNA
CNBr	cyanogen bromide
COP	coatomer protein
Da	dalton
DAB	diaminobenzidine
DMEM	Dulbecco´s modified Eagles medium
DNA	deoxyribonucleic acid
EAR	domain structure first identified in gamma-adaptin
EAST	EGFR-associated protein
ECL	enhanced chemiluminescence
ECV	endosomal carrier vesicle
EDTA	ethylenediaminetetra-acetic acid
EE	early endosome
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMEM	Eagles´s minimal essential medium with Earle´s salts
Eps15	EGFR pathway substrate clone 15
ER	endoplasmic reticulum
EST	expressed sequence tag
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GBM	glomerular basement membrane

GC	Golgi complex
GEF	guanine-nucleotide exchange factor
GGAs	Golgi-localized, γ ear-containing, ARF-binding proteins
GST	glutathione-S-transferase
GTP	guanosine 5ʹ-triphosphate
GTPase	guanosine 5ʹ-triphosphatase
HA	hemagglutinin
HES	human embryo skin fibroblast
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
IC	intermediate compartment
IEM	immunolabeling electron microscopy
IF	immunofluorescence
IHC	immunohistochemistry
ISH	*in situ* hybridization
kb	kilobase
kDa	kilodalton
LAMP	lysosomal associated membrane protein
LDL	low-density lipoprotein
LE	late endosome
LIMP	lysosomal integral membrane protein
LY	lysosome
MDBK	Madin-Darby bovine kidney cell
MDCK	Madin-Darby canine kidney cell
MHC	myosin heavy chain
MPR	mannose-6-phosphate receptor
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide hydrogenase
NSF	N-ethylmaleimide-sensitive fusion protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SG	secretory granule
SH3	src homology 3
SNAP	soluble NSF associated protein
SNARE	soluble NSF attachment receptors
STAM	signal transducing adaptor molecule
TGN	*trans*-Golgi network
TRITC	tetramethyl rhodamine isothiocyanate
VHS	domain present in VPS27, Hrs and STAM
VIP21	vesicular integral-membrane protein of 21 kD
VPS27	vacuolar protein sorting (gene) 27

List of original articles

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.

I   Poussu A, Lohi O & Lehto V-P (2000) Vear, a novel Golgi-associated protein with VHS and gamma-adaptin "ear" domains. J Biol Chem 275:7176-7183.

II Poussu A, Thompson P, Mäkinen M & Lehto V-P (2001) Vear, a novel Golgi-associated protein, is preferentially expressed in type I cells in skeletal muscle. Muscle & Nerve 24:127-129.

III Poussu A, Virtanen I, Autio-Harmainen H & Lehto V-P (2001) Podocyte-specific expression of a novel trans-Golgi protein Vear in human kidney. Kidney Int 60:626-634.

IV Mäkinen M, Poussu A & Lehto V-P (2001) Vear, a novel Golgi-associated protein is highly expressed in the secretory cells of the gastrointestinal tract. Manuscript.

Contents

1 Introduction

A characteristic feature of eukaryotic cell organization is compartmentalization, the formation and maintenance of a characteristic set of organelles and their protein and lipid compositions in order to optimize the unique functional and biochemical reactions that occur within and on them (Scales et al. 2000). The mechanism by which different compartments receive the correct set of components and communicate with each other is now understood in general terms, based largely on a recently discovered machinery responsible for the formation, targeting and fusion of transport vesicles (Rothman 1994, Rothman & Wieland 1996).

The secretory and endocytic pathways of a cell consist of several functionally different, membrane-bound compartments linked together by vesicular traffic. The vesicular transport system not only permits the transport of soluble and membrane-bound components from one compartment to another, but also determines and maintains the protein and lipid composition as well as the size and polarized distribution of the compartments (Le Borgne & Hoflack 1998). The initiation step of the transport process is the selection of the cargo followed by budding, i.e. generation and pinching off of the endocytic/secretory vesicle at the donor membrane. After its transit to the target area, the vesicle is docked to the target membrane where it delivers its contents by a membrane fusion (Rothman & Wieland 1996).

There are several classes of proteins that operate at the various stages of vesicular transport. At the budding stage, small soluble GTPases and their activators are needed (Donaldson & Jackson 2000). Activated GTPases then recruit cytosolic coat components to the cytosolic face of the vesicle wall (Chavrier & Goud 1999). Cytoskeletal structures provide efficient "tracks" for the movement of transport vesicles between different membranous compartments. Power for the translocation along the tracks is provided by various motor proteins (Kamal & Goldstein 2000). At the docking stage, specific transmembrane "pilot" proteins termed SNAREs (soluble NSF attachment receptors) and their regulators are needed both at the vesicle membranes (v-SNAREs) and target membranes (t-SNAREs). At the fusion stage, NSF (N-ethylmaleimide-sensitive fusion protein) and SNAPs (soluble NSF associated protein) mediate the fusion event between the vesicle membrane and the target membrane (Söllner et al. 1993, Söllner & Rothman 1996).

The coat proteins are cytosolic proteins needed both to shape the transport vesicle and also to select the correct set of cargo molecules into the vesicle through a direct or an indirect interaction with the cytosol-exposed portions of the transported transmembrane proteins. Presently, five types of coat complexes are known: clathrin and its adaptors AP-1 and AP-2, the adaptor-related AP-3 complex, the adaptor-related AP-4 complex, COP I, and COP II (Kirchhausen 2000, Scales *et al*. 2000).

In this work, we describe the discovery, initial characterization and tissue distribution of a novel *trans*-Golgi protein termed Vear (VHS and "ear" domains). Its structure shows similarity to several endocytosis-associated proteins in that it has a typical VHS domain in its N-terminus. It is also homologous to γ-adaptin, a heavy subunit of the AP-1 complex, in having in its C-terminus a typical "ear" domain. A tissue-specific role for Vear is suggested by its highly cell-type specific distribution detected in muscle, kidney and gastrointestinal tract.

2 Review of literature

2.1 Vesicle transport

Eukaryotic cells display a complex array of organelles. Many of them are connected with each other by a constant and bidirectional movement of small carrier vesicles whose purpose is to transport macromolecules between the membranes. For every compartment, an organelle-specific mechanism exists to recruit and package a correct set of proteins and lipids into vesicles that are destined for transport to an acceptor membrane (Schekman & Orci 1996, Lippincott-Schwartz *et al.* 2000). The movement of the cargo between different compartments is mediated primarily by coated vesicles that are detached from the "donor" membrane by a process commonly called "budding" and incorporated to the target membrane by "docking" and fusion. The vesicles at the budding process have a distinct coat, a complement of proteins that are instrumental for the budding and the release from the donor membrane to occur. Fully formed vesicles usually lose their coat soon after their emergence from the membrane (Rothman 1994, Söllner & Rothman 1996). In addition to coated vesicles, there are probably also endocytic transport systems in which non-coated vesicles operate (Hansen *et al.* 1991, Bursztajn *et al.* 1993). Significant progress has recently been made in the identification of different transport steps and the molecular machineries involved in the vesicle transport. This has been achieved especially through studies utilizing yeast genetics and cell-free assays (Schekman 1992, Rothman 1994).

Operatively, two major vesicle transport machineries are delineated: secretory and endocytic. The secretory pathway starts from the endoplasmic reticulum (ER) from which freshly synthesized proteins, lipids and carbohydrates are transported through the Golgi apparatus to the cell surface or to the endosomes/lysosomes. The endocytic pathway, on the other hand, mediates transport from the plasma membrane (PM) to the endosomes and from there to lysosomes, or back to the plasma membrane (Le Borgne & Hoflack 1998). In general, the vesicle transport system provides a mechanism by which a cell is able to transfer soluble proteins (e.g. albumin) in transport vesicles to the extracellular space. It also provides a system in which transmembrane proteins are transferred and inserted into different membranes, for instance, to the PM or to lysosomes (lysosomal proteins). Addi-

tionally, membrane lipids are able to move from one endomembrane compartment to another as a part of a vesicle membrane.

2.1.1 Coat complexes

Vesicle formation at the donor membrane is a multi-step event. It includes the recruitment of the ADP ribosylation factors (ARF) onto the membrane, subsequent recruitment of the coat proteins to the bud site, packaging of the cargo into the vesicles, and finally, pinching off of the coated vesicle (Söllner & Rothman 1996). ARFs are a family of structurally and functionally conserved proteins of approximately 21 kDa. They belong to a larger family of small Ras-like GTPases divived functionally into ARFs and ARF-like (ARL) proteins (Clark *et al.* 1993, Boman & Kahn 1995). Like other GTPases, ARFs cycle between an active GTP-bound and an inactive GDP-bound conformation; ARF-GDP is mostly cytosolic whereas ARF-GTP is membrane-bound (Helms & Rothman 1992).

A pivotal step in the budding process is the accumulation at the prospective budding site of coat proteins, a class of proteins which are targeted to the membrane from the cytosol. At the membrane, they coassemble to demark the vesicle-forming area (bud site) (Springer *et al.* 1999). Coats are spherical protein shells of fixed composition, consisting of many copies of the same subunit (Rothman 1994). The first coat to be identified on a vesicle was the clathrin coat (Roth & Porter 1964, Kanaseki & Kadota 1969). Clathrin, like other coat proteins, co-operates and coassembles with accessory proteins, such as various adaptors, to form coat complexes. The mechanisms of budding relative to concentrating the cargo at the bud site vary; that is, the cargo may recruit coats, the cargo may be recruited into emerging bud sites by other factors, or the cargo and coat may be recruited co-ordinately (Schmid 1997).

Five types of coat complexes, each mediating different transport steps, are presently known: clathrin coat with its adaptors AP-1 and AP-2, the AP-3 complex, the AP-4 complex, COP I and COP II (for review, see Scales *et al.* 2000). AP-1 drives transport vesicle formation at the *trans*-Golgi network (TGN). AP-2 drives endocytic vesicle formation at the plasma membrane (Kirchhausen 1999, and references therein). The AP-3 complex mediates vesicle transport from the TGN to the endosomal/lysosomal compartment (Simpson *et al.* 1996, Dell´Angelica *et al.* 1997, Simpson *et al.* 1997, Le Borgne *et al.* 1998). Furthermore, AP-3 is implicated in the reconstitution of synaptic vesicles from the early endosomes, suggesting that AP-3 is probably involved in several transport pathways, i.e. secretory and endocytic pathways (Faundez *et al.* 1998). The role of the recently cloned AP-4 complex in vesicle traffic is unknown (Dell´Angelica *et al.* 1999, Hirst *et al.* 1999, Rohn *et al.* 2000). COPI denotes a protein complex called "coatomer". COPI-coated vesicles mediate transport in the early secretory pathway within Golgi and between Golgi and ER (Letourneur *et al.* 1994, Kreis *et al.* 1995, Orci *et al.* 1997). A set of COPI coat proteins are involved in the biogenesis of endosomal carrier vesicles (ECVs) in the endosomal pathway (Gu & Gruenberg 1999, and references therein). Endosomal and secretory COPs differ, however, in their composition (Anieto *et al.* 1996).

Another class of non-clathrin coated vesicles, COPII vesicles, bud at the ER and mediate the transport of cargo from ER to Golgi (Barlowe *et al.* 1994).

Also other, less well known clathrin- and non-clathrin-containing coat complexes mediating different transport events have been described. For instance, VIP21 (Dupree *et al.* 1993), also known as caveolin (Rothberg *et al.* 1992), is involved in non-clathrin mediated endocytosis from the PM (reviewed by Anderson 1998). A schematic representation of the different coat complexes participitating in various transport routes is shown in Figure 1.
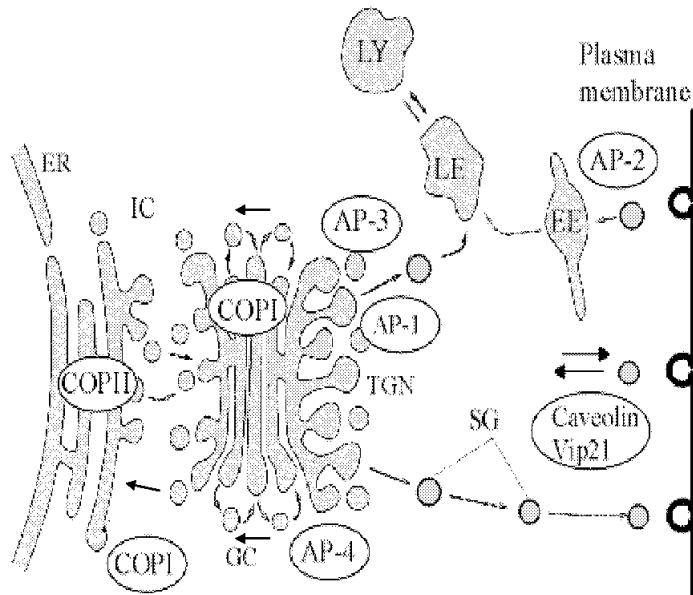
Fig. 1. A schematic representation of the coat complexes in the endocytic and exocytic pathways. AP, adaptor protein complex; COP, coatomer protein; EE, early endosome; ER, endoplasmic reticulum; GC, Golgi complex; IC, intermediate compartment; LE, late endosome; LY, lysosome; SG, secretory granule; TGN, *trans*-Golgi network; VIP, vesicular integral membrane protein (modified from Alberts *et al.* 1998).

2.1.1.1 Clathrin

Clathrin-coated vesicles (CCVs) are central players in the receptor-mediated endocytosis. They also mediate the transport of cargo from the TGN to the endosomal/lysosomal compartment (Robinson 1994, reviewed by Kirchhausen 1999). Originally clathrin was identified as a major component in purified coated vesicle extracts (Pearse 1975). Further purification of clathrin from the vesicle extracts showed that clathrin alone or with its adaptors is able to assemble *in vitro* into lattice- or basket-like structures of hexagonal and pentagonal architecture (Crowther & Pearse 1981, Pearse & Crowther 1987). As isolated from vesicles, clathrin is a trimer consisting of three copies of a clathrin-heavy chain (192 kDa) (Kirchhausen 2000). Each heavy chain in its turn associate with a clath-

rin-light chain (~ 30 kDa). Together they form a three-legged structure called a triskelion (Robinson 1994).

