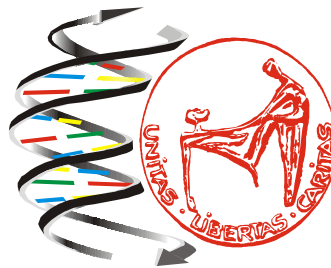


ZIB

ZENTRUM FÜR INFektionsBIOLOGIE

Functional properties of the Coated-Vesicle-Associated-Kinase of 104 kDa (CVAK104) in clathrin-mediated vesicular trafficking

-While kinase may be its name, phosphorylation may not be its game -*



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Abstract

Clathrin-coated vesicles (CCVs) are involved in fundamental intracellular transport pathways such as receptor-mediated endocytosis at the plasma membrane and the sorting of lysosomal enzymes at the *trans*-Golgi network (TGN). The formation of CCVs is a dynamic and complex process that is mediated and controlled by the coat protein clathrin and a battery of accessory proteins.

Recently, a novel CCV-associated protein with a serine/threonine kinase homology domain was identified in a tandem MS analysis of rat brain CCVs and termed CVAK104 (coated-vesicle-associated kinase of 104 kDa) (Blondeau *et al.*, 2004; Conner and Schmid, 2005). In this study CVAK104 was characterized by biochemical and cell biological methods. Although CVAK104 is capable of binding to ATP, sequence analysis of the kinase domain and data from *in vitro* kinase assays indicated that CVAK is catalytically inactive and can therefore be classified as a pseudokinase. Instead, it was demonstrated that a C-terminal segment of CVAK104 interacts directly with the N-terminal domain of clathrin and with the α -appendage domain of the adaptor protein AP2. Fluorescence microscopy examinations revealed that CVAK104 localizes predominantly to the perinuclear region of HeLa and COS-7 cells but it is also present on peripheral vesicular structures that are accessible to internalized transferrin. The distribution of CVAK104 overlaps extensively with that of AP1, AP3, the mannose 6-phosphate receptor and perinuclear clathrin but not at all with that of its *in vitro* binding partner AP2. RNAi-mediated clathrin knockdown reduced the membrane association of CVAK104. The recruitment of CVAK104 to perinuclear membranes of permeabilized cells is enhanced by GTP γ S. Moreover, upon treatment of cells with Brefeldin A, CVAK104 redistributes into the cytosol. Both observations suggest a direct or indirect requirement for GTP-binding proteins in the membrane association of CVAK104. Live-cell imaging showed colocalization of GFP-CVAK104 with endocytosed transferrin and with RFP-clathrin on rapidly moving endosomes. Like AP1-depleted COS-7 cells, CVAK104-depleted cells missort the lysosomal hydrolase cathepsin D. Taken together, the present data suggest a function for CVAK104 in clathrin-dependent pathways between the TGN and the endosomal system.

Key words: clathrin-coated vesicles, endocytosis, lysosomal enzymes, adaptor proteins, AP1, AP2, CVAK104, RNA interference, phosphorylation

*) The subtitle of the thesis (“While kinase may be its name, phosphorylation may not be its game”) is taken and modified from (Morrison, 2001).

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Abbreviations

AAK1	Adaptor-associated kinase 1
ANTH	AP180-N-terminal homology
AP	Adaptor protein (e.g. AP1, AP2)
AP180	Assembly protein with apparent molecular weight of 180 kDa
Arf1	ADP-ribosylation factor 1
ATP	Adenosine triphosphate
BFA	Brefeldin A
BLAST	Basic logical alignment search tool
BSA	Bovine serum albumin
CALM	Clathrin assembly lymphoid myeloid leukemia protein
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
CCV	Clathrin-coated vesicle
cDNA	Complementary DNA
CHC	Clathrin heavy chain
CIP	Calf intestinal alkaline phosphatase
CLASP	Clathrin-associated sorting protein
CLC	Clathrin light chain
CME	Clathrin-mediated endocytosis
COP	Coatamer protein
CVAK104	Coated vesicle associated kinase of 104 kDa
DMEM	Dulbeccos modified Eagle's medium
DNA	Deoxyribonucleic acid
dT	Desoxythymidin
DTT	Dithiothreitol
ECL	Enhanced Chemoluminescence
EDTA	Ethylene diamine tetraacetic acid
EE	Early endosome
EEA1	Early endosomal antigen 1
EGF	Epidermal growth factor
EH	Eps15 homology
ENTH-	Epsin N-terminal homology domain

domain	
Eps15	EGF-receptor protein substrate 15
Epsin	Eps15-interacting protein
ER	Endoplasmatic reticulum
FCS	Fetal calf serum
GAK	Cyclin G-associated kinase
GBP	Golgi binding buffer
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGA	Golgi-localizing, γ -adaptin ear homology domain, Arf -binding
GPCRs	G protein-coupled receptors
GSH	Glutathione
GST	Glutathione-S-transferase
GT	Galactosyltransferase
GTP	Guanosine 5-triphosphate
GTP- γ -S	Guanosin-5'-[γ -thio]triphosphate
GTPase	Guanosin triphosphatase
H ₆	6 x histidine
HEPES	Hydroxyethyl-piperazino-ethansulphonic acid
Hip1	Huntingtin interacting protein 1
Hip1R	Hip1-related
Hsc70	Cognate heat shock-protein with a MW of 70 kDa
IPTG	Isopropyl- β -D-thiogalactopyranoside
kDa	Kilodalton
KGAK	Kinase domain of GAK
LB-Medium	Luria-Bertani medium
LDL	Low-density lipoprotein
M6PR	Mannose 6-phosphate receptor
MBP	Myelin basic protein
MHH	Hannover Medical School
mRNA	Messenger ribonucleic acid
MW	Molecular weight
nt	Nucleotide
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDB	Protein database
PFA	Paraformaldehyde
PIPK	Phosphatidylinositol 4-phosphate 5-kinase
PMSF	Phenyl-methyl-sulfonyl fluoride
PONDR	Predictor of Naturally Disordered Regions
PtdIns	Phosphatidylinositol
PtdIns(4) <i>P</i>	Phosphatidylinositol 4-monophosphate
PtdIns(4,5)P ₂	Phosphatidylinositol-4,5-bisphosphate
RE	Recycling endosome
RFP	Red fluorescent protein
RME	Receptor-mediated endocytosis
RPM	Rounds per minute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
Snx 9	Sorting nexin 9
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TD	Terminal domain
TE	Tris-EDTA
TE	Tris-extract
Tf	Transferrin
TfR	Transferrin receptor
TGN	<i>trans</i> -Golgi-network
T _m	Melting temperature
Tris	Tris(hydroxymethyl)-aminomethane
U	Units
UIM	Ubiquitin interacting motif
UV	Ultra violet

1. Introduction

1.1 Intracellular transport processes

A defining feature of all eukaryotic cells is compartmentalization. Vesicle-mediated transport takes place between these membrane-bound compartments and also between these compartments and the plasma membrane. The vesicles bud from the donor organelle membrane, are transported across the cytosol, tether and fuse with the membrane of the target organelle. This membrane traffic allows the exchange of membrane constituents and also of soluble as well as receptor-bound cargo molecules. Dependent on the sort of cargo, the location of origin and on the target compartment the vesicle formation is mediated by different coat proteins. The most well studied type of coat protein is the macromolecule clathrin. Clathrin forms a characteristic, cage-like structure around the vesicles, which are easily identifiable by electron microscopy. Clathrin-coated vesicles (CCVs) are involved in several intracellular transport processes like receptor-mediated endocytosis (RME) at the plasma membrane and sorting of proteins at the *trans*-Golgi network (TGN) during the biogenesis of lysosomes and secretory granules. (for an overview see (Brodsky *et al.*, 2001; Mousavi *et al.*, 2004; Edeling *et al.*, 2006b). Since these processes are of special importance for this work, they will be discussed in more detail in the following sections.

CCVs are not unique in their ability to select protein cargo for the transport from one membrane compartment to another. Coatamer protein-II (COPII) -coated vesicles, for instance, are responsible for the anterograde transport of proteins from the Endoplasmatic reticulum (ER) to the Golgi apparatus and COPI vesicles mediate the retrograde transport of resident proteins to the ER (Barlowe, 2000). However, coat proteins that facilitate the formation of vesicles derived from endosomes and destined for secretion are still not well characterized.

1.2 Endocytosis

The mechanism by which cells internalize macromolecules and particles into transport vesicles derived from the plasma membrane is called endocytosis. Endocytic pathways can be subdivided into two major types: phagocytosis and pinocytosis (Besterman and Low, 1983; Conner and Schmid, 2003). **Phagocytosis** (cell eating) describes the uptake of large particles

like bacteria or yeast and occurs in a few specialized mammalian cell types including macrophages and neutrophils (Aderem and Underhill, 1999). The uptake of fluids and solutes occurs in all mammalian cell types and is termed **pinocytosis** (cell drinking). In respect to the molecular mechanisms by which cells internalize fluid and solutes, pinocytosis can be subdivided into additional classes: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis.

Macropinocytosis results from the actin-driven formation of membrane protrusions, which engulf large volumes of extracellular milieu and then fuse with the plasma membrane to generate large endocytic vesicles called macropinosomes. Dendritic cells use this mechanism to screen the extracellular milieu for antigens (Mellman and Steinman, 2001).

Caveolae are flask-shaped invaginations of the plasma membrane that contain cholesterol- and sphingolipid-rich microdomains on the plasma membrane where many transporters and signaling molecules are concentrated. The caveolin coat on the surface of the membrane invaginations is formed by the cholesterol-binding protein caveolin. Caveolae-mediated endocytosis is of great importance for epithelial cells where this mechanism is assumed to mediate the transcellular shuttling of serum proteins from the bloodstream into tissues across the endothelial cell layer. Although caveolin knockout mice have no overt phenotype they exhibit some tissue-specific defects that suggest possible functions of caveolae in regulation of specific signaling cascades. Analysis of lung epithelia of caveolin-null mice, e.g., revealed that caveolae negatively regulate the endothelial nitric oxide synthase (eNOS), which is involved in the control of vasodilatation (Anderson, 1998; Razani *et al.*, 2002).