2.1.1.2 Adaptor proteins (APs)

Adaptor proteins (APs) were first identified as factors promoting clathrin assembly into cages and recruitment of clathrin to membranes (Keen *et al.* 1979, Robinson 1994). AP-1 and AP-2 interact with clathrin triskelions, while the interaction of AP-3 and AP-4 with clathrin is still controversial (Simpson *et al.* 1996, Dell´Angelica *et al.* 1998, Kirchhausen 1999). Structural studies indicate that clathrin-associated APs are located between the clathrin lattice and the vesicle membrane (Vigers *et al.* 1986). All the APs share structural similarities: they are heterotetrameric complexes consisting of two heavy chains of ~100 kDa (generally called adaptins), a medium-sized subunit of ~50 kDa (μ) and a small subunit of ~20 kDa (σ) (Kirchhausen 1999).

The AP-1 complex consists of large subunits γ- and $\beta1$-adaptin, the medium subunit $\mu1$, and the small subunit $\sigma1$. The AP-2 complex consists of α, $\beta2$, $\mu2$, and $\sigma2$ subunits, the AP-3 complex consists of δ, $\beta3$, $\mu3$, and $\sigma3$ subunits, and the AP-4 complex of ϵ, $\beta4$, $\mu4$, and $\sigma4$ subunits (reviewed by Schmid 1997, Dell´Angelica *et al.* 1999, Kirchhausen 1999). Most studies to resolve the domain organization of the adaptors have been done with AP-2. Electron microscopy revealed that APs consists of a globular core or a "head" domain flanked by two symmetrically globular appendages or "ears" (Heuser & Keen 1988), the "head" domains corresponding to the N-termini of the large chains, medium chain and small chain, while the "ear" domains correspond to the C-termini of the large subunits (Zaremba & Keen 1985, Kirchhausen *et al.* 1989). The "ears" are attached to the "head" via flexible "hinges" (Hirst & Robinson 1998). In Figure 2, the subunit composition and a schematic representation of the structure of different APs are shown.

The exact functions of different subunits of the adaptor protein complexes have not been established as yet. The α- and γ-adaptins have many protein-protein interactions. There is strong evidence implicating that β-adaptins play a major role in clathrin binding not only by interacting with clathrin, but also by promoting clathrin assembly (Gallusser & Kirchhausen 1993, Shih *et al.* 1995). An association between clathrin and α-adaptin was suggested in a study in which the interaction between clathrin and α-adaptin using soluble clathrin triskelions was shown (Prasad & Keen 1991). Another role for α- and γ-adaptins is probably the targeting of the corresponding adaptor complexes (AP-2 and AP-1) to correct membrane-compartments (Page & Robinson 1995, Hirst & Robinson 1998).

The "ear" domain of the $\beta2$-chain contains a binding site for clathrin, and a removal of this domain from APs inhibits their ability to form clathrin coats (Shih *et al.* 1995). Another study also supports the idea that the "ear" domain is necessary for the normal incorporation of AP complexes into clathrin coats (Clairmont *et al.* 1997). The "ear" domain of γ-adaptin (AP-1) interacts with γ-synergin (Page *et al.* 1999), while the "ear" domain of α-adaptin (AP-2) interacts with dynamin, Eps15, epsin and amphiphysin, accessory proteins participating in receptor-mediated endocytosis (Wang *et al.* 1995, Benmerah *et al.* 1996, David *et al.* 1996, Chen *et al.* 1998). At the moment, it is not known whether these interactions are responsible for targeting the APs to appropriate membranes. However, at

least γ-synergin is suggested to follow AP-1 to the TGN membranes rather than leading it there (Page *et al.* 1999). The function of α- and γ-adaptin "ear" domains was initially thought to be to control the specificity of adaptor binding (Matsui & Kirchhausen 1990). A more recent study, however, proposed only a minor role for the "ears" in adaptor localization (Robinson 1993).

The medium chains, μ1 and μ2, seem to have roles in receptor binding and in sorting signal recognition, which are important in cargo recognition (Hirst & Robinson 1998). The function of the σ subunits remains currently unknown.
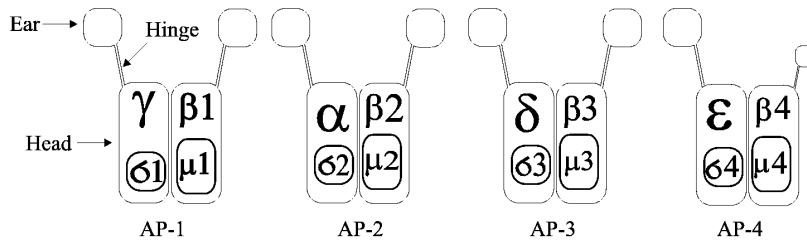
Fig. 2. A schematic representation of the subunit composition and structural features of the adaptor protein complexes AP-1, AP-2, AP-3 and AP-4 (modified version from Dell´Angelica *et al.* 1999).

Clathrin-associated adaptor complexes play multiple roles in controlling the vesicle formation. Upon binding to membranes, AP-1 and AP-2 trigger the assembly of the clathrin lattice. They also interact directly with the cytoplasmically exposed sorting signals of the transmembrane proteins and probably have a role in concentrating cargo molecules into the coated pits (Marks *et al.* 1997, Schmid 1997).

2.1.1.3 Coatomer protein (COP) and COPII

COPI is a multimolecular complex composed of seven COPs (for <u>co</u>at <u>p</u>roteins): α, β, β´, γ, δ, ε, and ξ. The seven COP polypeptide complex is generally called a coatomer (Waters *et al.* 1991, Schekman & Orci 1996). The eighth component of the COPI coat is ARF1, a small GTP-binding protein (Serafini *et al.* 1991). The mammalian COPII coat consists of small GTPase hSar1p and the heterodimeric protein complexes hSec23/24p and hSec13/31p (Barlowe *et al.* 1994, Wieland & Harter 1999). The subunit composition, localization and function of COP- and AP-coated vesicles is presented in Table 1.

2.1.1.4 Other types of coat complexes

Coat proteins other than AP- or COP-related ones have also been described. VIP21/caveolin is involved in the biogenesis of caveolae and in the formation of vesicles which are formed independently of clathrin at the PM or at the exocytic pathway (Rothberg *et al.* 1992, Dupree *et al.* 1993). p200 is a cytoplasmic phosphoprotein which is found in non-

clathrin vesicles at TGN and suggested to be a coat protein (Narula & Stow 1995). Whitney *et al.* reported a variant of COPI associated with vesicle trafficking between endosomal membranes (Whitney *et al.* 1995). Ladinsky *et al.* visualized at TGN a lace-like coat whose structure differs significantly from that of the coatamer or clathrin coats (Ladinsky *et al.* 1994).

2.1.1.5 ARFs

Mammalian ARFs are divided into three subclasses based on their sequence similarities: class I ARFs (ARFs 1-3), class II ARFs (ARF4 and ARF5) and class III ARF (ARF6) (Jackson & Casanova 2000). Highly homologous to ARFs are ARL-proteins, e.g. human ARL2 (Clark *et al.* 1993).

The best characterized ARF, ARF1, is localized to Golgi membranes and regulates the recruitment of COPI, AP-1, and AP-3 onto membranes (Chavrier & Goud 1999). The switch from ARF-GDP to ARF-GTP ("activation"), and the concominant recruitment of coat proteins onto membranes, is mediated by specific guanine-nucleotide-exchange factors termed GEFs. All GEFs share a domain called Sec7 (Morinaga *et al.* 1997, Jackson & Casanova 2000). Examples of GEFs in humans are ARNO, Cytohesin-1, EFA6, and GBF1 (Chardin *et al.* 1996, Kolanus *et al.* 1996, Claude *et al.* 1999, Franco *et al.* 1999). The switch from ARF-GTP to ARF-GDP ("inactivation"), on the other hand, is mediated by GTPase-activating proteins (GAPs) (Donaldson & Jackson 2000).

Table 1. The subunit composition, localization and function of different coat complexes. AP, adaptor protein complex; ARF, ADP ribosylation factor; COP, coatomer protein; ER, endoplasmic reticulum; PM, plasma membrane; TGN, trans-Golgi network.

Coated vesicle	Subunits of coat [1]	GTPase [2]	localization [3]	Function[4]
AP-1	clathrin γ, β1, μ1, σ1	ARF1	TGN, early endosomes	transport from TGN to endosomes/lysosomes
AP-2	clathrin α, β2, μ2, σ2	ARF6? Rab5?	PM, endosomes	endocytosis
AP-3	δ, β3, μ3, σ3	ARF1	TGN endosomes	transport from TGN to lysosomes
AP-4	ε, β4, μ4, σ4	ARF?	TGN?	?
COPI	α,β, β′, γ, δ, ε,ξ	ARF1	ER-Golgi	bidirectional Golgi-ER transport, intra-Golgi traffick
COPII	hSec23/24p hSec13/31p	hSar1p	ER-Golgi	ER-Golgi transport

[1] (Serafini *et al.* 1991, Waters *et al.* 1991, Barlowe *et al.* 1994, Simpson *et al.* 1997, Dell´Angelica *et al.* 1999)

[2] (Donaldson *et al.* 1990, Robinson & Kreis 1992, Wong & Brodsky 1992, Stamnes & Rothman 1993, Rothman & Wieland 1996, Simpson *et al.* 1997, West *et al.* 1997 Dell´Angelica *et al.* 1999)

[3] (Serafini *et al.* 1991, Waters *et al.* 1991, Barlowe *et al.* 1994, Simpson *et al.* 1997, Kirchhausen 1999, Wieland & Harter 1999)

[4] (Letourneur *et al.* 1994, Schekman & Orci 1996, Le Borgne *et al.* 1998, Kirchhausen 1999, Hirst *et al.* 1999, Wieland & Harter 1999, Scales *et al.* 2000)

2.1.2 Coat assembly and vesicle budding

Several groups have explored the biochemical requirements for the recruitment of adaptor and coat proteins to isolated membranes. Identification of novel coat proteins and characterization of their binding to membranes have been studied especially by using yeast genetic assays, GTPγS (a non-hydrolysable analogue of GTP), and brefeldin A (BFA), a fungal metabolite known to inhibit the action of ARF (Donaldson *et al.* 1990, Robinson & Kreis 1992, Schekman 1992, Rothman 1994). BFA causes disassembly of the Golgi complex and a redistribution of Golgi resident enzymes to ER, thus resulting in a block in protein secretion (Yan *et al.* 1994). The detailed mechanism for BFA action is presumably the inhibition of the ARF GEFs and, thus, the prevention of the nucleotide exchange from ARF-GDP to ARF-GTP (Peyroche *et al.* 1996).

Binding of AP-1, AP-3, and COPI to Golgi membranes is blocked by BFA and, on the other hand, enhanced by GTPγS. This strongly suggests that the recruitment event onto the membranes is mediated through the activation of a specific small GTPase (Donaldson *et al.* 1990, Robinson & Kreis 1992, Wong & Brodsky 1992). *In vitro* studies suggest that this GTPase is most likely ARF1 (Stamnes & Rothman 1993). Currently, little is known about the mechanism by which cytosolic AP-2 complex is targeted to the plasma membrane. Some results indicate that it is probably also controlled by an as yet unknown small GTPase (Traub *et al.* 1996, West *et al.* 1997).

Vesicle budding is a multifactorial event. At the moment, the best information is available from experiments made with the coatomer protein COPI. Golgi-localized GEF specific for ARF1 converts ARF1-GDP into ARF1-GTP which is recruited to the membrane. Then it probably triggers the activation of phospholipase D and the binding of the coatamer to the Golgi membranes, followed by a formation of coatamer-coated COPI-buds and vesicles (Serafini *et al.* 1991, Nickel & Wieland 1998). The assembly of coat proteins deforms the donor membrane and captures cargo molecules, resulting in the formation of a bud (Rothman & Wieland 1996). Membrane cargo is sorted into the buds by a direct interaction between the cytoplasmic domain of the cargo protein and the coat components. The soluble cargo, on the other hand, requires the involvement of a transmembrane receptor to mediate the interaction (Wieland & Harter 1999). In the cargo molecules there are sorting signals to ensure that the cargo molecules they carry become trapped and packed into correct transport carriers. For instance, dilysine motif (KKXX) in the membrane proteins and tetrapeptide sequence KDEL in the soluble proteins mediate the sorting of these proteins into COPI-vesicles (Townsley *et al.* 1993, Cosson & Letourneur 1994, Letourneur *et al.* 1994). Members of the p24 family of proteins are also involved in the budding and the formation of COPI-vesicles as putative cargo receptors or as coat receptors (Sohn *et al.* 1996, Wieland & Harter 1999).

The budding of CCVs appears to be generally similar to that of COPI vesicles (Rothman & Wieland 1996). An especially well explored example is the transport of lysosomal enzymes from the TGN to lysosomes by CCVs. It involves the binding of soluble proteins in the TGN to transmembrane mannose-6-phosphate receptors (MPRs). MPRs interact further by their cytoplasmic tails with AP-1 and sequester the cargo into CCVs (see also 2.3.3.1) (Traub & Kornfeld 1997).

The vesicle pinches off in a process called periplasmic fusion which, in the case of COPI vesicles, requires fatty acyl coenzyme A (Ostermann *et al.* 1993). The other components mediating this last step are not known (Nickel & Wieland 1998). Periplasmic

fusion of endocytic CCVs at the PM and probably also at the TGN is aided by the GTPase dynamin (for review, see Damke 1996). After this, ARF-bound GTP is hydrolysed to GDP (Wieland & Harter 1999), thus resulting in dissociation of the ARF from the membranes. Thereafter the remaining coat proteins dissociate from the fully formed transport vesicle (Rothman 1994).

For both AP-1 and AP-3, binding of the adaptor proteins to membranes and the coat assembly is triggered by ARF-GTP, most probably by ARF1 (Stamnes & Rothman 1993, Ooi *et al.* 1998). ARF1 mediates the activation process either by a direct interaction with the coat components or alternatively/additionally by activation of phospholipase D (Le Borgne & Hoflack 1998). In addition to small GTPases, the lipid composition of membranes plays a central role in coat recruitment and vesicle formation (Roth 1999).

2.1.3 Targeting, docking and fusion of transport vesicles

There are several mechanisms for the transport of vesicles from the donor membrane to the acceptor membrane. For instance, the vesicle may simply diffuse if the distance is short (e.g. intra-Golgi transport). If the distance is long, the transport is mediated by vesicle-associated components interacting with the cytoskeleton (e.g. from TGN to PM). In large and polarized cells, vesicle transport utilizes cytoskeletal "tracks" consisting of microtubules and actin filaments and is probably powered by motor proteins belonging to the nonmuscle myosin, kinesin and dynein superfamilies (McNiven & Marlowe 1999, Kamal & Goldstein 2000). However, the involvement of the cytoskeleton is not always necessary for the transport (Kamal & Goldstein 2000, and references therein).

It is generally accepted that there must be signals on the vesicles which are recognized by a receptor on the target membrane. Such a recognition is most probably mediated by specific SNAREs. They are a group of proteins located on the transport vesicle (v-SNARE) or on the target membrane (t-SNARE) (Söllner *et al.* 1993, Söllner & Rothman 1996). v-SNAREs include e.g. the protein synaptobrevin/VAMP present on synaptic vesicles of *Torpedo californica* (Trimble *et al.* 1988). t-SNAREs include e.g. the proteins syntaxin (Bennett *et al.* 1992) and SNAP-25 present at the presynaptic plasma membrane (Oyler *et al.* 1989). The term "SNARE complex" refers to a machinery including four components: v-SNARE, t-SNARE, SNAP, and NSF. Proteins that regulate SNAREs include Rabs which facilitate SNARE complex formation (Sogaard *et al.* 1994, Schimmöller *et al.* 1998, Gerst 1999) and Sec1 proteins which prevent their binding/assembly (Pevsner *et al.* 1994, Jahn 2000).

The Rab proteins belong to the Ras superfamily of GTPases. In mammals, over 30 Rabs are known (reviewed by Chavrier & Goud 1999). Like ARFs, Rabs also cycle between an inactive GDP-bound and an active GTP-bound conformation with GEFs catalyzing the GDP/GTP exchange reaction. Rabs link transport vesicles or membranes to cytoskeleton, trigger the recruitment of docking complexes, and probably regulate the assembly of SNARE complexes (Chavrier & Goud 1999). However, data so far implicate that Rabs do not act alone. Instead, the mechanism in which they regulate membrane traffic and vesicle targeting seems to be more involved in the recruitment of "tethering" proteins (see below) and docking factors (Guo *et al.* 2000).

The detailed mechanism of the fusion event between the transport vesicles and their target membranes is not fully understood at the moment. During the fusion, the vesicle delivers its content and adds the vesicle membrane to the target membrane. To start the fusion at the target membrane, components such as NSF and SNAPs are needed (Söllner & Rothman 1996). The fusion does not require coat proteins. The features of the SNARE complex suggest that it "draws" the vesicle to target membrane, thus inducing fusion, but does not drive the membrane fusion to completion (Bajjalieh 1999). Each v-SNARE/t-SNARE-complex binds several (3 to 6) SNAP proteins. The subsequent binding of an ATPase NSF leads to ATP hydrolysis and to the dissociation of the complex (Söllner & Rothman 1996). The rearrangement triggered by the ATP hydrolysis leads to a release of SNAP proteins, presumably then allowing the fusion of membranes to occur (Rothman 1994, Hayashi *et al.* 1995, Rothman & Wieland 1996). Figure 3 presents the different steps and components of vesicle budding, docking/targeting and fusion.

2.1.4 Membrane tethering

Recent findings indicate that Rabs and SNAREs are not the only players responsible for the targeting of transport vesicles to their correct membranes. Molecular motors and actin/microtubule-based cytoskeleton are also required to transport vesicles a long way from one compartment to another. Recent studies have revealed "tethering" proteins as new components of the targeting machinery. They are probably responsible for bringing the vesicles close to their target membranes (Pfeffer 1999).

The tethering factors seem to collaborate with Rabs and SNAREs to generate targeting specificity (Waters & Pfeffer 1999). They bind or "tether" membranes of the incoming vesicle and the target organelle. This is then followed by the formation of a SNARE complex and fusion of the membranes (Waters & Pfeffer 1999). The potential tethering factors include, in endosomes, the protein EEA (Mills *et al.* 1998) and, within intra-Golgi transport, a protein complex consisting of at least four proteins: giantin, p115, GM130 and GRASP65 (Barr *et al.* 1998, Waters & Pfeffer 1999). A common feature of many proteins involved in tethering is their propensity to form highly extended, coiled-coil structures (Pfeffer 1999). While the detailed mechanism of vesicle targeting is still unknown, it is apparent that the specificity is most probably achieved through co-operation between several different regulatory components.
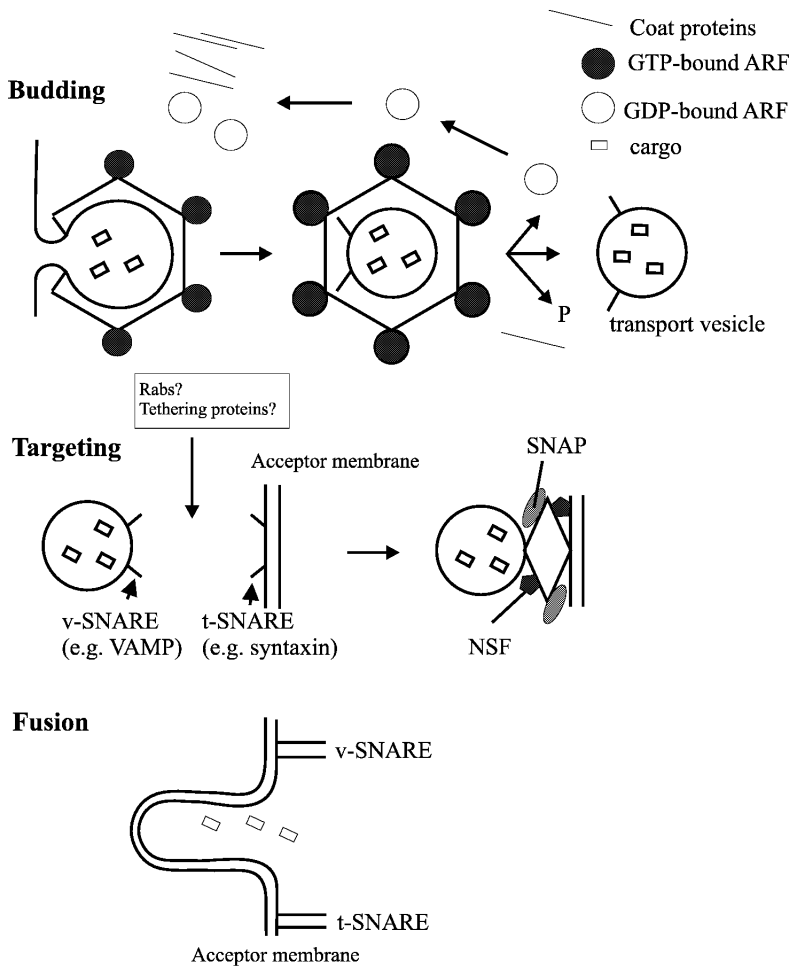
Fig. 3. The basic molecular machinery involved in the budding, docking/targeting, and fusion of the transport vesicles. At the budding stage, membrane-bound ARF-GTP triggers the recruitment of coat proteins onto membrane, resulting in the shaping of the membrane into a coated bud. After periplasmic fusion, the fully formed vesicle soon looses its coat and after this it is targeted and "docked" to the target membrane by specific "pilot" proteins. v-SNARE present on the vesicle membrane interacts with t-SNARE present on the acceptor membrane. Before fusion, SNAP binds to the SNARE complex then recruiting NSF. ATP hydrolysis by NSF dissociates the SNARE complex then inducing membrane fusion. ARF, ADP ribosylation factor; NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF associated protein; SNARE, soluble NSF attachment receptor (mofified from Söllner & Rothman 1996).

2.2 Endocytosis

Many critical functions of a cell are mediated by endocytic mechanisms, including the uptake of extracellular nutrients, regulation of cell-surface receptor expression, mainte-

nance of cell polarity, and antigen presentation. Additionally, pathogens such as viruses, toxins, and different micro-organisms utilize endocytic pathways to get inside the cell (reviewed by Mukherjee *et al.* 1997).

Endocytic machinery has a wide capacity to internalize anything from small molecules to entire cells. Remodeling of the PM by endocytosis enables a cell to control its responses to extracellular stimuli (Marsh & McMahon 1999). The majority of the endocytosed plasma membrane components (lipids and membrane proteins) are recycled back to the PM from the early endocytic compartment to maintain the normal composition of the PM (Mukherjee *et al.* 1997).

At least five different pathways for endocytic internalization are known: the clathrin-dependent pathway, macropinocytosis, the caveolar pathway, a clathrin- and caveolin-independent pathway, and phagocytosis (Riezman *et al.* 1997). The most common and best characterized endocytic pathway is clathrin-mediated endocytosis.

2.2.1 Endocytic pathway and its machinery

One of the best studied endocytic mechanisms is the receptor-mediated endocytosis via clathrin-coated pits, which is known in detail e.g. for many growth factor receptors with tyrosine kinase activity. The binding of a growth factor to its specific receptor on the plasma membrane causes the initiation of the signal transduction cascade which results in e.g. cell proliferation. Endocytosis of the ligand-receptor complexes from the cell surface also results in receptor down-regulation which negatively regulates cell signalling (Sorkin & Waters 1993). After internalization, the ligand dissociates from its cognate receptor and the free receptor is transported back to the PM for reutilization (Gruenberg *et al.* 1989).

After budding, vesicles rapidly lose their coats which leaves them unstable, which in turn facilitates fusion of the vesicles with sorting or early endosomes (EE), the first station of the endocytic pathway (Kornfeld & Mellman 1989, Mellman 1996). EEs are structurally seen as a network of tubules and vesicles throughout the cytoplasm, and their basic function includes the sorting of received molecules (Mellman 1996). From EEs the membrane-bound receptors are recycled efficiently back to the PM and the free ligands are further transported for degradation to late endosomes (LE) or lysosomes (Mukherjee *et al.* 1997). Additionally, material (e.g. glucose transporter GLUT4) can also be transported from EEs to the regulated secretory pathway (Wei *et al.* 1998, Simpson *et al.* 2001)

Transport of free receptors back to the PM from EEs is mediated by recycling compartment or recycling vesicles (RV). The recycling compartment is seen as a physically separate portion of the EE system and thought to arise from the tubular extensions of EEs (Mellman 1996, Mukherjee *et al.* 1997). Movement of material from the early endosomes to the late endosomes is generally thought to be mediated by transport vesicles which pinch off and transfer endosome contents to LEs. However, in the so-called maturation model, it is proposed that the EEs as such are transformed into LEs (Mukherjee *et al.* 1997).

Late endosomes are responsible for the accumulation and concentration of the cargo after receiving it from the EEs. LEs contain hydrolytically active lysosomal hydrolases

and they are considered to be a starting point for the degradative process of foreign material. MPRs concentrate in LEs from which they are recycled back to the TGN (Griffiths *et al.* 1988). Delivery of endocytosed material from LEs to lysosomes is thought to occur by fusion of LEs with lysosomes, resulting in the digestion of cargo in the lysosomes by low pH (≈5.0) and lysosomal enzymes (Gruenberg *et al.* 1989, Mellman 1996). The resulting degradation products are transferred out from the lysosomes to cytosol, where they can be utilized by the cell or alternatively transported out of the cell (Mellman 1996).

Clathrin-independent phagocytosis ("cell eating") refers to the internalization of large (> 0.5 µm diameter) particles which usually bind to their corresponding plasma membrane receptors triggering the formation of F-actin-driven pseudopods for internalization of the bound particles. Micro-organisms are also internalised by phagocytosis (Mellman 1996, Mukherjee *et al.* 1997). In mammals, phagocytosis is seen preferentially in cells with specialized roles, such as e.g. macrophages and neutrophils (Mukherjee *et al.* 1997).

In contrast to phagocytosis, pinocytosis ("cell drinking") mediated by clathrin-coated pits is commonly seen in eukaryotic cells. It refers to a constitutive formation of smaller vesicles (< 0.2 µm) through which extracellular fluid and macromolecules bound to plasma membrane are internalized for further processing. The best characterized type of pinocytosis is receptor-mediated endocytosis which provides a selective uptake of specific macromolecules (Mellman 1996).

2.2.2 Receptor-mediated endocytosis

Epidermal growth factor receptor (EGFR) is an epitome of receptor tyrosine kinases, transmembrane receptors which carry a kinase domain in their cytoplasmic tail (Boonstra *et al.* 1995). The endocytosis of EGFR with its ligand epidermal growth factor (EGF) represents one of the best characterized systems of signaling upon a soluble ligand binding to its cognate receptor. At the first step, binding of EGF to EGFR promotes clustering of the receptor (Sorkin & Waters 1993). AP-2 is then recruited from the cytosolic pool to PM to initiate clathrin assembly, vesicle formation, and internalization of the ligand-receptor complex in CCVs. AP-2 interacts directly with the tyrosine-based recognition sequence in the cytosolic tail of EGFR (Sorkin & Carpenter 1993, Ohno *et al.* 1995, Sorkin *et al.* 1996). Recent studies suggest that cytoplasmic receptor tails are not the only sequences responsible for the recruitment. Instead, there are probably also specific docking mechanisms which are currently unknown. Candidates for this are e.g. synaptotagmin (Zhang *et al.* 1994) and Eps15 which binds directly to the cytoplasmic tail of EGFR (Torrisi *et al.* 1999).