The **clathrin- and caveolin-independent** molecular mechanisms are poorly understood and are often simply defined only by the endocytic components that are not involved. E.g. the interleukin-2 (IL-2) receptor is associated with lipid microdomains, but its internalization is independent of caveolin and clathrin. Moreover, in neuroendocrine cells, endocytosis occurs even when a non-functional form of dynamin -a GTPase required for budding and release in both CME and caveolae-mediated endocytosis- is used (Damke *et al.*, 1994; Artalejo *et al.*, 2002; Pelkmans and Helenius, 2002).

The best characterized type of endocytosis is **clathrin-mediated endocytosis** (CME). CME occurs in all mammalian cells and carries out the internalization of receptors and extracellular ligands, the recycling of plasma membrane components, and the retrieval of surface proteins destined for degradation. It is responsible for the constitutive uptake of essential ligands such as transferrin and low-density lipoprotein (LDL) as well as for the ligand-triggered receptor

uptake of proteins such as activated epidermal growth factor receptor (EGFR). In nerve terminals clathrin-coated vesicle biogenesis serves as the major pathway for the recycling of synaptic-vesicle components after release of neurotransmitter in response to action potentials. And even some pathogens are known to hijack the clathrin machinery to penetrate their host cell. Examples are the influenza virus, the Ebola virus or the Semliki Forest virus that bind to specific receptors on the cell surface and are then internalized by CME and delivered to endosomes (Marsh and Helenius, 2006). A schematic overview of the above mentioned internalization- and trafficking processes shows figure 1.1.

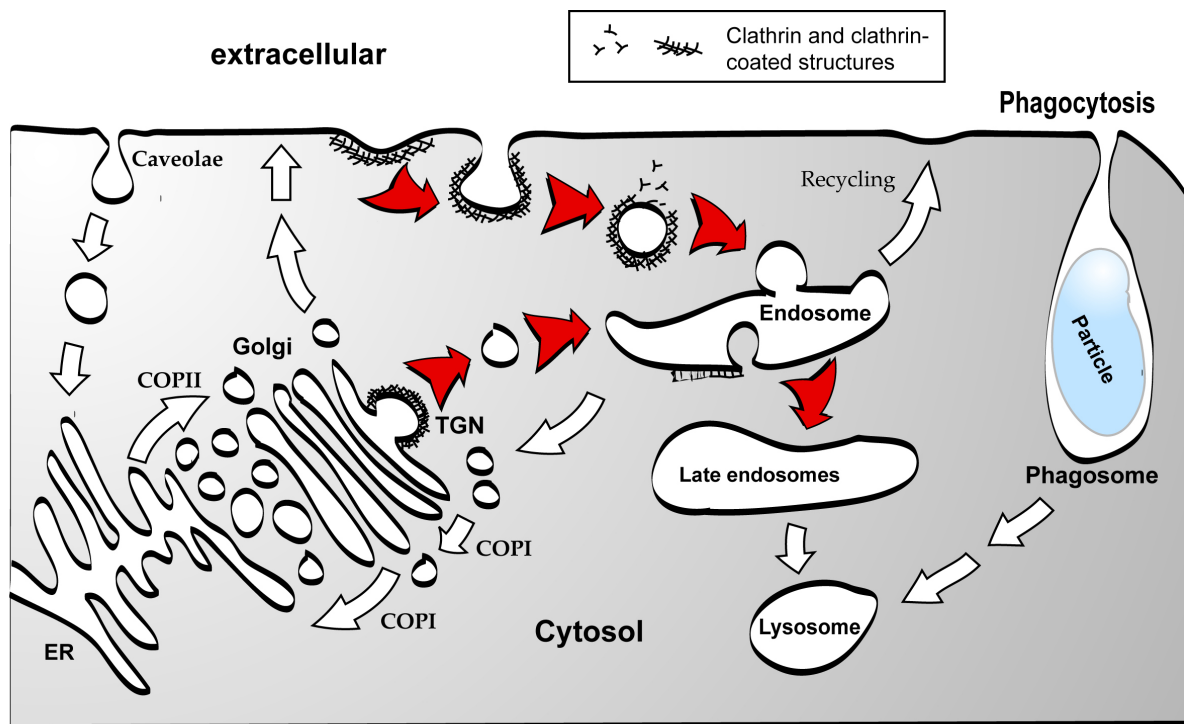


Figure 1.1: Schematic overview of intracellular transport processes. Three uptake mechanisms are illustrated: phagocytosis, caveolae- and clathrin-dependent endocytosis. Clathrin-coated vesicles derived either from the plasma membrane or the TGN shed up their coat and fuse with endosomes. Cargo can then be sorted to the cell surface or to late endosomes/lysosomes for degradation. COPI- and COPII-coated vesicles transport proteins between ER and Golgi. Figure is taken and modified from (Hinrichsen, 2005).

The internalization frequency (constitutive or ligand-induced) and the post-endocytic fate of a receptor (recycling, retention or degradation) depend on the type of the receptor and the state of the cell. The endocytic pathway is primarily comprised of three types of endosomes: the early, recycling, and late endosomes. Endocytosed cargos were believed to first enter a common pool of early endosomes/sorting endosomes (EE/SE). Subsequently, receptors and ligands destined for recycling are delivered back to the cell surface; cargos bound for

degradation are sorted to late endosomes (LE) and lysosomes. Two receptors, the transferrin receptor (TfR) and the EGF-receptor (EGFR), are exemplary for two possible endocytic routes: the glycoprotein transferrin is used to provide cells with iron. Fe³⁺-laden transferrin (ferrotransferrin) binds to the **TfR**, and the receptor/ligand-complex is subsequently internalized by CME. Endocytosed vesicles fuse primarily with early/sorting endosomes in the peripheral cytoplasm. Due to the low pH in EE (pH 5.9) generated by vacuolar proton ATPases, the iron dissociates from transferrin. The iron-free transferrin (apotransferrin) remains associated with the TfR and is sorted to recycling endosomes (RE) from where the ligand/receptor-complex is finally recycled back to the plasma membrane. The neutral pH in the extracellular milieu reduces the affinity of apotransferrin for its receptor. Thus the ligand dissociates from the TfR and is available for another round of iron-binding. While the TfR is important for the basal maintenance of the cell with iron, the **EGFR** is a signal transducer that -upon stimulation with EGF at the plasma membrane- is able to induce the proliferation of cells. A permanent active receptor could have fatal consequences for the cell like uncontrolled cell growth and division. Hence the endocytosis of the EGF/EGFR-complex serves to regulate the density of active EGFRs and therefore the signal duration of the receptors at the plasma membrane. For this reason the internalized EGFRs have a different fate than the TfRs: the EGFRs are sorted from the EE into LE for subsequent degradation in lysosomes and only a small number of receptors is recycled to the plasma membrane through recycling endosomes.

1.3 Sorting of lysosomal proteins at the TGN

The sorting of newly synthesized lysosomal enzymes from the TGN to endosomal/lysosomal compartments is mediated by mannose 6-phosphate receptors (M6PRs). The M6PRs bind the common mannose 6-phosphate recognition marker on soluble lysosomal enzymes in the TGN. In this way the lysosomal enzymes are physically separated from proteins destined for secretion. The ligand-receptor complex then exits the Golgi in a clathrin-coated vesicle and is delivered to an endosomal acidified compartment where the M6PRs unload their bound ligands. The released lysosomal enzymes are packaged into a lysosome while the receptor either returns to the Golgi to repeat the process or moves to the plasma membrane where it functions to internalize exogenous lysosomal enzymes (Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998; Rouille *et al.*, 2000).

Two different M6PRs have been identified. Both are type-I integral membrane glycoproteins but differ in their size and their dependence on divalent cations for high-affinity ligand binding: the 300-kDa cation-independent mannose 6-phosphate receptor (CI-M6PR) contains two high affinity Man-6-P-binding sites whereas the 46-kDa cation-dependent M6PR (CD-M6PR) binds only one molecule of Man-6-P/polypeptide. Furthermore, only the CI-M6PR functions at the cell surface in the binding and internalization of exogenous ligands (Dahms *et al.*, 1989; Ghosh *et al.*, 2003). The sorting of M6PRs from the compartments they visit (e.g. plasma membrane, TGN, early, recycling and late endosomes) is directed by various sorting motifs in their cytoplasmic domain. These sorting motifs are recognized by components of the vesicular trafficking machinery (Mellman, 1996; Ghosh *et al.*, 2003). At the TGN the M6PRs are sorted into clathrin-coated vesicles and this sorting is mediated amongst others by the adaptor protein AP1 and by proteins of the GGA-family (Golgi-localized, γ -ear-containing, ARF-binding proteins). The recycling of M6PRs from early endosomes to the TGN is thought to be coordinated by AP1 and the cytosolic sorting protein PACS-1. Due to maturation of the early endosomes some M6PRs reach the late endosomes where they are prevented from being delivered to the lysosome by the cargo selection protein TIP47. Together with the small GTPase Rab9, TIP47 also mediates the recycling of M6PRs from late endosomes to the TGN. Finally, either by missorting at the TGN or by recycling from early and late endosomes M6PRs can be transported to the cell surface where they are internalized rapidly in an AP2 and clathrin-dependent manner (for an overview of M6PR trafficking pathways see figure 1.2 and (Ghosh *et al.*, 2003).

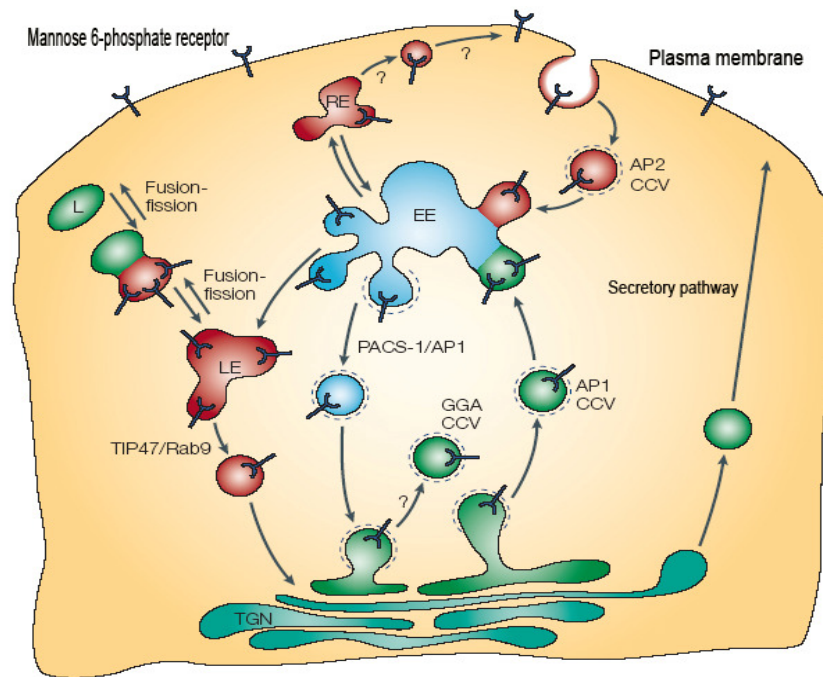


Figure 1.2: Schematic overview of the subcellular localization trafficking itinerary of M6PRs. Lysosomal hydrolases with mannose 6-phosphate moiety are engaged by M6PR in the TGN and transported via clathrin-coated vesicles to acidified early endosomes where the ligand/receptor-complex dissociates. The lysosomal enzymes are further processed and finally transported to lysosomes, whereas the M6PRs in early and late endosomes are sorted either back to the TGN or to the plasma membrane. As a consequence of missorting M6PRs are occasionally transported directly from the TGN to the cell surface where they are internalized rapidly by clathrin-mediated endocytosis. Figure taken and modified from (Ghosh *et al.*, 2003).

1.4 Clathrin-coated vesicles

The formation of clathrin-coated vesicles (CCVs) is a complex process, which occurs through the interactions of multifunctional adaptor proteins with components of the donor membrane as well as with clathrin and several accessory proteins. Before discussing the stages of CCV biogenesis in detail, a brief review about clathrin and the adaptor protein complexes as the main components of the basic CCV machinery will be given.

1.4.1 Clathrin

The protein clathrin is responsible for the remarkable morphology of the cage-like structure that surrounds the internalized vesicle. The assembly unit of clathrin is called triskelion and consists of three identical clathrin heavy chains (CHC) each of which is associated with a clathrin light chain (CLC) (Ungewickell and Branton, 1981). The mammalian CHC comprises

1675 residues with calculated molecular weight of 192 kDa. Each CHC can be subdivided structurally and functionally into several distinct regions (see also figure 1.3): the globular N-terminal domain (TD) followed by a distal domain (DD) that is connected by a flexible bend (knee) to the proximal (PD) and finally to the trimerization domain that contributes to the triskelion hub (Brodsky *et al.*, 2001). The globular TD, which is connected to the distal leg via a flexible linker region, forms a seven-blade β -propeller structure with binding sites for a number of endocytic proteins including the adaptor proteins AP2 and AP180. Co-crystallization experiments revealed that the majority of clathrin-interacting proteins bind to a site in the groove between blade 1 and 2 of the propeller through the consensus sequence L(L/I)(D/E/N)(L/F)(D/E). This binding motif is generally known as 'clathrin-box'-motif (Dell'Angelica *et al.*, 1998; ter Haar *et al.*, 2000). However, in recent years the mapping of clathrin binding sites in several clathrin interacting partners revealed that these sites do not completely fit the original definition of the 'clathrin-box'-motif. Some of these motifs are degenerated variants of the clathrin-box such as the tripeptide DLL, which was shown to likewise support the interaction of the adaptor protein AP180 or the uncoating ATPase auxilin with the TD of clathrin (Morgan *et al.*, 2000). More recently the so called W-box motif PWDLW was discovered in the amphiphysins and in Snx9 for instance. This W-box motif interacts with the β -propeller of the clathrin TD in a way that is not competitive with the classical clathrin-box (Miele *et al.*, 2004).

Structural analysis revealed that the TDs point towards the membrane of the CCV, what makes them accessible for the interaction with adaptors and other accessory proteins. The proximal and the distal domain of CHC in contrast contribute to intermolecular contacts in order to establish a stable basket structure of assembled clathrin. The proximal domain also contains binding sites for the CLCs. There are two forms of CLC (CLCa and CLCb) in vertebrate species, which are encoded on different chromosomes in humans and share about 60% protein sequence identity (Brodsky *et al.*, 1987; ter Haar *et al.*, 2000). Due to tissue specific splicing the CLCa and CLCb vary from 25-29 kDa. However, the N-terminus is rich in negatively charged residues and this composition results in an anomalous electrophoretic mobility in SDS-PAGE, which has led to estimated molecular masses of 32-36 kDa (Scarmato and Kirchhausen, 1990). Although there are differences in the relative expression levels of both CLCs in cells from different tissues, triskelions comprise a random distribution of CLCa and CLCb. It is noteworthy that only CLCb is subjected to phosphorylation *in vitro* by the protein kinase CK2 (Bar-Zvi and Branton, 1986).

The assembly of the clathrin coat is the key ability of CCVs to selectively capture cargo into a membrane vesicle. The CLC has been suggested to contribute to the pH sensitivity of clathrin assembly (Jiang *et al.*, 2000). Without CLC bound, clathrin triskelions self-assemble into a cage-like structure at physiological pH (Ungewickell and Ungewickell, 1991). In contrast, with bound CLCs the polymerization of triskelions will only occur in a slight acidic milieu. This CLC-dependent inhibition at physiological pH can be reversed by adaptor proteins such as AP1 and AP2 (Ybe *et al.*, 1998). Thus CLCs counteract the self-assemble tendency of clathrin triskelions and enable the regulation of the polymerization process by other factors.

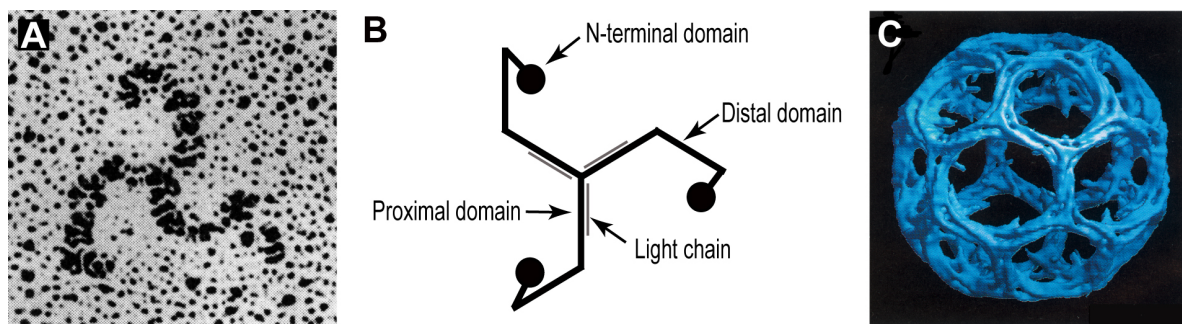


Figure 1.3: Clathrin. A) Electron microscopic picture of a clathrin triskelion (taken from (Ungewickell and Branton, 1981)). B) Schematic representation of a clathrin triskelion. Three clathrin heavy chains (CHCs) with a terminal-, a distal- and a proximal domain each and a clathrin light chain (CLC) bound to each CHC form one triskelion. C) Computer-animated model of a clathrin cage (taken from (Smith *et al.*, 1998)).

1.4.2 Adaptor protein complexes

Adaptor protein complexes are heterotetramers that play important roles in cargo selection as well as in vesicle formation in clathrin-mediated trafficking processes (Nakatsu and Ohno, 2003). The interaction of clathrin with membrane lipids and receptors destined for internalization is not direct but mediated by the adaptors AP1 (γ -, β 1-, μ 1- and σ 1-subunits) and AP2 (α -, β 2-, μ 2- and σ 2-subunits). Furthermore, these polypeptides are absolutely required for the assembly of purified clathrin triskelions into clathrin coats under physiological conditions. Due to their ability to recognize and to interact with a select subset of membrane constituents and to induce clathrin assembly, AP1 and AP2 initiate CCV formation at the TGN (AP1) or at the plasma membrane (AP2), respectively.

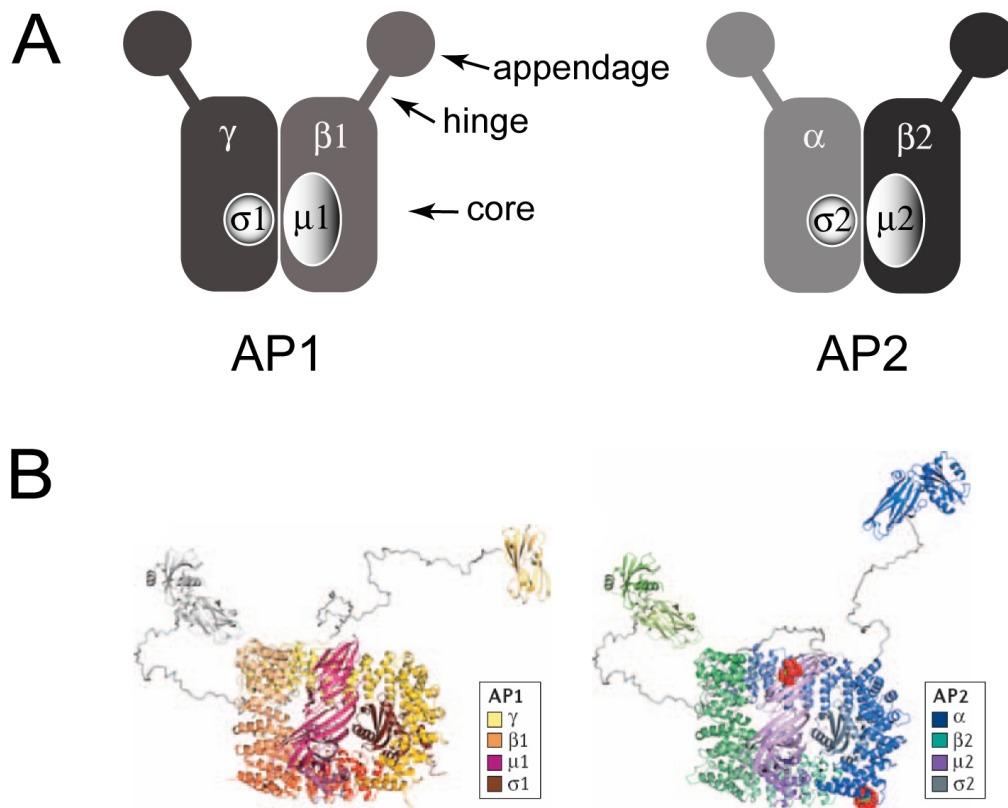


Figure 1.4: Schematic overview of the adaptor protein complexes AP1 and AP2. A) AP1 and AP2 both consist of two large subunits ($\gamma/\beta1$ and $\alpha/\beta2$), a middle subunit ($\mu1$ and $\mu2$) and a small subunit ($\sigma1$ and $\sigma2$). The core domains are connected to the appendage domains by a flexible hinge domain. B) Structure models of AP1 and AP2 (colored by subunit as indicated) based on X-ray crystallography data. The linkers and the $\beta1$ -appendage of AP1 were modeled, because their structures are unknown. The position of a phosphoinositide mimic that was bound to AP2 is shown in spacefill representation. Figure 1.4B was taken from (Edeling *et al.*, 2006a).