The 100-kDa GTP-hydrolyzing protein dynamin has a central role in the constriction of the coated pits. In its GTP-bound form, dynamin assembles into a collar around the neck of deeply invaginated pits. Hydrolysis of GTP bound to dynamin drives the closing of the collar´s neck, resulting in the dissociation of dynamin and pinching off of an isolated clathrin-coated vesicle (Damke 1996, Yang & Cerione 1999).

There are several regulatory proteins that either interact with EGFR or alternatively affect its endocytosis. They include e.g. Eps15, a recently identified substrate of EGFR. It plays an important role in receptor-mediated endocytosis (Fazioli *et al.* 1993). Eps15

associates with both clathrin and AP-2, and a number of studies have linked Eps15 to coated-pit mediated internalization (Torrisi *et al*. 1999). A recently cloned and characterized EGFR- and Eps15-associated protein, EAST, contains SH3 -, TAM - and VHS domains, and colocalizes partly with clathrin (Lohi *et al*. 1998). The name VHS derives from its occurrence in <u>V</u>PS-27, <u>H</u>rs and <u>S</u>TAM (Lohi & Lehto 1998). The specific function of the VHS domain remains poorly known at the moment, but its occurrence in proteins involved in vesicular trafficking and endocytosis suggests that it could serve as a functional domain involved in membrane targeting and association (Lohi & Lehto 1998, Lohi *et al.* 1998, Schultz *et al.* 1998, Misra *et al.* 2000).

2.3 Organization of the biosynthetic/secretory pathway

The biosynthetic/secretory pathway was originally described in pancreatic exocrine cells as early as in 1975 (Palade 1975). The pathway consists of several functionally and structurally different membrane-bound compartments. They include the endoplasmic reticulum ER, the intermediate compartment (IC), *cis*-Golgi network (CGN), the Golgi stacks and *trans*-Golgi network as a final sorting station (Saraste & Kuismanen 1992, Harter & Wieland 1996). The basic function of this pathway includes transport of proteins destined for secretion from the ER to Golgi and further to the PM. Synthesized material can also be recycled to ER from the Golgi (Pelham 1996). The compartments are connected with each other by vesicular traffic which mediates the transport of cargo between different organelles and also controls the composition and homeostasis of the structures (Rothman & Wieland 1996).

The early secretory pathway transports biosynthetic material from the ER to Golgi complex. Lipids, proteins and carbohydrates are modified and transported through Golgi to TGN in which they are sorted and packed into vesicles for further transport to various destinations. At TGN, specific sorting signals in the cargo molecules and the cellular sorting machineries are responsible for directing the cargo either to the PM, to regulated secretory granules, or to the endosomal/lysosomal system (for review, see Ikonen & Simons 1998, Le Borgne & Hoflack 1998). The bulk flow secretory pathway operates in all cells and it leads to a continuous unregulated secretion or transport to the PM. Some specialized cells also possess a distinct regulated secretory pathway in which certain specific proteins (e.g. hormones or neurotransmitters) are secreted to extracellular space in response to external signal(s) (Traub & Kornfeld 1997). In general, the secretory pathway links organelles together to provide a framework by which proteins undergo a series of post-translational modifications including proteolytic processing, folding and glycosylation (Storrie *et al*. 2000).

2.3.1 Endoplasmic reticulum

Endoplasmic reticulum is the entry station for all proteins of the synthetic/secretory pathway consisting of nuclear envelope, rough ER (rER), smooth ER (sER), transitional ER (tER), and intermediate compartment (IC) (Saraste & Kuismanen 1984, Saraste & Kuis-

manen 1992, Lippincott-Schwartz *et al.* 2000, and references therein). Different subcompartments of ER have characteristic biochemical and physiological properties and they serve specific subcellular functions. Structurally ER is seen as a three-dimensional, reticular network of continuous tubules and sheets creating the largest membranous organelle of the cell (Vertel *et al.* 1992). Functionally ER is responsible for the synthesis and processing of secreted proteins, membrane proteins and organelle resident proteins. ER is also seen as a compartment participitate in the assembly, sorting, and degradation of proteins as well as in the regulation of the level of intracellular calcium (Vertel *et al.* 1992). ER also has a role in lipid synthesis and detoxification of substrates by UDP-glucuronyl transferases (Harter & Wieland 1996).

From ER, properly processed, folded and assembled proteins are further transported via IC to the Golgi complex by specific carrier vesicles which bud on the ER and move to *cis*-Golgi membranes (Kaiser & Ferro-Novick 1998). Folded proteins may remain in the ER if it is their home compartment or else they are transported to the secretory pathway. COPII is a coat complex which forms a main structure of transport vesicles responsible for forward transport of cargo from the ER to the Golgi complex (Barlowe *et al*. 1994). COPII vesicles carry several classes of material: secretory cargo being transported from the ER to the Golgi, components of the folding and sorting machinery, and the components of the targeting and docking machinery (Scales *et al.* 2000). COPI vesicles in their turn carry cargo between Golgi and ER and are also involved in intra-Golgi transport (Letourneur *et al*. 1994, Orci *et al*. 1997). Sorting of synthesized proteins at the ER occurs by selective incorporation of secretory and membrane proteins into vesicles that bud from the ER (Pelham 1996, Wieland & Harter 1999). The lumenal soluble proteins of the ER carry a specific carboxyterminal KDEL signal which prevents the secretion of these proteins. Those ER-resident KDEL proteins which have escaped the ER, are recycled back from the Golgi to ER by COPI vesicles (Donaldson & Lippincott-Schwartz 2000).

2.3.2 Golgi complex

The Golgi complex plays an important role at the crossroads of the secretory pathway. It receives freshly synthesized proteins and lipids from the ER, modifies them, and then distributes cargo to various destinations. The Golgi has earlier been viewed as a static station for the processing of secretory material, but now it seems more like Golgi undergoes continuous remodeling.

Traditionally, Golgi has been viewed as a series of stable compartments, named the *cis*-, medial- and *trans*-Golgi, as well as TGN (Glick 2000). Proteins coming from ER to Golgi enter the organ on its tubulovesicular *cis*-face, travel across the stacks, and leave the Golgi on its *trans*-face. CGN not only receives the material from the ER but is also involved in sorting and recycling of lipids and proteins to the ER (Hsu *et al.* 1991, Saraste & Kuismanen 1992, Lippincott-Schwartz *et al.* 2000). On the way through the Golgi, newly synthesized glycoproteins are subjected to several posttranslational modifications such as ordered remodeling of their N-linked oligosaccharide side chains and biosynthesis of O-linked glycans. To effect such modifications, the Golgi complex is organized as

polarized stacks of flattened cisternae enriched in transmembrane processing enzymes (Mellman & Simons 1992). To be able to send cargo even long distances through the cytoplasm, the Golgi complex is closely associated with the cytoskeleton. It is situated around the microtubule organizing center and is surrounded by actin cytoskeleton and actin-binding proteins (Donaldson & Lippincott-Schwartz 2000). Recent studies have shown a novel Golgi-associated spectrin-based network which could provide a structural framework for the Golgi complex (Holleran & Holzbaur 1998, and references therein). The close relationship between the Golgi and the cytoskeleton indicates that the structural network not only maintains the Golgi morphology, but also participates in the vesicle traffic to and from the Golgi.

There are two different models to explain and understand the movement of material within the Golgi: in the so-called "stable compartments" model, membrane-bound carriers (vesicles) transport cargo, while in the "cisternal maturation" model, ER-derived membranes coalesce to form new cis-cisternae (Glick 2000, Weiss & Nilsson 2000). COPI-coated vesicles have been implicated in the vesicle budding and fission events at the Golgi membranes and they also participate in the anterograde or bi-directional transport of material across the Golgi stack (Letourneur *et al.* 1994, Schekman & Orci 1996). In a directing process of cargo to different destinations (see also 2.1.2) within intra-Golgi traffic, there are probably multiple classes of different COPIs involved. Also, "tethering" proteins presumably direct various types of COPI vesicles to correct destinations (Glick 2000).

2.3.3 Trans-Golgi network

The *trans*-Golgi network is the site of the sorting and final exit of cargo from the Golgi. It refers to the *trans*-side of the Golgi and structurally it is seen as a sacculotubular network. The structure and the size of TGN varies remarkably from one cell type to another: in cells with a low number of secretory granules but with an extensive lysosomal system, TGN is massive, while secretory cells showing small or large secretory granules typically possess small TGN or even lack it (Clermont *et al.* 1995). The size of TGN also varies depending on the level of the activity of protein secretion in a cell (Griffiths *et al.* 1989).

At TGN, cargo molecules are sequestered into coated vesicles and directed to their correct destinations. For example, proteins with specific recognition signals are packed into CCVs and transported to endosomal/lysosomal system in a selective pathway (Marks *et al.* 1997). Proteins carrying specific sorting signals are targeted and transported to plasma membrane through a so-called constitutive pathway (Kornfeld & Mellman 1989, Pearse & Robinson 1990). In specialized cells producing large quantities of particular products in response to extracellular stimuli (e.g. hormonal or neural stimuli), there exists another secretion pathway leading to cell surface called the regulated secretory pathway (Traub & Kornfeld 1997).

2.3.3.1 Sorting of cargo at TGN

At TGN, sorting of cargo to different destinations is regulated by many means. First, bio-chemically distinct coats are likely to specify protein sorting. Second, cytosol-oriented sorting signals of cargo proteins direct them to the appropriate export site. Third, TGN might be organized into discrete subdomains dedicated to assemble specific coat population (Traub & Kornfeld 1997).

The constitutive pathway in polarized epithelial cells (e.g. MDCK cells) includes the apical- and basolateral routes. Sorting to the basolateral pathway is mediated by cytoplasmic sorting signals of the cargo molecules. They include tyrosine residues, the dileucine motif, or "adders" that contains neither dileucine- or tyrosine-motifs (Keller & Simons 1997). The machinery responsible for the basolateral sorting is currently unknown while some data implicate that it could be mediated by AP-1 and clathrin (Futter *et al*. 1998). The apical sorting signals of the cargo include glycosylphosphatidyl inositol (GPI)-anchor, N-glycosylation and the transmembrane domain (Zurzolo *et al*. 1993, Scheiffele *et al*. 1995, Keller & Simons 1997). Sorting signals involved in the targeting to selective pathway have also been reported: the mannose 6-phosphate (M-6-P) residues in soluble proteins (e.g. lysosomal hydrolases) and tyrosine- and di-leucine-based sorting determinants in membrane proteins direct them into CCVs and further into the endosomal/lysosomal system (Kirchhausen *et al*. 1997, Marks *et al*. 1997, Rohn *et al*. 2000) The membrane proteins are e.g. the lysosomal associated membrane protein (LAMP) and the lysosomal integral membrane protein (LIMP). For regulated secretory pathway, a conformation-dependent motif is suggested to destine the proteins for secretion (Keller & Simons 1997).

At TGN, the sorting and transport of a group of soluble proteins (≈ 50) via a selective pathway to lysosomes relies on the existence of mannose 6-phosphate residues on their oligosaccharides (Le Borgne & Hoflack 1998). They serve as recognition signals for MPRs. Sorting of MPRs and their bound ligands to their destinations is mediated preferentially by the interaction of tyrosine- and dileucine-based sorting signals present in their tails with the adaptor protein complex AP-1 and by transport in CCVs (Le Borgne *et al*. 1996, Le Borgne & Hoflack 1998). The pinching off of the CCVs from the membranes is effected by dynamin (Hinshaw & Schmid 1995, Jones *et al*. 1998, Kasai *et al*. 1999). The transport vesicles move to acidic endosomes releasing the MPRs to recycle back to TGN, and subsequently, moving the cargo to lysosomes (Le Borgne & Hoflack 1998).

The lysosomal membrane glycoproteins such as LAMP and LIMP are targeted and transported from the TGN to lysosomes preferentially via an AP-3-dependent pathway. In contrast to MPRs, LAMP and LIMP do not recycle back to this compartment (Rohn *et al*. 2000). In the transport, also AP-1 and clathrin are probably involved (Höning *et al*. 1996). The cytoplasmic tyrosine- and dileucine-based motifs of LAMP and LIMP serve as sorting signals by interacting with AP-3 (Höning *et al.* 1996, Le Borgne & Hoflack 1998, Rohn *et al.* 2000).

Clathrin, AP-1, and AP-3 are the central components of the coat complexes of the selective pathway. The coat components of the other secretory pathways are not yet known (Traub & Kornfeld 1997). In Figure 4, a schematic representation is given of the different vesicle transport pathways originating from TGN.
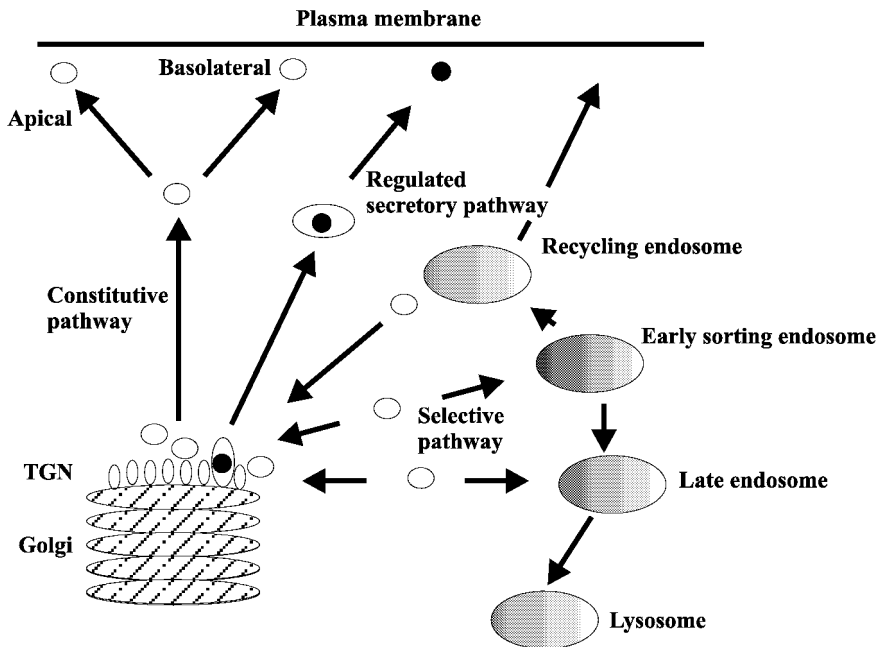
Fig. 4. A schematic representation of the different vesicle transport pathways originating from the *trans*-Golgi network. TGN, *trans*-Golgi network (modified from Le Borgne & Hoflack 1998).