The large subunits of the complexes ($\gamma/\beta1$ and $\alpha/\beta2$) exhibit a molecular mass of ~ 100 kDa. Electron microscopic analysis has revealed that the adaptors have a characteristic morphology resembling a head with ears (Figure 1.4) (Heuser and Keen, 1988). The head, or core domain, comprises the N-terminal thirds of the large subunits whereas the ears, or appendages, are formed by the C-terminal part. Head and ear domains are linked by a proline-rich hinge domain. The hinge regions of $\beta1$ and $\beta2$ have a ‘clathrin-box’-motif and mediate interaction with clathrin. Moreover, the hinge-appendage domain of $\beta2$ has been shown to stimulate the assembly of purified clathrin triskelions *in vitro* (Greene *et al.*, 2000). The structure of the α - and $\beta(1+2)$ -appendages exhibit a similar overall architecture, which is composed of an amino-terminal 8-stranded β -sandwich and a carboxy-terminal ‘platform’ subdomain. By contrast, the structure of the γ -appendage domain is similar only to that of the N-terminal β -sandwich subdomain of the other appendages (an overview of AP structures gives (Edeling *et al.*, 2006b)).

The core domain of one of the large subunits in each AP complex (γ and α) mediates binding to the target membrane through the recognition of phosphoinositides. In the case of AP2 the interaction with phosphoinositides is supported by a positively charged patch on the μ 2-subunit whereas μ 1 lacks this concentration of positive charges (Rohde *et al.*, 2002; Heldwein *et al.*, 2004). Different phosphoinositides are generally found on only one or a small subset of organelles. Phosphoinositides are therefore important determinants of membrane identity (Behnia and Munro, 2005). The α -subunit of AP2 primarily mediates the interaction with phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂), which is enriched at the plasma membrane. Thus, PtdIns(4,5)P₂ drives the specific recruitment of AP2 to the plasma membrane (Gaidarov *et al.*, 1996). PtdIns(4,5)P₂ is generated by phosphatidylinositol 4-phosphate 5-kinase (PIP₂K) and the kinase is stimulated by AP2 that is associated with transmembrane cargo molecules. This scenario provides a positive feedback loop for the formation of CCP at the plasma membrane (Krauss *et al.*, 2006). The localization of AP1 at the Golgi depends in part on phosphatidylinositol 4-monophosphate (PtdIns(4)P), which is the most prominent phosphoinositide at the TGN and interacts with the γ -subunit of AP1 (Heldwein *et al.*, 2004).

The ear domain of the adaptors act in addition to the N-terminal domain of clathrin as the major interacting-platform for accessory proteins in the nascent CCV (an overview gives (Brodsky *et al.*, 2001). As in the case of binding to the TD of clathrin, short linear peptide motifs are responsible for the interaction of proteins with the appendages. It is known that the α -appendage domain of AP2 bears binding sites for the motifs DPF/W and FxDxF (x is any amino acid) through an overlapping site in the platform subdomain (Brett *et al.*, 2002). Additionally, the sandwich subdomain interacts with proteins containing the motif WXX(F/W)X(D/E) (Praefcke *et al.*, 2004; Ritter *et al.*, 2004). As in the case of clathrin TD binding-partners also appendage-binding partners usually display multiple copies of the above mentioned short, linear peptide motifs in unstructured regions of the protein. However, recently, two groups identified a novel binding motif that engages the β 2-appendage platform subdomain of AP2 in an α -helical conformation rather than in an extended conformation like other peptide ligands. The motif [DE]_nX₁₋₂FXX[FL]XXXXR occurs and is functional in the ARH (autosomal recessive hypercholesterolemia) protein, in β -arrestin and in the epsins (Edeling *et al.*, 2006a; Schmid *et al.*, 2006). All three proteins are important for the internalization of specific families of transmembrane receptors such as the LDL receptor (ARH), G protein-coupled receptors (β -arrestin) and ubiquitinated receptors (Barriere *et al.*, 2006; Hawryluk *et al.*, 2006). Furthermore, both studies demonstrated that the AP2 β 2-

appendage sandwich subdomain has a second independent interaction motif for proteins such as Eps15 and clathrin (Edeling *et al.*, 2006a; Schmid *et al.*, 2006).

The medium subunits ($\mu 1$ and $\mu 2$, ~50 kDa) as well as the small subunits ($\sigma 1$ and $\sigma 2$, ~20 kDa) are associated with the large subunits within the core domain. $\sigma 1$ and $\sigma 2$ are thought to be involved in the stabilization of the complexes whereas the medium subunits play an important role in the sequestering of cargo molecules into nascent CCVs. In particular, the μ -subunits directly recognize tyrosine-based sorting signals within the cytoplasmic tail of cargo membrane proteins with the consensus motif Yxx $\varnothing$ (x is any amino acid and $\varnothing$ is an amino acid with a bulky hydrophobic side chain) (Ohno *et al.*, 1995; Owen and Evans, 1998). The so called dileucine motif ([DE]xxxL[LI]) is present in other cargo proteins and probably recognized by an as yet unknown surface upon an $\alpha/\sigma 2$ (AP2) and $\gamma/\sigma 1$ (AP1) subunit hemicomplex (Marks *et al.*, 1996; Janvier *et al.*, 2003).

As mentioned above AP1 is involved in the formation of CCVs at the TGN and AP2 promotes clathrin-dependent endocytosis at the plasma membrane. In addition to these adaptors two more complexes with subunit composition similar to AP1 and AP2 have been identified and characterized (Robinson and Bonifacino, 2001). AP3 (δ -, $\beta 3$ -, $\mu 3$ - and $\sigma 3$ -subunits) and AP4 (ϵ -, $\beta 4$ -, $\mu 4$ - and $\sigma 4$ -subunits). Although AP3 is able to interact with clathrin through a conserved clathrin-binding domain in the $\beta 3$ -subunit and colocalizes with clathrin on endosomal membranes in HeLa cells (Dell'Angelica *et al.*, 1998), AP3 does not copurify with CCVs and AP3-mediated pathways in yeast were not impaired in clathrin mutants (Simpson *et al.*, 1996; Vowels and Payne, 1998). Genetic studies revealed that AP3 is involved in the sorting pathway to pigment granules in fruit fly (Ooi *et al.*, 1997). Due to the relationship between pigment granules and lysosomes regarding their origin, this data indicates a role for AP3 in the sorting of membrane proteins to lysosomes and lysosome-related organelles. In fact, in a human patient suffering from Hermansky-Pudlack syndrome (HPS), which accompanies defects in the function of lysosomes such as prolonged bleeding and few melanosomes, Dell'Angelica *et al.* discovered a mutation in the δ -subunit of AP3 (Dell'Angelica *et al.*, 2000). The functional characterization of AP4 has revealed so far that its expression is limited to a low level and is localized to the TGN region of cells (Dell'Angelica *et al.*, 1999). Interestingly, AP4 is the only adaptor protein in the group of the four complexes that is presumed to have no binding-affinity for clathrin, indicating that AP4 might interact with a non-clathrin-coat protein. More probable functions of AP3 and AP4 are discussed in (Nakatsu and Ohno, 2003).

1.5 Molecular mechanism of clathrin-coated vesicle formation

This chapter focuses on the formation of clathrin-coated vesicles (CCVs) at the plasma membrane because at this site our understanding of the molecular mechanisms is currently the most advanced. Major defined stages in the life of a CCV are: (1) concentration of transmembrane receptors and their bound ligands into clathrin-coated pits (CCP), which are formed by the recruitment of adaptor proteins and assembly of cytosolic clathrin triskelions at the inner leaflet of the plasma membrane, (2) invagination of the CCP, (3) scission of the CCV and finally (4) the dissociation (uncoating) of the adaptor proteins and the clathrin coat from the vesicle that allows the recycling of CCV components for subsequent rounds of endocytosis (for an overview see (Brodsky *et al.*, 2001; Mousavi *et al.*, 2004; Owen, 2004).

1.5.1 Formation of clathrin-coated pits

The life cycle of CCV-formation begins with the formation of coated pits by the initial recruitment of AP2 adaptors, accessory proteins and clathrin triskelions to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂)-enriched plasma membrane regions. It has become evident that mainly AP2 is responsible for the nucleation of coated pits and that AP2 therefore specifies the localization of clathrin assembly (Brodsky, 1988). AP2 harbours two high-affinity PtdIns(4,5)P₂-binding sites, one on the α - and one on the μ 2-subunit, and both contribute to the association of AP2 with the plasma membrane. Recent results also indicate that the targeting of AP2 to the plasma membrane is regulated through the activation of the small GTPase ARF6 (ADP-ribosylation factor 6) by the GTPase activating protein (GAP) SMAP1. ARF6 promotes the nucleation of AP2 directly by interacting with AP2 and indirectly by targeting the phosphatidylinositol 4-phosphate 5-kinase (PIP5K) to the plasma membrane. Moreover, the PIP5K is stimulated by AP2 that is associated with cargo molecules. The PtdIns(4,5)P₂ that is thus generated increases the affinity of AP2 for the membrane (Krauss *et al.*, 2003; Paleotti *et al.*, 2005; Tanabe *et al.*, 2005; Krauss *et al.*, 2006).