2.4 Golgi complex in skeletal muscle cells

Muscle is a heterogeneous tissue constituting up to 40 to 45% of the total body weight of adult man. Skeletal muscle is composed of a large number of muscle fibers with their width and length varying from one muscle to another (Kakulas & Adams 1986). Each fiber is actually a large multinucleated syncytium. Several types of fibers can be distinguished based on their functional, metabolic and ultrastructural features: type I and type IIA, IIB and IIC fibers. Type I fibers are aerobic, slow-contracting and capable of long, continued activity. Type II fibers are typically anaerobic, fast-contracting and phasic (Hoh 1992). Type I fibers are rich in mitochondria and myoglobin while poor in adenosine triphosphatase (ATPase). Type II fibers are poor in mitochondria but rich in myofibrillar ATPase and phosphorylase (Kakulas & Adams 1986). In normal skeletal muscle, different fiber types are distributed in a mosaic pattern. The relative abundance of each fiber type differs from one anatomical region to another. Variability in the fiber type composition in the same muscle is also seen between different species (Gunning & Hardeman 1991). Basic differences seen between type I and type II fibers are listed in Table 2.

Fiber-type diversity seen histochemically is mostly based on the differential expression pattern detected for the specific isoforms of myofibrillar and other related proteins (Pette & Staron 1990). Myosin is a central component of skeletal muscle. It consists of two heavy chains and four light chains. Usually, a single muscle fiber expresses only a single

isoform of a myosin heavy chain (MHC) which correlates with the contractile properties of the fiber and histochemical ATPase staining characteristic of the fiber. Thus, the ATPase activity is a useful indicator of the fiber type and its determination is utilized in fiber-type classification (Talmadge *et al.* 1993).

Different abundances and subcellular distribution patterns correlating to the different fiber-types have been reported for e.g. mitochondria, T-tubules, sarcoplasmic reticulum, microtubules and the Golgi complex (Ralston *et al.* 1999). In skeletal muscle, GC does not display the same morphological features as in many other cells. In fact, only a very limited amount of information about its organization in muscle is currently available. On the other hand, in myoblasts, representing immature muscle cells, GC is seen as a distinct and compact structure like in many other mammalian cells. During differentiation of the myoblast, GC reorganizes into a network of smaller elements (Tassin *et al.* 1985a, Tassin *et al.* 1985b).

In skeletal muscle fibers, the total number of GC per length unit is approximately the same in different fiber types. Its organization, however, is fiber-type dependent. Thus, in type I fibers the Golgi elements accumulate more along the surface of the fibers, while in fast-twitch type II fibers they show a more even distribution between the surface and the core (Ralston *et al.* 1999). Additionally, Ralston *et al.* suggested that microtubules could be responsible for the differential distribution pattern of GC seen between different muscle fibers (Ralston *et al.* 1999).

Vesicular trafficking pathways in multinucleated skeletal muscle fibers differ considerably from those of mononucleated cells. During myogenesis, specialized vesicular trafficking pathways develop and the exocytic pathway is reorganized (Ralston 1993, Rahkila *et al.* 1998). Currently, little is known about the endocytic pathway and its components in the skeletal muscle. Earlier it was suggested that endocytosis occurs predominantly in the neuromuscular junctions (NMJ) (Libelius & Tagerud 1984), but recently it was shown that endocytosis can be seen over the entire muscle fiber (Elmquist *et al.* 1992, Kaisto *et al.* 1999).

Table 2. Physiological and functional features of different types of skeletal muscle fibers.

	TYPE I	TYPE II
Contraction	prolonged	rapid, powerful
Physiology	slow-twitch	fast-twitch
Glycogen	low content	high content
Mitochondria	many	few
Oxidative enzymes	high	low
Myoglobin	high	low
Phosphorylase	low	high
Lipids	high	low
Enzyme-histochemical reaction		
NADH	strong	weak
ATPase at pH 9.4	weak	strong

2.5 Golgi complex in podocytes, specialized epithelial cells of the glomeruli

The kidney consists of about one million nephrons as its basic functional units. The primary functions of the kidneys include the removal of waste products from the plasma and controlling the fluid, electrolyte, and acid-base balance of the circulation system. The kidneys have a high content of epithelial cells with an extensive intracellular membrane trafficking machinery (Lehtonen *et al.* 1999).

Urine production starts in the renal glomerulus which consists of Bowman´s capsule and capillary tuft (Mundel & Kriz 1995). The glomerular capillary wall is permeable for water, small molecules, and ions, but inpenetrable for plasma proteins and other large molecules (Reiser *et al.* 2000). In the glomerulus, four major cell types are found: endothelial cells, mesangial cells, podocytes, and parietal epithelial cells of Bowman´s capsule (Mundel & Kriz 1995). In Figure 5, a schematic representation of the structure and the different cell types of the glomeruli are presented.
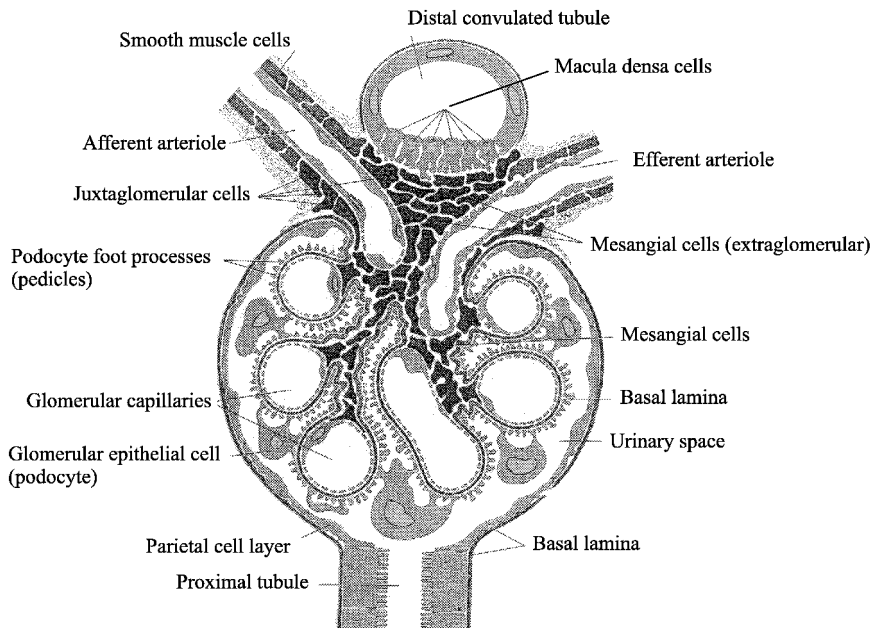
Fig. 5. A schematic representation of the structure and the different cell types of the glomeruli (modified from Ross & Romrell 1989).

Podocytes form a crucial component of the glomerular filtration barrier by covering the exterior basement surface of the glomerular capillary and maintaining a massive filtration surface composed of the slit diaphragms (Pavenstadt 2000). To pass the filtration barrier, the filtrate first goes through the open pores of the glomerular endothelium, then passes the glomerular basement membrane (GBM), and finally the filtration slits (Reiser *et al.* 2000). Functionally, podocytes are responsible for the GBM turnover, maintenance of

the filtration barrier, support of the capillary tuft and regulation of the glomerular filtration. They also mediate some immunological functions (Mundel & Kriz 1995).

Structurally and functionally podocytes can be divided into three different segments: cell body, major processes, and foot processes. The foot processes or pedicles abut the outer surface of the GBM. Between the pedicles there are filtration slits. The cell bodies and major processes float freely in Bowman´s space and are not in direct contact with GBM (Mundel & Kriz 1995). They possess, on their luminal surface, a well-developed and negatively charged glycocalyx which is composed of several sialoglycoproteins, e.g. podocalyxin and podoendin (Kerjaschki *et al*. 1984, Huang & Langlois 1985). This structure has several functions. It forms a negatively-charged surface of the filtration barrier and plays an important role in maintaining the complex architecture of the pedicles by dissociating the pedicles from each other. It also prevents the attachment of podocytes to the parietal epithelium of Bowman´s capsule (Schnabel *et al*. 1989, Kerjaschki 1994, Mundel & Kriz 1995). Figure 5 depicts ultrastructural features of a podocyte as seen in electron microscopy.

Several structural features of podocytes point to a high rate of vesicular traffic in these cells. First, many coated vesicles and coated pits can be seen along the basolateral domain of the podocytes. Second, in their cell body, podocytes possess a well-developed ER and a large Golgi apparatus, indicative of a high capacity for protein synthesis and post-translational modifications. Third, a high endocytic activity is suggested by the large number of multivesicular bodies and other lysosomal components seen in these cells (Mundel & Kriz 1995). The major cellular processes of podocytes contain a well-organized microtubular network which is necessary for normal formation of the cellular processes (Kobayashi *et al*. 1998).
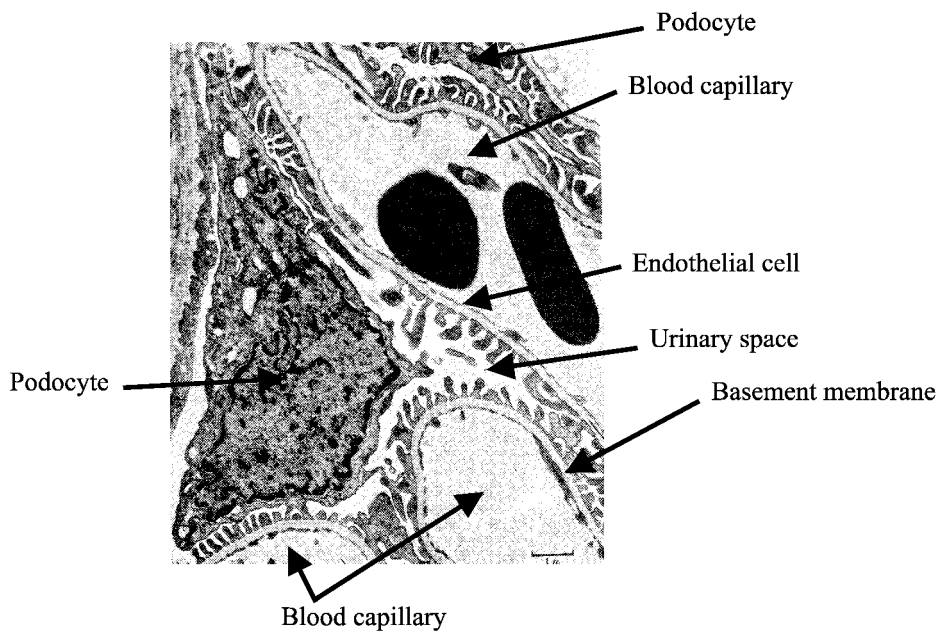

Fig. 6. Podocytes and capillary loops seen at the ultrastructural level (modified from Williams *et al.* 1989).

Four different developmental stages can be discerned during glomerulogenesis in developing fetal kidney: vesicle phase, S-shaped body stage, capillary loop stage, and mature juxtamedullary stage (Reeves *et al.* 1978). The renal vesicle stage is characterized by clusters of epithelial cells which resemble vesicular structures. At this stage, it is not possible to distinguish the future podocytes from those cells that form the epithelium of the proximal tubule. At the S-shaped body stage, a cleft is formed in the vesicular cell aggregates leading to segregation of podocytes to one side of the cleft, and the cells destined to become proximal tubule epithelium to the other side of the cleft. The evolving endothelial and mesangial cells arise from the mesenchymal cells in the cleft region. The formation of capillaries is the expressive feature of the capillary loop stage with the progressive capillarization leading to formation of the maturing glomeruli (Reeves *et al.* 1978). In the fetal kidney, a gradient of glomeruli representing different developmental stages can be seen across the renal cortex. In the cortex, the maturing glomeruli are located near the medulla and the most immature are seen close to the renal capsule (Abrahamson 1991).

2.6 Golgi complex in gastrointestinal epithelia

The epithelial cells of the gastrointestinal (GI) tract are active in various exocytic and endocytic functions. Typically, these functions include e.g. the secretion of proteins or other components into the blood stream via ducts or directly into the intestinal lumen. On the other hand, they actively take up material from the intestinal lumen by endocytosis. These events are known to be mediated by an active vesicle transport machinery (McNiven & Marlowe 1999). Typically, in polarized epithelial cells, such as are found for instance in the intestinal surface epithelium, their nuclei are located basally and the Golgi complex lies between the nucleus and the apical (luminal) cell surface (Simons & Fuller 1985, Schnabel *et al.* 1989).

Compared with many other cell types, the function and the organization of the GC in the cells of the GI tract remains poorly known. The organization and the size of GC and TGN varies considerably between various cell types (Clermont *et al.* 1995). In cells with a high secretory activity, e.g. goblet cells and Brunner´s gland cells, GC is a large ribbon-like supranuclear organelle (Rambourg *et al.* 1987, Rambourg & Clermont 1990), which in confocal microscopy exhibits a dome-like organization in the supranuclear region (Suzaki & Kataoka 1999). TGN, on the other hand, appears small or as residual fragments in the cells of Brunner´s gland (Clermont *et al.* 1995).