In addition, sorting motifs in the cytoplasmic region of surface receptors that use the coated pit pathway for internalization are engaged by the μ 2-subunit of AP2, finally resulting in the concentration of these receptors in the nascent CCV (Pearse and Crowther, 1987).

It is of note that the $\mu 2$ -subunit with its binding sites for tyrosine-containing sorting motifs and for phosphoinositides, respectively, is buried in the AP2 complex and that a conformational change is required for its exposure. This conformational change is triggered by the phosphorylation of $\mu 2$ (at Thr156) by the adaptor-associated kinase 1 (AAK1). Interestingly, the activity of this kinase is enhanced by polymerized clathrin, thus promoting cargo engagement at sites of clathrin lattice assembly (Conner and Schmid, 2002; Conner *et al.*, 2003). After association with endocytic sites, AP2 mediates the assembly of a clathrin lattice.

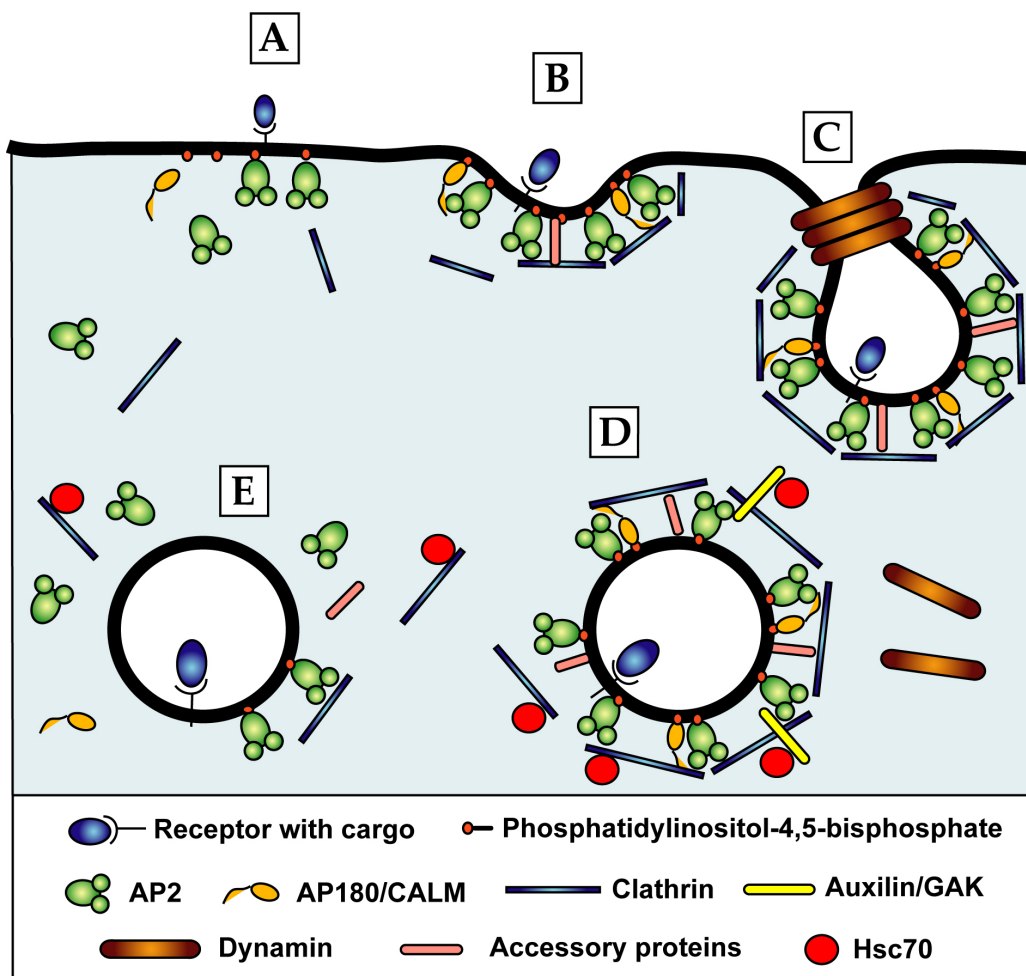


Figure 1.5: Model for clathrin-dependent endocytosis

- A) AP2 binds to phosphoinositides as well as to transmembrane proteins and initiates along with alternative adaptors the formation of a clathrin-coated pit (CCP).
 - B) Clathrin-assembly and further recruitment of accessory proteins. The membrane starts to invaginate.
 - C) Assembly of the GTPase dynamin around the neck of the deep invaginated CCP and scission of the clathrin-coated vesicle (CCV).
 - D) ATP-dependent uncoating of the clathrin-lattice by the chaperone hsc70 and the co-factor auxilin/GAK.
 - E) The uncoating of the adaptor coat is promoted by the synaptojanin-catalyzed dephosphorylation of $\text{PtdIns}(4,5)\text{P}_2$ and by phosphorylation of AP2.
- (Adopted and modified from (Hinrichsen, 2005).

The ongoing characterization of other components of the CME machinery revealed that additional proteins are involved in the initial nucleation step of coated pits. The alternate adaptors AP180/CALM and Epsin, for instance, contain binding sites for phosphoinositides, AP2 as well as for clathrin and create multi-point attachment sites for the recruitment and assembly of clathrin (see figure 1.5 A+B).

1.5.2 Invagination of clathrin-coated pits

The continuing assembly of triskelions at the donor membrane results in the formation of a flat hexagonal clathrin lattice (Heuser, 1989; Heuser and Anderson, 1989). However, in a second step, some hexagons are converted into pentagons, transforming the lattice into a closed sphere while the membrane starts to invaginate. In fact, based on the mathematical requirements for sphere formation, the presence of at least 12 pentagons is necessary for the curvature of the clathrin coat. Until recently, it seemed plausible that the introduction of pentagons can only occur at the edge of the growing hexagonal lattice (Kirchhausen, 2000) but recent results indicate that local rearrangements of the coat driven by the ATP-dependent exchange of lattice-embedded clathrin triskelions with cytosolic clathrin is an alternative scenario (Nossal, 2001).

There is an ongoing debate about the question if the formation of the clathrin coat can provide the driving force for membrane invagination. Not doubting that clathrin is essential for driving coated pit invagination, it becomes more and more likely that the clathrin coat has more a fixing and stabilizing function, rather than providing the driving force for membrane budding (Nossal, 2001). In agreement with this hypothesis in the meantime several other endocytic proteins like epsin and endophilin have been suggested to capture a supporting role in membrane curvature (Farsad *et al.*, 2001; Ford *et al.*, 2002). An intriguing illustration of clathrin-coated structures at the inner leaflet of the plasma membrane gives the electron microscopic analysis of ‘unroofed’ HeLa cells as demonstrated in figure 1.6.

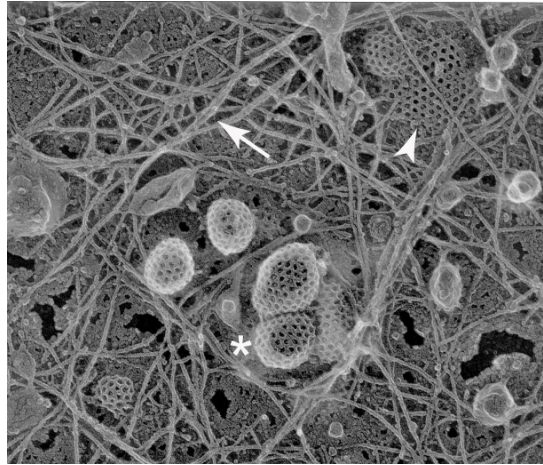


Figure 1.6: Visualization of clathrin coat structures at the plasma membrane. The exposed plasma membranes of 'unroofed' HeLa cells were fixed, critical point dried, rotary shadowed with platinum/carbon and analyzed by electron microscopy. The picture shows flat clathrin lattices (arrow head) as well as invaginated clathrin-coated pits (asterisk). The arrow denotes cytoskeleton bundles underneath the membrane. (own picture, 2005; for a description of the precise method see (Meyerholz et al., 2005)).

1.5.3 Membrane fission and formation of free coated vesicles

Coated-vesicle detachment from plasma membrane requires the GTPase-containing protein dynamin, which is recruited by amphiphysin to the nascent CCV (Kosaka and Ikeda, 1983; Shpetner and Vallee, 1989; Chen *et al.*, 1991). In the *shibire* (a dynamin homolog) mutant of *D. melanogaster*, neuronal synapses contain coated pits with essentially completely formed clathrin lattices but with a thin collared bilayer neck connecting the vesicle to the plasma membrane (Sever *et al.*, 2000). The same is true in cells expressing a dynamin mutant that lacks its GTPase activity (Takei *et al.*, 1995). Although it is generally accepted that dynamin self-assembles into a helical collar around the neck of an invaginated coated pit and thereby stimulating its own GTPase activity (Figure 1.5C), there are currently two views of how dynamin promotes the scission of CCVs. The most current view suggests that the self-assembly of dynamin around the neck of the deep invaginated CCP and GTP hydrolysis is the mechanical force behind vesicle scission. The alternative view holds that dynamin functions as a regulatory GTPase and that it recruits other mediators needed for vesicle scission. It is likely that the role of dynamin in CCV scission involves both of these mechanisms (Hinshaw and Schmid, 1995; McNiven, 1998; Schmidt *et al.*, 1999; Marks *et al.*, 2001). The role of dynamin as a mechanoenzyme was recently further supported by a study of De Camilli and co-workers, who monitored the effect of nucleotides on dynamin-coated lipid tubules in real time (Roux *et al.*, 2006). However, this study also implied that the dynamin-mediated

constriction of the neck is not sufficient for fission of the vesicle. They proposed that the actin cytoskeleton mediates the membrane tension that is necessary for scission and therefore cooperates with dynamin in this process. Moreover, there are accumulating evidences that actin plays important roles at different steps along the endocytic pathway, which have been reviewed recently in (Kaksonen *et al.*, 2006).

1.5.4 Coat disassembly

A prerequisite for the fusion of the internalized vesicle with an endosomal compartment is the dissociation of the clathrin coat and the adaptor protein complexes. Clathrin and adaptors dissociate from CCVs in separate steps (Hannan *et al.*, 1998). The disassembly of the clathrin coat is an energy-requiring process that is mediated by the ATP-dependent chaperone hsc70. Given that hsc70 has a low affinity for clathrin, it has to be recruited either by the neuron-specific co-chaperone auxilin or the ubiquitously expressed homolog auxilin2/GAK (cyclin G-associated kinase) (Ungewickell *et al.*, 1995; Holstein *et al.*, 1996; Greener *et al.*, 2000; Umeda *et al.*, 2000). In addition to numerous, tandemly arranged binding motifs for clathrin and AP2, auxilin harbours a J-domain (named after DnaJ, the partner of the *Escherichia coli* hsp70, DnaK) (Cyr *et al.*, 1994) by which it interacts with hsc70 in its 'ATP-bound conformation'. Interestingly, in contrast to auxilin, GAK harbors a serine/threonine kinase domain at its N-terminal end, which was shown to phosphorylate the medium (μ) subunits of AP1 and AP2 *in vitro* (Korolchuk and Banting, 2002). Auxilin1+2 not only target hsc70 to clathrin cages but in addition stimulate the ATPase-activity of the chaperone. ATP hydrolysis results in a conformational change in the hsc70 molecule and finally in the formation of a tight clathrin-hsc70 complex. This complex formation weakens the intermolecular interactions of clathrin within the coat with the consequence that triskelions with bound hsc70 are released into the cytosol, where they refresh the pool of clathrin available for subsequent CCP formation. Furthermore, the dephosphorylation of PtdIns(4,5) P_2 by the endocytic phosphatase synaptojanin may destabilize the interaction of AP2 and AP180/CALM with the vesicle (Cremona *et al.*, 1999). The dissociation may be further promoted by the adaptor-phosphorylation in the clathrin-binding domain of the β -subunits (Wilde and Brodsky, 1996).

1.5.5 Differences in CCV formation at the TGN and at the plasma membrane

Although there are broad mechanistically similarities in the formation of CCVs at the plasma membrane and the TGN, the main differences are based on the usage of different adaptor proteins. As mentioned above, the adaptor protein complexes AP1 and AP2 display a specificity to their cellular localization and mediate distinct vesicle-formation processes either at the TGN (AP1) or at the plasma membrane (AP2). But what are the prerequisites for the specific recruitment of AP1 and AP2 to distinct donor membranes? One striking difference is the absolute requirement for the small GTPase ADP-ribosylation factor 1 (ARF1) in its GTP-bound form in the translocation of AP1 to Golgi membranes (Stamnes and Rothman, 1993). ARF1 uses a myristoylated N-terminal helix as membrane anchor and associates with lipids in its GTP-bound form (Goldberg, 1998). The switch of GTP for GDP, which is necessary for the association of ARF1 with membranes, is catalyzed by a guanine nucleotide exchange factor (GEF). As a result, the administration of brefeldin A, a fungal inhibitor of GEF activity, averts the activation of ARF1 and therefore causes a redistribution of AP1 into the cytosol (Robinson and Kreis, 1992). Interestingly, another member of the ARF family, ARF6, seems to have an equivalent importance for clathrin-mediated endocytosis at the plasma membrane, either indirectly by targeting lipid-modifying enzymes to the membrane or directly by recruiting AP2 for CCP nucleation (Paleotti *et al.*, 2005; Tanabe *et al.*, 2005).

Another factor that provides specificity for the distinct recruitment of AP1 and AP2 is the different lipid composition of the membranes. AP2 binds to $\text{PtdIns}(4,5)\text{P}_2$, which is more prominent at the plasma membrane, whereas phosphatidylinositol 4-monophosphate ($\text{PtdIns}(4)\text{P}$) is more prominent at the TGN and binds AP1 (Haucke, 2005; Di Paolo and De Camilli, 2006). The specificity provided by phosphoinositides is not only given by direct interactions with AP1 and AP2 but also by establishing a platform for other accessory proteins that can stabilize the AP1 and AP2 coat, respectively. It must be noted that the affinity as well as the specificity of the APs to the phosphoinositides is relatively modest. But the interaction with different ARF-proteins in combination with local phosphoinositide recognition promotes the optimal association of AP1 and AP2 with the TGN and the plasma membrane, respectively. Thus, this avidity phenomenon, which is also referred to as coincidence detection, restricts adaptor and clathrin assembly to precise compartment locations. Once on the membrane the adaptor coat is stabilized by the polymerized clathrin scaffold and by interaction with accessory proteins and cargo molecules.

Sorting motifs in the cytosolic domain of transmembrane proteins are in principle recognized by different AP complexes so that *a priori* they do not contribute to a specific recruitment of adaptors to distinct membranes. But the affinity of AP complexes for a specific binding motif might differ, depending on the membrane-organelle environment. Nevertheless, the cargo engagement stabilizes the adaptor at the membrane and it is noteworthy that – like in the case of AP2 – once on the membrane the μ 1-subunit of AP1 undergoes phosphorylation, which results in an increased binding affinity for sorting signals of cargo molecules (Ghosh and Kornfeld, 2003).

AP1 is not the only coat constituent that specifically functions at the Golgi during CCV formation. One important example is another family of adaptor proteins, the GGAs (Golgi-localized, γ -ear-containing, ARF-binding proteins; see also chapter 1.3). The GGAs are also recruited to membranes by ARF-GTP and operate along with AP1 in the generation of a subset of CCVs (Bonifacino, 2004). Moreover, it is proposed that the GGAs bind to specific receptors such as the M6PRs and transfer the cargo to AP1. Thus, the GGAs promote the incorporation of these cargo molecules into AP1-containing CCVs (Doray *et al.*, 2002; Ghosh *et al.*, 2003). In addition to the GGAs several more proteins have been identified that specifically function at the Golgi during clathrin/AP1-dependent sorting processes (for an overview see (Traub, 2005).

1.6 Accessory proteins in CCV formation

The model of clathrin-coated vesicle formation given in chapter 1.5 is rather simplified. It has become evident that this process is not a chain of distinct monocausal events but instead is a highly cooperative and concerted action of numerous proteins. Indeed, the discovery and characterization of many accessory factors of the CCV machinery led to a more detailed understanding of this process and updated the oversimplified cargo-adaptor-clathrin central trinity model. A common feature of these ‘accessory proteins’ is that they are able to interact with clathrin and/or with at least one of the two adaptor proteins AP1 and AP2, respectively. In many cases the interactions are mediated by tandemly arrayed short peptide motifs in unstructured regions of the accessory proteins with folded regions of the adaptor proteins such as the appendages or with the N-terminal domain of clathrin. Moreover, by associating with other proteins or lipids in addition accessory factors build up a complex interaction network (Figure 1.7). The accessory proteins can be broadly classified into three functional categories:

alternate adaptors, mechanically/assembly and regulatory proteins. **Alternate adaptors** are necessary because the adaptor proteins AP1 and AP2 are not capable of recognizing all sorting motifs of transmembrane proteins known to be internalized in CCVs. The CME of G protein-coupled receptors (GPCR), for instance, is dependent on the alternate adaptor β -arrestin. The engagement of an activated GPCR triggers a conformational change in β -arrestin, which results in an exposure of clathrin- as well as AP2-binding motifs. Eventually, the interaction of β -arrestin with clathrin and AP2 promotes the uptake of the GPCR in CCVs (Kim and Benovic, 2002; Santini *et al.*, 2002). Alternate adaptors at the TGN are the GGA proteins, which have already been mentioned in chapter 1.5.5.

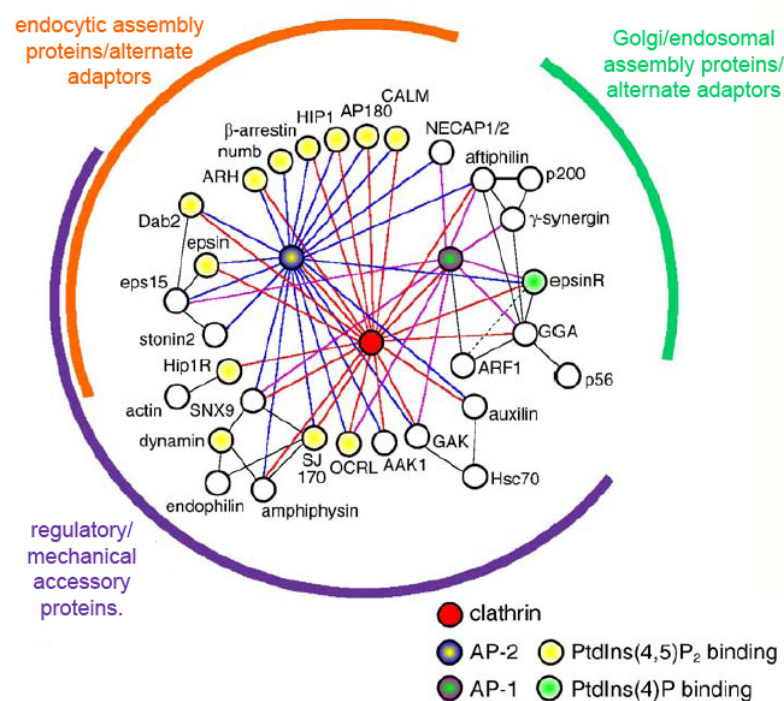


Figure 1.7: The clathrin protein-protein interaction network. Overview of protein-protein and protein-lipid interactions that contribute to CCV formation. Adopted and modified from (Traub, 2005).

Several members of the **mechanically/assembly accessory group** like AP180/CALM and epsin bear a PtdIns(4,5)P₂-binding module (the AP180-N-terminal homology (ANTH) domain in AP180 and the epsin N-terminal homology (ENTH) domain in epsin for instance) in combination with interaction motifs for AP2 and clathrin. Therefore these proteins facilitate the association of AP2 and the assembly of clathrin at the inner leaflet of the plasma membrane (Ford *et al.*, 2001; Itoh *et al.*, 2001; Ford *et al.*, 2002). Epsin, however, can additionally be classified as an alternate adaptor since the protein harbors two UIMs (ubiquitin-interacting motifs) and is involved in the endocytosis of ubiquitinated cargo molecules (Sigismund *et al.*, 2005). Another member of the mechanically accessory group is

the GTPase dynamin, which triggers the scission of a nascent CCV. Dynamin uses a PH (pleckstrin homology) domain for binding to PtdIns(4,5)P₂-containing membranes and a PRD (proline-rich domain) for the interaction with SH3 (Src homology 3) domain-containing proteins like amphiphysin and endophilin (Schmid *et al.*, 1998).