The main function of the Golgi complex in the GI-tract involves posttranslational modifications of proteins, carbohydrate synthesis, and glycosylation of proteins and lipids (Donaldson & Lippincott-Schwartz 2000). In the mucus-producing cells of Brunner´s gland, specific functions of the GC include protein glycosylation, sulphation of mucins, and formation of components of intestinal mucosubstances such as proteoglycans and glycosaminoglycans (Rios-Martin *et al.* 1993, Yang *et al.* 1996). Goblet cells are polarized epithelial exocrine cells and functionally responsible for the secretion of mucins, which are high molecular weight (> 200 kDa) glycoproteins. After secretion, mucins mix with e.g. water, serum, electrolytes, and micro-organisms to form mucus (Radwan *et al.* 1990). In rabbit goblet cells, GC is composed of numerous cisternae which are concentrated in the supranuclear region. Mucin granules are closely packed in the apical portion of the cell (Radwan *et al.* 1990).

3 Aims of the present study

Previously, we reported the discovery and characterization of EAST, an EGFR-associated protein characterized by a novel VHS domain. Later on, the VHS domain was found to be typical of proteins involved in vesicle trafficking. Since it was obvious, based on the existing literature, that there are as yet unknown proteins involved in the presently only partially characterized vesicle transport pathways, we decided to use the newly discovered VHS domain as a "signature" to identify new components of these pathways.

The specific aims of this study were:

1. to search for novel VHS domain containing proteins using computer analysis and EST (expressed sequence tag) databanks (I),

2. to further characterize one of the novel VHS and ear domain containing proteins named Vear by molecular- and cell biological methods (I), and

3. to determine the distribution of Vear in different tissues (II, III, IV).

4 Materials and methods

Materials and methods are described in detail in the original articles I-IV.

4.1 General procedures and computer analysis (I)

Standard solutions, buffers and procedures for purification, cloning, digestion, and precipitation of DNA were as described in Sambrook *et al.* (Sambrook *et al.* 1989). Synthetic oligonucleotides for polymerase chain reaction (PCR) were obtained from Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden). For analysis of the expressed sequence tag (EST) databanks, BLAST searches (Altschul *et al.* 1997) were carried out on a World Wide Web server at NCBI. Sequence alignments and analyses were performed by using the Genetics Computer Group Wisconsin Package Program suite 9.0 (Genetics Computer Group 1995) and Clustal X program (Thompson *et al.* 1997). For prediction of α-helical content, a COILS program on a network server http://www.ch.embnet.org/software/COILS_form.html was used.

4.2 EST clones and DNA sequencing (I, III, IV)

The VHS domain of EAST (nucleotides 13-420; GenBank[TM] accession number AJ224514) (Lohi *et al.* 1998) was used as a query to search EST databases to identify novel proteins carrying a VHS domain. Several matching EST clone sequences were identified and the corresponding cDNAs were obtained from the Human Genome Mapping Project Research center (Hinxton, Cambridge, UK) and handled as recommended by the distributor. One of the clones, AA070902 (accession number AA070902), was chosen for further characterization. Sequencing of both strands of the cDNAs (I, III, IV) was done by using an automated ABI PRISM 377XL DNA Sequencer (Perkin Elmer, Branchburg, NJ, USA).

4.3 Purification of RNA and Northern analysis (I, III)

The presence of Vear mRNA in normal human tissues was studied by using a multiple-tissue Northern blot filter (Clontech, Palo Alto, CA, USA) (I). Total RNA from the cultured cells (I) and from the renal medulla and cortex (III) was purified using the Total SV RNA Isolation System (Promega, Madison, WI, USA). The RNAs, along with the RNA size markers (Promega), were resolved in 1,0 % formaldehyde-agarose gel and transferred thereafter to a Hybond-N nylon filter (Amersham Pharmacia Biotech). For hybridization, Vear cDNA corresponding to the nucleotides 501-950 of the open reading frame of Vear was radiolabeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) and used as a probe. Labeling was done by using an Oligolabeling Kit (Amersham Pharmacia Biotech). Prehybridization, hybridization with the Vear probe, and rehybridization with the control β-actin probe were carried out by using ExpressHyb solution (Clontech) following the manufacturer´s instructions. The blots were analyzed by using PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA, USA).

4.4 Cell culture (I)

COS-7 and MDBK cells were grown in Dulbecco´s modified Eagle´s medium (D-MEM) (Seromed, Biocrom KG, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT, USA), 2 mM glutamine, and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). For MDBK cells, the medium also contained amphotericin B (0.25 µg/ml). Hela and MDCK cells and human embryonic skin (HES) fibroblasts were grown in Eagle´s minimal essential medium with Earle´s salts (E-MEM) (Seromed) supplemented with 10% heat-inactivated FCS (Hyclone), 2 mM glutamine, and antibiotics (above).

4.5 Preparation of antibodies (I)

Polyclonal antibodies to Vear (anti-Vear) were generated in rabbits by immunization. The full-length Vear cDNA was amplified by PCR by using *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and cloned into a pGEX-4T1 expression vector (Amersham Pharmacia Biotech). The production of the GST-Vear fusion protein was induced in bacterial cells at room temperature (RT) by isopropyl-1-thio-β-D-galactopyranoside (IPTG; 0.25 mM) for 3-5 hours. The cells were pelleted and resuspended into ice-cold (– 0 °C) PBS (phosphate-buffered saline) containing 1% Triton X-100 and protease inhibitors. After incubation on ice for 10 min, the cell suspension was sonicated and centrifuged at 14000 x g for 10 min. The fusion protein was purified from the supernatant by using glutathione (GT)-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer´s instructions. Approximately 100 µg of the GST-Vear fusion protein was emulsified with an equal volume of Freund´s complete adjuvant and the emulsion was injected intradermally into three rabbits. Four booster injections of the GST-Vear with Freund´s incom-

plete adjuvant were injected into rabbits at three-week intervals. After boosters, antisera were collected, tested for the antibody titer, and affinity-purified on CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech) coupled with the GST-Vear fusion protein or Vear protein.

4.6 Immunoblotting (I, III)

Cultured cells grown to 90% confluence were first washed with sterile ice-cold PBS and then scraped into a lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 25 mM NaF, 10 µm ZnCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 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 then centrifuged at 14 000 x g for 10 min. The supernatants were collected and the proteins were electrophoretically separated on SDS-PAGE. They were then transferred onto nitrocellulose filters (Schleicher & Schuell, Dassell, Germany) using a Semi-Dry blotter (Kem-En-Tech, Copenhagen, Denmark). For immunodetection, anti-Vear antibodies (I, III), anti-HA antibodies (I) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and peroxidase-conjugated anti-rabbit immunoglobulins (Biosys, Compegnie, France) were used. The filters were developed by the ECL (enhanced chemiluminescence) method (Amersham Pharmacia Biotech).

The samples representing normal renal cortex and medulla were obtained from surgical nephrectomies. Protein lysate from the renal medulla was prepared as desribed elsewhere (Meriläinen *et al.* 1997). The immunoblotting procedure for the tissue lysates (III) was the same as described for the cultured cells (above).

4.7 Isolation of the human glomeruli (III)

The glomeruli were isolated from the human kidney tissue according to the method of Striker and Striker (Striker & Striker 1985) with minor modifications. The cortical tissue was first gently minced with a razor blade and then pushed through a steel sieve of 250-µm pore size by using a spatula. The pass-through was then filtered through a 150-µm pore size sieve and, finally, the glomeruli were collected by rinsing with PBS from the surface of a third sieve of 100-µm pore size. The preparation was examined under a light microscope for purity; regularly nearly 100% pure glomeruli were obtained. The homogenate passing the 100-µm pores was found to represent mainly cortical non-glomerular structures. It was used for immunoblotting and considered to represent a cortical fraction devoid of glomeruli.

4.8 Fluorescence microscopy (I, II, III)

Cultured cells grown on glass coverslips were first washed with Hank´s salt solution and then fixed in methanol or 4% paraformaldehyde as described in detail in article I. After washing in PBS, non-specific binding of the antibodies was blocked by incubating the coverslips in 10% FCS in PBS-glycine (PBS with 20 mM glycine) for 30 min, whereafter primary antibodies diluted in the blocking buffer (above) were applied for another 30 min. Following a wash in PBS, antibodies were visualized by incubating the cells with the appropriate Texas Red-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Oregon Green-conjugated (Molecular Probes, Eugene, OR, USA), or FITC-conjugated (Caltag Laboratories, San Francisco, CA, USA) secondary antibodies diluted in the blocking buffer for another 30 min. The cells were examined under an Olympus BH2 fluorescence microscope (Olympus, Tokyo, Japan) equipped with the appropriate filters. In transfection experiments (I), the polyclonal anti-hemagglutin (HA) antibody (sc-805) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was used. For double-labeling experiments, the mouse monoclonal antibody to γ-adaptin (A36120) (Transduction Laboratories, Lexington, KY, USA) was combined with the anti-Vear or the anti-HA and the corresponding secondary antibodies were added concurrently (I). For some experiments, the fungal metabolite brefeldin A (BFA) (Alexis Corp., San Diego, CA, USA) was added to a culture medium of MDCK cells at a final concentration of 5 µg/ml; the cells were fixed after various incubation times and double-staining for Vear and γ-adaptin was performed as described above (I).

For the immunofluorescence analysis of tissues (II, III), cryosections were made, air-dried (10 min at RT), and then fixed in acetone for 10 min at –20 °C. After incubation in the blocking buffer (20% FCS in PBS), the sections were washed in PBS. The slides were overlaid with the primary antibodies, washed, and incubated with the secondary antibodies as described above for the cultured cells. For double-staining labelings, the sections were overlaid with a solution containing either a mouse monoclonal antibody to vimentin (III) (a gift from Prof. Ismo Virtanen, Department of Anatomy, University of Helsinki, Helsinki, Finland), or a mouse monoclonal antibody to podocalyxin (III) (a gift from Dr. Dontscho Kerjaschki, University of Vienna, Vienna, Austria), combined with anti-Vear. After washing, the sections were overlaid with the corresponding fluorophore-carrying secondary antibodies, and viewed under an Olympus BH2 microscope. Control stainings included the substitution of primary antibodies with PBS or, alternatively, with anti-Vear preabsorbed with the bacterially produced Vear-protein or GST-protein.

4.9 Immunolabeling electron microscopy (III)

For immunoelectron microscopy (IEM) of the kidney, specimens were taken from kidneys removed at nephrectomies carried out at the Department of Surgery, Oulu University Hospital (Oulu, Finland). Small specimens (~ 1 mm^3) were cut and incubated in the fixation solution (4% paraformaldehyde in 0.1 M phosphate buffer with 2.3 M sucrose) for 24 hours, immersed in 2.3 M sucrose, and then frozen in liquid nitrogen. Ultrathin cryosections were cut by using Leica Ultracut UCT microtome (Leica, Vienna, Austria),

blocked in 5% bovine serum albumin (BSA) (Aurion, Wageningen, the Netherlands) together with 0.1 % gelatin in PBS (10 min). After this, the sections were overlaid with anti-Vear diluted in 0.1 % BSA, washed, and incubated with protein A-gold complex particles (size 10 nm) (Slot & Geuze 1985) for another 30 min. The sections were washed, embedded in methylcellulose, and viewed under a Philips 410 LS transmission electron microscope. Control labelings were done by substituting PBS for the primary antibody.

4.10 Transfection experiments (I)

For transfection experiments, both full-length and several truncated constructs of Vear cDNAs were used. For that purpose, they were cloned into the *Bam*HI/*Not*I-cloning site of the pRK5 eukaryotic expression vector (a gift from Dr. Joseph Schlessinger, New York University Medical Center, New York, USA). The cDNAs corresponding to the full-length Vear (aa 1-612), the VHS domain of Vear (aa 29-165), the C-terminal end of Vear (aa 170-612), and the ear domain of Vear (aa 483- 605) were amplified by PCR by using *Pfu* polymerase (Stratagene, La Jolla, CA, USA). The HA-epitope tag was added to the C-terminus of the PCR products by primer design. To verify the authenticity, the constructs were sequenced for both strands. Transient transfections were made by using a FUGENE 6-transfection reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer´s instructions. 20 hours after the transfection, the cells were fixed in methanol (- 20 °C for 10 min), and then double-stained with anti-HA and anti-γ-adaptin as described in article I.

4.11 Immunohistochemistry and *in situ* hybridization (II, III, IV)

For immunohistochemistry (IHC), the frozen sections (5 µm) of various freshly obtained human tissues were air-dried and fixed in – 20 °C acetone for 10 min. The slides were incubated for 5 min at 3% hydrogen peroxide in methanol to quench endogenous peroxidase. After washing in distilled water and PBS, the samples were incubated in the blocking buffer (20% FCS in PBS), and then overlaid with the anti-Vear diluted in the blocking buffer (above) for 2 hours at RT or, alternatively, overnight at + 4 °C. Following a rinsing in PBS, the slides were incubated with the biotinylated swine anti-rabbit IgG (DAKO, Klostrupp, Denmark) for 60 min and thereafter with Streptavidin ABComplex/ HRP (DAKO) for another 30 min according to the manufacturer´s instructions. Incubation in 3,3´-diaminobenzidine (Sigma, Steinheim, Germany) was according to the manufacturer´s instructions. The slides were counterstained and mounted with Enlitt (YA-Kemia, Helsinki, Finland). As controls, primary antibodies were preabsorbed with the bacterially produced Vear protein, or PBS was substituted for anti-Vear.

For *in situ* hybridization (ISH), both frozen sections (III) and paraffin sections (IV) were used. The protocol was that described earlier in detail by Autio-Harmainen *et al.* with minor modifications (Autio-Harmainen *et al.* 1991, Autio-Harmainen *et al.* 1993). For the generation of Vear cRNA probe, the N-terminal part of Vear (amino acids 1-192) was cloned into the *Bam* HI/*Hind* III–site of the pGEM-4Z vector (Promega). The

construct was sequenced and linearized, and the sense and anti-sense probes were genera-ted by using a riboprobe transcription kit (Promega) and $\alpha[^{35}S]$-UTP (Amersham Phar-macia Biotech). The anti-sense probe was used as a probe for specific signaling while, as a control for background hybridization, a cRNA-probe transcribed to sense orientation was used. The *in situ* hybridization was performed as described in the articles III and IV.