It is of particular importance that such a complex process like that of CCV formation, which involves series of dynamic protein-protein and protein-lipid interactions, has to be highly regulated. In recent years it became evident that the life-cycle of a CCV is coordinated by cycles of phosphorylation and dephosphorylation of interacting partners by **regulatory accessory proteins** (Korolchuk and Banting, 2003). An interesting example is a group of endocytic proteins designated as dephosphins that include dynamin 1, amphiphysin 1 and 2 and the adaptor protein AP180. The dephosphins are found in the phosphorylated state in resting nerve terminals and are coordinately dephosphorylated upon stimulation of the nerve terminals (Cousin and Robinson, 2001). In addition the clathrin heavy chain and one of the clathrin LCs (LCb) as well as the large and the medium subunits of AP1 and AP2 are subject to phosphorylation both *in vitro* and *in vivo* (Bar-Zvi and Branton, 1986; Ghosh and Kornfeld, 2003; Flett *et al.*, 2005). It is known that some of the kinases responsible for the phosphorylation mentioned above are associated with purified CCVs. As described in 1.5.1, the μ 2-subunit of AP2 for instance is phosphorylated *in vitro* by the CCV-associated kinases AAK1 and GAK/auxilin2, respectively (Greener *et al.*, 2000; Conner and Schmid, 2002).

Finally, many accessory proteins like Hip1R and dynamin interact directly or indirectly with both the CCV machinery and the actin cytoskeleton and therefore create a link between these two cellular systems. This is of special importance because it is now clear that focal actin polymerization of actin contributes to CCV assembly and movement at both plasma membrane and Golgi sites (reviewed in (Kaksonen *et al.*, 2006).

1.7 Aims of this study

In recent years it became evident that the formation of clathrin-coated vesicles (CCVs) is a spatially and temporally highly coordinated and complex process that involves -in addition to clathrin and adaptor protein complexes- the contribution of a diverse set of accessory factors. The characterization of accessory proteins and the definition of their precise function have deepened our understanding of clathrin-mediated trafficking processes.

In my diploma thesis I discovered that the uncoating co-factor auxilin is phosphorylated *in vitro* by a CCV-associated kinase-activity. Moreover, the data indicated that none of the so far characterized kinases found in CCV preparations (AAK1, GAK and CK2) is responsible for the phosphorylation of auxilin (Düwel, 2003). At the beginning of 2004 McPherson and co-workers discovered a novel protein in purified rat brain CCVs by mass spectroscopy (Blondeau *et al.*, 2004). Interestingly, this protein, later termed CVAK104 (coated-yesicle-associated kinase of 104 kDa), possesses a serine/threonine kinase homology domain. This let us speculate whether auxilin might be the substrate of CVAK104.

The first goal of this study was to confirm if auxilin is also phosphorylated *in vivo*. The second goal, which moved into the centre of my thesis, was the characterization of the novel CCV-associated protein CVAK104. By biochemical and cell biological approaches we aimed not only to determine, whether CVAK104 is an active kinase that targets auxilin, but also we wished to define the role of CVAK104 within the clathrin-machinery. The main questions in this context were (1) which interaction factors contribute to the association of CVAK104 with CCVs, (2) where exactly CVAK104 executes its function in the cell and (3) what consequences regarding clathrin-mediated transport processes result from the depletion of CVAK104 in cells.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Substance	Manufacturer
Agarose	BioRad (München, Germany)
Ampicillin	Sigma (Taufkirchen, Germany)
Bacto-agar	BD Biosciences (Heidelberg, Germany)
Brefeldin A	Sigma (Taufkirchen, Germany)
Brominephenol blue	Sigma (Taufkirchen, Germany)
Complete Proteaseinhibitor Cocktail	Roche (Mannheim, Germany)
Coomassie Brilliant Blue R250	Serva (Heidelberg, Germany)
Creatine phosphate	Sigma (Taufkirchen, Germany)
Desferroxamine	Sigma (Taufkirchen, Germany)
Desoxynucleotidtriphosphate mix	AGS-Hybaid (Heidelberg, Germany)
Digitonin	Wako industries (Japan)
Dithiothreitol (DTT)	Sigma (Taufkirchen, Germany)
EDTA	Sigma (Taufkirchen, Germany)
Ethidiumbromid	Sigma (Taufkirchen, Germany)
Fetal calf serum	Seromed, Berlin, Germany)
Ficoll 400	GE Healthcare (Piscataway, NJ, USA)
Glutathione	Sigma (Taufkirchen, Germany)
HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid)	Sigma (Taufkirchen, Germany)
Imidazol	Merck (Darmstadt, Germany)
Isopropyl-beta-D-thiogalactoside (IPTG)	Sigma (Taufkirchen, Germany)
L-Cysteine	Sigma (Taufkirchen, Germany)
Leibowitz's L15 cell culture medium	Invitrogen (Karlsruhe, Germany)
Low-fat milk powder	Uelzena (Uelzen, Germany)
NAMP 100	GE Healthcare (Piscataway, NJ, USA)

Nocodazole	Sigma (Taufkirchen, Germany)
OptiMEM	Invitrogen (Karlsruhe, Germany)
[³² P]-orthophosphate	Hartmann (Braunschweig, Germany)
Paraformaldehyde (PFA)	Fluka (Steinheim, Germany)
Penicillin / Streptomycin-solution	Invitrogen (Karlsruhe, Germany)
Phenyl-methyl-sulfonyl fluoride (PMSF)	Sigma (Taufkirchen, Germany)
Phosphatase inhibitor cocktail	Sigma (Taufkirchen, Germany)
Poly-L-lysine	Sigma (Taufkirchen, Germany)
Ponceau S	Sigma (Taufkirchen, Germany)
Redivue I-(³⁵ S)methionine	GE Healthcare (Piscataway, NJ, USA)
Saponin	Sigma (Taufkirchen, Germany)
Sodium azide	Merck (Darmstadt, Germany)
Sodium dodecyl sulfate (SDS)	BioRad (München, Germany)
Sodium pyruvate	Sigma (Taufkirchen, Germany)
Sodium pyrophosphate	Sigma (Taufkirchen, Germany)
Sucrose	Calbiochem (Darmstadt, Germany)
Tris (Trizma Base)	Sigma (Taufkirchen, Germany)
Triton X-100	Sigma (Taufkirchen, Germany)
Trypanblue	Sigma (Taufkirchen, Germany)
Trypsin-EDTA solution	Invitrogen (Karlsruhe, Germany)
Trypton	BD Biosciences (Heidelberg, Germany)
Yeast extract	BD Biosciences (Heidelberg, Germany)

Table 2.1 Chemicals and reagents.

2.1.2 Proteins and enzymes

Protein / Enzyme	Order #	Manufacturer
β-galactosidase	G-8511	Sigma (Taufkirchen, Germany)
Alcohol dehydrogenase	A-8656	Sigma (Taufkirchen, Germany)
Alkaline phosphatase, calf intestinal	713023	Roche (Mannheim, Germany)
Bovine serum albumin (BSA)	A-7306	Sigma (Taufkirchen, Germany)

Carboanhydrase	C-2273	Sigma (Taufkirchen, Germany)
Creatine kinase	C-3755	Sigma (Taufkirchen, Germany)
DNA-polymerase I Klenow-fragment	1008412	Roche (Mannheim, Germany)
Ovalbumin	A-7642	Sigma (Taufkirchen, Germany)
<i>Pfu</i> Turbo DNA polymerase		Stratagene (Amsterdam, Netherlands)
Phosphorylase b	P- 4649	Sigma (Taufkirchen, Germany)
Restriction endonucleases	diverse	MBI Fermentas (St.Leon-R., Germany)
T4-DNA-Ligase	EL0335	MBI Fermentas (St.Leon-R., Germany)
<i>Taq</i> DNA polymerase	EP0403	MBI Fermentas (St.Leon-R., Germany)
Transferrin	616424	Calbiochem (Darmstadt, Germany)
Transferrin (Texas-Red labeled)	T-2875	Molecular Probes (Leiden, Netherlands)
Transferrin (I-125 labeled)	NEX212	PerkinElmer (Boston, MA, USA)

Table 2.2 Proteins and enzymes.

If not mentioned otherwise, restriction enzymes and other reagents for molecular biology were purchased from MBI Fermentas (St. Leon-Roth, Germany).

2.1.3 Commercial systems

Aim	System	Manufacturer
Agarose gel extraction	<i>QIAquick Gel Extraction Kit</i>	Qiagen (Hilden, Germany)
Protein concentration detection	<i>BCA protein Assay</i>	Pierce (Rockford, IL, USA)
Chemoluminescence-detection	<i>Renaissance Western blot Chemiluminescence Reagent Plus</i>	NEN (Boston, MA, USA)

F-actin co-sedimentation assay	<i>non-muscle actin-binding protein spin-down biochem kit</i>	Cytoskeleton (Denver, CO, USA)
Mounting of glass coverslips on object slides	<i>ProLong Antifade Kit</i>	Molecular Probes (Leiden, Netherlands)
Plasmid-Miniprep	<i>QIAprep Spin Miniprep Kit</i>	Qiagen (Hilden, Germany)
Preparation of endotoxin-free DNA	<i>EndoFree Plasmid Maxi Kit</i>	Qiagen (Hilden, Germany)
RNA isolation	<i>RNeasy Micro Kit</i>	Qiagen (Hilden, Germany)
RT-PCR	<i>RevertAid™ H Minus First Strand cDNA Synthesis Kit</i>	MBI Fermentas (St.Leon-R., Germany)
Site directed mutagenesis	<i>QuickChange Site Directed Mutagenesis Kit</i>	Stratagene (Amsterdam, Netherlands)
Transfection of DNA and siRNA in eukaryotic cells	<i>LipoFect Transfection Reagent</i>	Invitrogen (Karlsruhe, Germany)

Table 2.3 Commercial systems.

2.1.4 Cells and tissues

<i>Escherichia coli</i>-strain	Supplier
BL21 (DE3)	Novagen (Madison, WI, USA)
DH5α	Invitrogen (Karlsruhe, Germany)
XL1-Blue	Stratagene (Amsterdam, Netherlands)

Table 2.4 *Escherichia coli*-strains.