4.12 Cell fractionation (I)

COS-7 cells grown to 70% confluence were transfected with the full-length Vear cDNA as described above. 20 hours after the transfection, the cells were scraped into ice-cold PBS. They were pelleted by centrifugation (1000 x g for 5 min at + 4 °C) and resuspend-ed into the ice-cold homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitors), sonicated, and centrifuged at 1000 x g for 15 min at + 4 °C. The supernatant was further centrifuged for 60 min at 100 000 x g to separate membrane and cytosolic fractions. The protein content of the fractions was determined by using Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA). Thereafter, equal amounts of protein samples of cytosolic and membrane fractions were mixed with Laem-mli´s sample buffer and subjected to SDS-PAGE and immunoblotting for anti-Vear as described above.

5 Results

5.1 Search for novel vesicle trafficking-associated proteins (I)

EST databases, computer analysis, and sequence alignments were utilized to identify novel cDNA clones which could qualify as encoding for novel proteins involved in vesicular trafficking based on their structural features. We were especially interested in proteins possessing a VHS domain, a domain named due to its occurrence in proteins V̲PS27, H̲rs and S̲TAM (Schultz *et al.* 1998). Searching of EST banks with the query sequence corresponding to the VHS domain of the epidermal growth factor receptor (EGFR)-associated protein (EAST) (Lohi *et al.* 1998) and by using a BLAST algorithm yielded several VHS domain-containing cDNAs. Six overlapping EST clones were found which together encompassed an open reading frame of a novel VHS domain-containing protein. This sequence was selected for further characterization. Additionally, we found also other EST clones which represent novel and still uncharacterized cDNAs encompassing novel VHS domains (unpublished results).

5.1.1 Primary and secondary structure of Vear (I)

The sequence analysis and alignment of the six overlapping and matching EST clones AA671009, AA279107, AA235896, AA307157, AA070902, and AI017433 suggested the presence of an open reading frame encoding a protein of 613 amino acids with a calculated molecular mass of 67,149 daltons (Da). The plasmids carrying these cDNAs were purchased from the Human Genome Mapping Project Research Center (Hinxton, Cambridge, UK). Restriction enzyme digestion analysis of the clone AA070902 revealed the presence of an approximately 2-kb insert with an open reading frame putatively encompassing the entire sequence for a 67 kDa protein. Therefore, this clone was selected for further characterization. Sequencing of both strands of AA070902 yielded a nucleotide sequence that was identical to that derived from the aligned sequences of the six EST clones described above. Together, the insert length was 1997 bp consisting of a 5´-untranslated region of 21 bp, an open reading frame of 1842 bp, and a 3´-untranslated

region of 134 bp. The sequence surrounding the methionine initiation codon (nucleotides 22-24 of the clone) partially conforms to Kozak´s consensus sequence (Kozak 1987). The open reading frame ends with the TAA termination codon at nucleotides 1861-1863 followed by a poly-A tail.

5.1.2 Domain structure of Vear (I)

A schematic representation of the domain structure of Vear is seen in Figure 7. The deduced amino acid sequence of Vear was compared to the protein sequences in the databases. The results showed the presence of a VHS domain, an evolutionary well-conserved domain of ~ 140 amino acids, in the N-terminal part of Vear (aa 29-165). The VHS domain of Vear showed a high degree of similarity with the VHS domains of e.g. EAST, Hrs, Hrs-2, STAM, and Tom1 (article I, Figure 2A) (for references, see article I). In the C-terminal part of Vear, sequence comparisons showed a close similarity with the "ear" domains of γ- and γ$_2$ _adaptins (Robinson 1990, Takatsu *et al.* 1998), suggesting the presence of an "ear" domain in Vear at the position of amino acids 483-605 (I, Figure 2B). With a COILS program, a region between amino acids 200-250 was predicted to possess a high α-helical content and a propensity to coiled-coil organization. Based on these structural similarities with other proteins, we named the protein Vear (VHS domain and ear domain of γ-adaptin). The middle region of Vear encompassing the amino acids 170-480 showed no significant similarity to known proteins.

The tripartite and unique domain structure of Vear encompassing both a VHS domain, an α-helical area, and an "ear" domain suggests that Vear participates in vesicle trafficking between different intracellular membranous compartments. This can be surmised on the basis of the predominant occurrence of the VHS domain in the proteins participitating in endocytosis/vesicular trafficking (Lohi & Lehto 1998) and, on the other hand, the occurrence of the "ear" domain in adaptins present in adaptor protein complexes (Kirchhausen 1999). Recently, proteins either similar or identical to Vear have been described. These proteins are termed GGA (Golgi-localized, γ-adaptin ear-containing, ARF-binding) proteins. It is suggested that GGAs mediate ARF-regulated vesicle transport from the *trans*-Golgi network (Boman *et al.* 2000, Dell´Angelica *et al.* 2000, Hirst *et al.* 2000) onwards.

The domain structure of Vear is of special interest because no proteins carrying a similar domain composition have been characterized earlier. On the other hand, ear domains outside of adaptins, components of vesicle coat complexes, have not been described before.
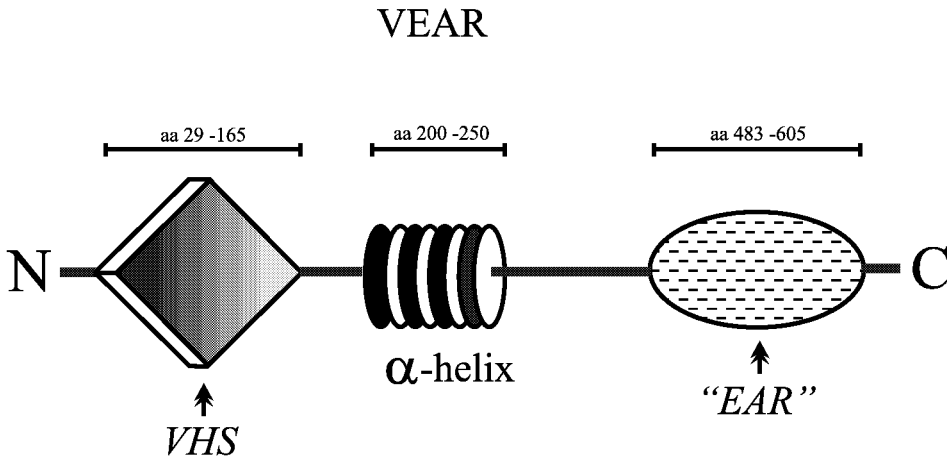
VEAR

Fig. 7. A schematic representation of the domain organization of Vear.

5.1.3 Northern blot analysis of Vear (I, III)

The presence of Vear RNA in different tissues and cultured cells was studied by Northern blot analysis. In human tissues a ubiquitous expression of Vear mRNA was seen with two major messages of 3.5 kb and 3.9 kb (I). A weaker but clear signal of 5.2 kb was also detected. The multitude of messages suggests the presence of related gene products, or the presence of differently spliced isoforms of Vear. A high amount of Vear mRNA was seen in the cardiac muscle, skeletal muscle, and kidney. A low amount of message was detected in brain, colon, and small intestine (I). Northern hybridization for Vear also revealed the presence of major messages in COS-7, MDBK, MDCK, and HeLa cells (I). Northern analysis for Vear in the kidney revealed that Vear RNA was present predominantly in the cortex but not in the medulla (III).

5.1.4 Subcellular distribution of Vear (I)

The cellular and ultrastructural localization of Vear was determined by using immunofluorescence microscopy, transfection studies, and subcellular fractionation. By immunofluorescence microscopy, Vear was seen in most cells in a perinuclear structure closely resembling the Golgi complex. The signal was slightly tubular or tubulovesicular and typical for the Golgi complex both in appearance and localization. The Golgi-associated localization was further confirmed by double-immunostaining experiments with a monoclonal antibody to γ-adaptin, a well-established marker for the *trans*-Golgi network (Robinson 1990). A close co-localization for Vear and γ-adaptin could be seen in different cell

types (I, Figure 5). Apart from the strong perinuclear reaction, some faint binding of anti-Vear in the cytosolic compartment could be seen in vesicular structures (unpublished observation).

The subcellular distribution of Vear and its role in cellular physiology was also probed by overexpressing Vear and its truncated parts in cultured cells. Overexpressed full-length Vear and Vear lacking the VHS domain localized to the Golgi area as visualized by anti-HA antibodies. Associated with the overexpression, there were changes in the Golgi morphology; instead of regularly spaced stacks, the Golgi in the cells overexpressing full-length Vear displayed a distinct "compaction". Compaction could also be visualized by staining with anti γ-adaptin, indicating that the redistribution of Vear is due to the reorganization of the underlying Golgi-architecture. No such change in the Golgi organization could be seen when the VHS domain or the ear domain alone were overexpressed.

The preferential Golgi-membrane association of the endogenous Vear was also supported by the experiments done with a fungal metabolite brefeldin A. Treatment with BFA brings about rapid dispersal of the Golgi membranes/TGN and their fusion with the ER (Donaldson *et al.* 1990). Fast dissolution of both Vear and γ-adaptin after BFA treatment from their perinuclear structures to more dispersed cytosolic vesicles (article I, Figure 6) strongly suggests that Vear is localized to Golgi- or TGN-membranes. Also, a rapid response of Vear to BFA suggests that the recruitment/assembly of Vear to Golgi membranes is regulated by an ARF-dependent manner. This can be concluded based on the known mechanism of BFA to inhibit the exchange of GDP for GTP on ARF, thus preventing the assembly of ARF-regulated proteins to Golgi membranes (Donaldson *et al.* 1992, Helms & Rothman 1992, Peyroche *et al.* 1996). The association of Vear with membranes was also demonstrated by cell fractionation in which the overexpressed Vear protein was almost exclusively present in the fraction containing membranous organelles of cultured cells.

5.2 Cell type-specific distribution of Vear in tissues (I-IV)

Western blot analysis was used to determinate the size of the Vear polypeptide present in cultured cells (I) and in the kidney (III). A single band of ~ 75 kDa was detected in total cell lysate. This corresponds well to the calculated molecular mass of Vear and to the major RNA signal of 3.5 kb detected by Northern blot (I). Also, a distinct band corresponding to the size of 75 kDa could be detected in the total protein lysates made from the isolated glomeruli of the kidney (III) and from the skeletal muscle (unpublished data).

Both Northern and Western blotting analysis showed that there was widespread distribution for Vear and that the Vear-immunoreactivity and the presence of Vear RNA varied considerably in a tissue- and cell-type specific manner.

Localization of Vear in different tissues was studied by immunohistochemistry (II, IV), immunofluorescence microscopy (II, III), immunolabeling electron microscopy (III), and *in situ* hybridization (III, IV). These studies were motivated by the observations by Northern hybridization indicating that Vear mRNA is abundant in the kidney, cardiac muscle, and skeletal muscle. Additionally, the gastrointestinal tract was selected for further stud-

ies because it contains several cell types which display a high secretory activity and, correspondingly, possess a well-developed vesicle trafficking machinery and Golgi complex (McNiven & Marlowe 1999).

5.2.1 Vear in the skeletal muscle (II)

The distribution of Vear in the skeletal muscle was studied by using IHC and IF. The fluorescence/color reaction pattern for Vear in the muscle was compared to that seen for ATPase and NADH (nicotinamide adenine dinucleotide hydrogenase), commonly used to differentiate the different types of muscle fibers. By both IHC and IF, antibodies for Vear were seen to decorate in a chessboard pattern with intensively stained and negatively or faintly stained fibers intermixed with each other. The serial cross-sections studied for Vear, ATPase, and NADH revealed the presence of Vear immunoreactivity preferentially in type I fibers (NADH-positive). Reaction for Vear was slightly positivite also in some type II fibers (ATPase ph 9.4 positive), but the signal was clearly more intense in type I fibers. From these results we conclude that in the skeletal muscle Vear is localized in a fiber type-dependent manner, being preferentially present in type I cells. It seems that Vear plays a cell-type specific role in skeletal muscle and is involved in the fiber-type specific metabolic and physiological functions of type I muscle cells.

5.2.2 Vear in the kidney (III)

In kidney, immunofluorescence stainings revealed a distinct Vear-positivity in glomerular structures. Apart from the faint signal seen in the smooth muscle cells of renal arteries, no binding of antibodies outside the glomeruli was present as judged by immunofluorescence microscopy. The positive immunoreaction for Vear in the glomeruli resembled the typical distribution pattern of the glomerular visceral epithelial cells, podocytes. The podocyte-associated localization was established by double-staining experiments which showed that Vear colocalized with the podocyte-marker podocalyxin and with the intermediate filament-protein vimentin, both preferentially expressed in podocytes (Kerjaschki *et al.* 1986, Stamenkovic *et al.* 1986, Virtanen *et al.* 1988). Moreover, double stainings for Vear and the mesangial cell marker α-SMA (Skalli *et al.* 1986) verified the lack of Vear-reactivity in glomerular mesangial cells (unpublished results).

   The distribution of Vear protein was also studied in the developing fetal kidney. In the histological sections, various developmental stages of the glomeruli could be discerned by conventional light microscopy after staining with hematoxylin and eosin. In IF, a preferential podocyte-associated signal for Vear was seen. The positive signal detected correlated with the maturation of the podocytes. Thus, Vear was detected weakly in the luminal cells in the vesicle phase which represents the earliest stage of glomerulogenesis. A more prominent staining was seen in the developing podocytes at the S-shaped body-stage and capillary loop stage, which represent more advanced stages of glomerulogenesis.

The subcellular localization of Vear in adult kidney was also examined by IEM. Vear was seen in podocytes which can be recognized in electron microscopy by their characteristic ultrastructural features. In podocytes, Vear was present in the Golgi vesicles and tubulovesicular structures connected to the Golgi complex. Some labeling was also seen in the foot processes (pedicles) of the podocytes.

Also by ISH, Vear mRNA could be localized to glomerular structures. The distribution of the labeling in the glomeruli was compatible with the distribution of podocytes. No specific labeling above the background level could be seen in other structures or cell types of the kidney.

The presence of Vear polypeptide in the isolated glomeruli vs. renal medulla and cortical fraction devoid of glomeruli was shown by immunoblotting. Vear was seen in total protein lysates prepared from the isolated glomeruli but not in the lysate prepared from the renal medulla or from the cortical fraction devoid of glomeruli.

5.2.3 Vear in the gastrointestinal epithelium (IV)

Biopsy specimens from stomach, small intestine, and large intestine were used to study the expression of Vear in the gastrointestinal tract by IHC and ISH. An abundant expression of Vear was seen throughout the epithelial cell compartment, while a low level of expression for Vear was seen in smooth muscle, peripheral nerves, and blood vessels.

In stomach, ISH revealed a high amount of mRNA in the glandular epithelium of the antrum, and a moderate expression level in the surface/foveolar epithelium. IHC showed strong immunoreactivity in the glandular and foveolar epithelium of the antrum and in the foveolar epithelium in the corpus. Also the parietal cells showed a strong signal. A somewhat weaker staining was seen in the chief cells.

In duodenum, by ISH, an intense accumulation of grains was seen in Brunner´s glands and in the crypt and villous epithelium. Also by IHC, Brunner´s glands were strongly positive as well as the Paneth cells and the goblet cells.

In colon, ISH showed the densest labeling in the surface epithelium and in the upper half of the crypt compartment. A moderate or a weak labeling was seen in the basal half of the crypts, endothelium, M cells, and fibroblasts. With IHC, practically the same staining pattern as by ISH was seen in colon. The strongest reactivity localized to the surface epithelium, the upper half of the crypts, and the goblet cells.

Some immunohistochemical stainings were also made on pancreas in which Vear also localized in a cell-type dependent manner so that a reaction for Vear was strongly positive in Langerhan´s cells (unpublished results).

6 Discussion

6.1 Characterization of novel vesicle traffic-associated proteins

In this study, we utilized a novel and non-classical method to search for novel vesicle transport-associated proteins. It was based on sequence queries and analysis performed on EST databanks. Such an approach has come to be known as "dry cloning" or "cloning *in silico*", based on the procedure carried out by utilizing computers without making traditional "wet" cloning experiments. The fast growing number of freely available uncharacterized DNA information in the databanks and the working out of the sequence of the human genome has created a whole new aspect and pathway to find/characterize novel genes and, through that, to analyze their corresponding functions.

It has been suggested, based on functional and morphological studies, that there are at least 12 different sorting events in vesicular transport systems which utilize specific coat proteins (Lewin & Mellman 1998). At the moment, however, not so many different coat complexes are characterized calling for novel, currently uncharacterized coat components. Recently, many new proteins to serve these functions have been reported while the detailed relationship of these coats to various transport events is in many cases poorly known. Also, there are some vesicular transport pathways, e.g. constitutive or regulated secretory pathways, in which the components of the vesicle coat are mostly unknown.

The recent characterization of the VHS domain (Schultz *et al.* 1998) and the primary report suggesting its possible functions (Lohi & Lehto 1998) were the initiation steps for us to search for novel VHS domain-containing proteins. There are several characterized proteins possessing VHS domain and many of them seem to be involved in endocytosis and vesicular trafficking (Lohi & Lehto 1998). However, it is unknown whether the VHS domain itself has a specific function in endocytosis or in other vesicular trafficking events. Also, interacting partners for VHS are not currently known and its function is still a mystery.

The search of EST databanks by using the VHS domain sequence of EAST (Lohi *et al.* 1998) as a query yielded several cDNAs which, when superimposed, revealed an open reading frame of a novel VHS domain-containing protein. Sequencing of the composite cDNAs and one long, continuous cDNA showed the expected VHS domain in the N-terminal part of a putative open reading frame and, surprisingly, the presence of a typical

domain structure called "ear" domain in the C-terminal region. The ear domains are typical of adaptins which are components of the protein complexes involved in clathrin-mediated (AP-1 and AP-2) and non-clathrin mediated (AP-3 and AP-4) vesicle transport (Hirst & Robinson 1998, Scales *et al.* 2000). Based on its specific structural features carrying both these domains (VHS- and ear), we named the protein Vear.

The deduced domain structure of Vear carries some specific and unique features. Firstly, VHS and ear domains have not been described earlier in the same protein. Secondly, ear domains, normally found in the AP complexes, have not been found outside of adaptins before.

6.2 Possible functions of Vear

In this study, we identified and partially characterized Vear. Based on its structural features and localization studies we suggest that Vear is involved in vesicle trafficking originating from the Golgi apparatus. The preferential Golgi-localization for Vear was demonstrated at cellular level for both endogenous Vear and exogenously expressed Vear. The predominant membrane-association was further shown by cell fractionation and BFA studies. Vear mRNA shows widespread distribution. On the other hand, distinct differences in the expression levels could be seen between different cell types and tissues.

The exact function of Vear is not clear at the moment, but some aspects based on its structure and subcellular localization studies may be pointed out. First, the tripartite domain structure of Vear suggests different targeting functions for its domains. This was supported by the demonstration that, when overexpressed separately, the VHS and ear domains clearly associated with different subcellular sites. The VHS domain showed a diffuse, mainly vesicle- and membrane-associated distribution without any significant connection to Golgi, while overexpressed ear domain showed a Golgi-associated staining. Our results regarding the function of the VHS domain are in good agreement with the studies published so far speculating the functions of this novel domain in vesicular trafficking (Komada *et al.* 1997, Lohi *et al.* 1998, Misra *et al.* 2000). Some earlier studies have suggested a targeting function for the ear domain of adaptins (Matsui & Kirchhausen 1990, Page & Robinson 1995). They have not been fully supported, however, by some other studies (Peeler *et al.* 1993, Traub *et al.* 1995). However, our results showing that ear domain-containing constructs associate with the Golgi complex raise the question that a specific ear-binding ligand(s) may occur in the Golgi membranes.

Another interesting aspect regarding the possible functions of Vear is based on the observations made from the changes in the Golgi complex morphology subsequent to overexpression of the full-length Vear and the C-terminal part of Vear. As visualized by TGN-marker γ-adaptin, a "compaction" of the Golgi complex was seen in the cells transfected with the full-length Vear or the C-terminal Vear lacking the VHS domain. "Compaction" implies that Vear could mediate vesicle traffic in the Golgi or in the immediate vicinity of the Golgi, and the mechanism of compaction could be due to either a block in the physiological dispersal of the Golgi structures or in the coalescence of the scattered vesicles on the Golgi membranes. Results similar to ours were recently reported also by other groups (Boman *et al.* 2000, Hirst *et al.* 2000, Takatsu *et al.* 2000).

The function of Vear can also be surmised by thinking about the changes seen in the subcellular distribution of Vear in the cultured cells after treatment with the fungal metabolite BFA. After BFA treatment, Vear becomes rapidly (~ 2 min) dissociated from the perinuclear Golgi membranes into discrete cytosolic vesicles. This response strongly suggests that endogenous Vear in its physiological state is mainly peripherally associated within the Golgi membranes and that the recruitment of Vear onto membranes is regulated by an ARF-dependent manner. An ARF-regulated assembly of Vear together with the localization studies showing the presence of Vear in the vesicles and tubulovesicular structures strongly support the idea that Vear plays a role in vesicle traffic either as a structural component or as a regulatory factor. Most of the BFA-sensitive membrane proteins are either ARFs, vesicle coat proteins (e.g. AP-1 and coatamer) or proteins associated with the coats (e.g. γ-synergin). Thus, this finding suggests that Vear could be a novel type of coat protein or a coat component, mediating its role at the Golgi complex. The possible role as a novel coat is also supported by the structural features of Vear, especially the presence of an ear domain in its C-terminus. Ear domains are seen in large subunits (adaptins) of adaptor protein complexes. While results so far suggest a role for Vear as a novel vesicle coat protein, important questions may be raised: what is the pathway for Vear-coated vesicles? What are the other partners (in addition to ARF) of Vear-coated vesicles? What is the cargo of the Vear-coated vesicles?

Is Vear a part of the major and best characterized intracellular vesicle transport system, clathrin-mediated traffic? While the answer is currently unknown, some findings deserve mention. In co-immunoprecipitation studies for Vear and clathrin we were not able to demonstrate any association between Vear and clathrin in cell lysates (unpublished data). However, this does not necessarily prove that there could not be any association between clathrin and Vear *in vivo*. Our ultrastructural studies by IEM also suggest that Vear is present in non-clathrin coated vesicle (unpublished results).

We also determined the distribution of Vear at tissue level using several organs and IHC, IF, IEM, and ISH (II, II, IV). As a major finding, the localization of Vear was strongly cell-type specific. A shared property of the cells showing positivity for Vear immunoreactivity abundantly was their high endocytic/secretory activity and a well-developed vesicular machinery. However, it is obvious that metabolic rather than structural features underlie the distinct differences seen in the distribution of Vear between different cell types of the tissues. This is based on the observation that, for example, in the skeletal muscle the total number of Golgi elements per unit length is approximately the same between the metabolically and functionally distinct fiber types which display highly different levels of Vear immunoreactivity (Ralston *et al.* 1999). Similarly, in the kidney, Vear expression was seen only in the podocytes, although a well-developed Golgi machinery can also be found in many other cell types (Mundel & Kriz 1995). In the gastrointestinal tract, the strongest Vear-expression was seen in cells mainly responsible for secretory activity. However, the physical size of vesicular machinery, e.g. the size of the Golgi complex and *trans*-Golgi network (Clermont *et al.* 1995) had no correlative relationship to the Vear expression. So, the distinctive expression of Vear in certain cell types is thought to be due to Vear-specific functional features of those cells. In future, more studies are needed to clarify the detailed mechanism by which Vear is involved in cell-type specific functions.

6.3 Proteins similar or identical to Vear (GGAs)

Recently, several proteins with similar or identical sequence to Vear have been reported and suggested to represent a novel family of proteins involved in vesicle transport. These proteins are generally termed GGAs for their properties to be Golgi-localized, gamma-adaptin ear containing, and ARF-binding proteins. The family includes three mammalian members GGA1, GGA2, and GGA3 and two yeast homologues Gga1p and Gga2p (Boman *et al.* 2000, Dell´Angelica *et al.* 2000, Hirst *et al.* 2000). Protein GGA2 is identical to Vear.

The results presented about GGAs are in good agreement with ours regarding e.g. the tissue distribution of Vear RNA, but also the subcellular localization of Vear as studied by transfections, polyclonal antibodies, and a immunofluorescence microscopy. Also, strong support for our results suggesting the possible role for Vear in the vesicle traffic at the Golgi membranes was received from results presenting the preferential localization of Vear (GGA2) at the TGN membranes/vesicles (Hirst *et al.* 2000). A study by Hirst *et al.* also demonstrated that Vear did not co-immunoprecipate with the AP-1 complex and it also confirmed our observations of the absence of Vear in clathrin-enriched membrane compartment (Hirst *et al.* 2000). On the other hand, Black and Pelham reported results in which they suggest, based on yeast mutation studies, that Vear is probably involved in clathrin-dependent transport (Black & Pelham 2000). Yeast cells deficient in both Gga1p and Gga2p carry normal endocytosis (Hirst *et al.* 2000). Both Vear and ARF can be seen preferentially concentrated in the same subcellular compartment at *trans*-Golgi network (Boman *et al.* 2000). It was also shown that the recruitment of Vear to membranes is regulated by direct GTP-dependent interaction with ARF (Boman *et al.* 2000, Dell´Angelica *et al.* 2000, Hirst *et al.* 2000).

7 Summary and conclusions

In this study, a novel protein termed Vear was found by utilizing EST databanks and computer analysis. Sequence analysis of selected cDNAs showed the presence of an open reading frame encoding a protein with 613 amino acids and with a calculated molecular mass of 67,149 daltons. The structure consists of the N-terminal VHS domain (amino acids 29-165), the middle part (aa 170- 480), and the C-terminal ear domain (aa 483-605). At tissue level, Vear showed high amounts of mRNA in the kidney, skeletal muscle, and cardiac muscle. Polyclonal antibodies to Vear (anti-Vear) were produced and affinity-purified. Western blot analysis with anti-Vear revealed the presence of a single polypeptide of 75 kDa in the cell solubilizates.

Vear was localized to the perinuclear structure resembling that of the Golgi complex in various cell types. The Golgi-association was verified by making double-labeling experiments with *trans*-Golgi marker γ-adaptin. The association with Golgi membranes showed brefeldin A sensitivity, suggesting an ARF-dependent recruitment onto Golgi membranes. In transfection experiments, overexpressed full-length Vear assembled on and caused a "compaction" of the Golgi, while the VHS domain alone showed diffuse membrane- and vesicle-associated distribution. The ear domain, on the other hand, localized to the diffuse perinuclear Golgi-like structure when overexpressed. By subcellular fractionation, presence of overexpressed Vear was predominantly seen in total membrane fraction of the cells.

Distribution of Vear in several tissues was studied. In skeletal muscle Vear was preferentially detected in type I cells. In the adult kidney Vear was exclusively present in glomerular visceral epithelial cells, podocytes. In the developing glomeruli, the expression of Vear protein corresponded to the developmental stages of podocytes and their cytoplasmic processes and foot processes. In the gastrointestinal tract Vear was found mostly in the epithelial compartment and specifically in the cells known to possess high secretory activity.

As a conclusion, a novel Golgi-associated protein was cloned and initially characterized. We suggest that this protein, named Vear, participates in vesicle traffic at the *trans*-Golgi. Distribution of Vear in tissues is highly cell-type specific.

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