Eukaryotic cell line	Origin	Supplier
COS-7	African green monkey kidney	See below
HeLa SS6	Human	See below
PtK2	rat kangaroo (Potorous tridactylus) kidney	See below

Table 2.5 Eukaryotic cell lines.

HeLa SS6-cells were obtained from Klaus Weber (Max-Planck-Institute for Biophysical Chemistry, Goettingen, Germany) and COS-7 cells by Miki Tsukada (Max-Planck-Institute for Experimental Endocrinology, Hannover, Germany). PtK2 cells were obtained from Beate Sodeik (Hannover Medical School, Hannover, Germany). Cultured cells were grown at 37 °C and 5% CO₂ in *Dulbeccos modified Eagle's medium* (DMEM) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were regularly passaged to maintain exponential growth.

Fresh pig brains were obtained from the local slaughterhouse (Hannover, Germany). Clathrin-coated vesicles were purified from pig brain by Huberta Ungewickell as described in (Lindner and Ungewickell, 1992). Fresh rat brains were provided by Peter Redecker (Hannover Medical School, Hannover, Germany).

2.1.5 Nucleic acids

The CVAK104 cDNA clone 6473586 (genbank accession # BC063798) in pCMV-sport6 was obtained from Open Biosystems (Huntsville, AL, USA).

Protein expression vectors

Vector	Expression of	In	Supplier
pQE30-32	H ₆ - fusion proteins	<i>E.coli</i>	Qiagen (Hilden, Germany)
pGEX-6P1-3	GST-fusion proteins	<i>E.coli</i>	GE Healthcare (Piscataway, NJ, USA)
pEGFP-C1-3	GFP- fusion proteins	Eukaryotic cells	Clontech (Clontech, Mountain View, CA, USA)

Table 2.6 Expression vectors.

Expression plasmids

Product	Vector	Source	Reference
GST-Clathrin TD	pGEX-2T	James Keen, Thomas Jefferson University (Philadelphia, PA, USA)	(Goodman <i>et al.</i> , 1997)
GST- α -appendage	pGEX-2T	Richard Anderson, University of Texas (Dallas, TX, USA)	(Wang <i>et al.</i> , 1995)
H ₆ - β 2-hinge/ear	pRSETc	Thomas Kirchhausen, Harvard Medical School (Boston, MA, USA)	(Shih <i>et al.</i> , 1995)
H ₆ -AP180 (328-745)	pQE31	Ruth Knorr, Hannover Medical School (Hannover, Germany)	(Kalthoff <i>et al.</i> , 2002)
mRFP-Clathrin LC	pEGFP-C3	Jürgen Wehland, GBF (Braunschweig, Germany)	(Benesch <i>et al.</i> , 2005)
FLAG-OCRL	pCMV-TAG2	Alexander Ungewickell, Washington University School of Medicine (St. Louis, MO, USA)	(Ungewickell <i>et al.</i> , 2004)

Table 2.7 Expression plasmids.

2.1.6 Oligodesoxynucleotides

Name	Sequence	Restriction-site
PAKfullforw	GCTCTGGAATTCAGCCATGGAGTCCATGC	<i>EcoRI</i>
PAKfullrev	CTTAGCGGCGCCGGTGGATCCCGGGCCCA	<i>NotI</i>
PAK697forw	GCTCTGGAATTCAGCAGCCTCTTAAACC	<i>EcoRI</i>
PAK697rev	CCTACTGTCGACAACACTATGCTGGCATGG	<i>Sall</i>

Table 2.8 Oligodesoxynucleotides used for PCR. The added restriction sites are marked in bold letters.

Name	Sequence	Restriction-site
PAK375STOP forw	GCAGAGAATTTTGCCTTGATAGAC <u>G</u> TCA GAATTTGTAAACCC	<i>AatI</i>
PAK375STOP rev	GGGTTTACAAATTCTGAC <u>G</u> TC <u>T</u> ATCAAG GCAAAATTCTCTGC	<i>AatI</i>
PAK697STOP forw	GCAGGCACAGAAGCTGTAAAGC <u>T</u> AGCA GCCTCTTAAACC	<i>NheI</i>
PAK697STOP rev	GGTTTAAGAGGCTGCTAGC <u>T</u> TTACAGCT TCTGTGCCTGC	<i>NheI</i>

Table 2.9 Oligodesoxynucleotides used for mutagenesis. Mutated bases are underlined; the added restriction sites are marked in bold letters.

All oligodesoxynucleotides were synthesized by Operon (Hilden, Germany).

2.1.7 siRNAs

The double-stranded siRNAs were obtained from Dharmacon (Lafayette, LA, USA) as a 20 μ M solution. With the help of the bioinformatic tool *BLAST* (Basic Local Alignment Search Tool; NCBI-database: <http://www.ncbi.nlm.nih.gov/BLAST/>) we ensured that the siRNA sequences were specific for the target mRNA and did not match the mRNA of other proteins. The selection was performed according to the criteria of the siRNA user guide of Dr. Thomas Tuschl from the Rockefeller University in New York: <http://www.rockefeller.edu/labheads/tuschl/sirna>. For an efficient knockdown the user guide recommends to use siRNA duplexes with 2-nt 3' overhangs composed of 21-nt sense and 21-nt antisense strands. We used to select siRNA sequences with dTdT in the 3' overhangs because 2'-deoxynucleotides are presumed to be more nuclease resistant but as efficient as ribonucleotides. The AA at the 5' end of the sense strand is a kind of traditional nomenclature for siRNAs and is not synthesized by the manufacturers. Taken together, when the sequence is indicated as "AAUGGGCUAGCUUGGAAGAUU" the corresponding siRNA duplex looks as follows:

UGGGCUAGCUUGGAAGAUUdTdT dTdTACCCGAUCGAACCUUCUAA
--

Target protein	Sequence	Denotation	Reference
γ -adaptin (AP1)	AAGCCCAGACATGCTTGCGCAUU	GADA	(Zhang <i>et al.</i> , 2004)
Clathrin (HC)	AACCUGCGGUCUGGAGUCAAC	Clathrin oligo I	(Hinrichsen <i>et al.</i> , 2003)
CVAK104	AAUGGGCUAGCUUGGAAGAUU	CVAK104 oligo I	(Duwel and Ungewickell, 2006)
CVAK104	AAAUGACAAAGAUUCCCUUCU	CVAK104 oligo II	(Duwel and Ungewickell, 2006)

Table 2.10 siRNAs.

2.1.8 Antibodies

Primary antibodies

Antibody	Antigen	Source/reference
Anti-AP2 (mouse)	α -adaptin (AP2)	Santa Cruz Biotechnology (Santa Cruz, CA, USA); catalog # sc-17771
mAb 100/4 (mouse)	Auxilin	(Ahle and Ungewickell, 1990)
Anti-GT (mouse)	β -1,4-galactosyltransferase	J. Rohrer; (Berger <i>et al.</i> , 1986)
Anti- δ -adaptin (mouse)	δ -adaptin (AP3)	BD Biosciences (Heidelberg, Germany); catalog # 611328

Anti-FLAG (rabbit)	FLAG	Sigma-Aldrich (St. Louis, MO, USA); catalog # F-7425
Anti-tubulin (mouse)	α -Tubulin	Sigma-Aldrich (St. Louis, MO, USA); catalog # T-9026
Anti-CD63 (H5C6) (mouse)	CD63	Developmental Studies Hybridoma Bank (Iowa City, IA)
Anti-GM130 (mouse)	GM130	BD Biosciences (Heidelberg, Germany); catalog # 610822
Anti-M6PR (rabbit)	Cation-independent mannose-6-phosphat receptor	B. Hoflack (TU Dresden, Germany); (Meresse and Hoflack, 1993)
Anti-EEA1 (mouse)	EEA1	BD Biosciences (Heidelberg, Germany); catalog # 610456
R461 (rabbit)	Clathrin light chain	E. Ungewickell; (Ahle <i>et al.</i> , 1988)
X22 (mouse)	Clathrin heavy chain (used for immunofluorescence analysis)	F.M. Brodsky; (Brodsky, 1985)
Anti-CHC (mouse)	Clathrin heavy chain (used for Western blot analysis)	Transduction Laboratories (Lexington, KY, USA); catalog # A59420
Anti-GST (goat)	GST	GE Healthcare (Piscataway, NJ, USA); catalog # 27-4577-01
Anti-GFP (mouse)	GFP	J. Wehland; Helmholtz Centre for Infection Research (Braunschweig, Germany)
Anti-CVAK104 (rabbit)	CVAK104	E. Ungewickell; (Duwel and Ungewickell, 2006)
R442-6 (rabbit)	Actin	E. Ungewickell; (Hinrichsen <i>et al.</i> , 2003)
Anti-syntaxin 6 (mouse)	Syntaxin 6	BD Biosciences (Heidelberg, Germany); catalog # 610635

AP.6 (mouse)	α -adaptin (AP2)	F.M. Brodsky; (Brodsky, 1985)
20D6 (mouse)	γ -adaptin (AP1)	E. Ungewickell; (Ahle <i>et al.</i> , 1988)

Table 2.11 Primary antibodies.

All primary antibodies for immunofluorescence and Western blots were diluted in PBS supplemented with 3% BSA and 0.02% NaN₃.

Secondary antibodies

Antibody	Antigen	Source
AMCA-conjugated IgG (goat)	Rabbit IgG	Dianova (Hamburg, Germany)
FITC-conjugated IgG (goat)	Mouse IgG	Dianova (Hamburg, Germany)
FITC-conjugated IgG (goat)	Rabbit IgG	Dianova (Hamburg, Germany)
HRP-conjugated IgG (goat)	Mouse IgG	MP Biomedicals (Solon, OH, USA)
HRP-conjugated IgG (goat)	Rabbit IgG	MP Biomedicals (Solon, OH, USA)
HRP-conjugated IgG (rabbit)	Goat IgG	MP Biomedicals (Solon, OH, USA)
Rhodamine-conjugated IgG (goat)	Mouse IgG	Dianova (Hamburg, Germany)
Rhodamine-conjugated IgG (goat)	Rabbit IgG	Dianova (Hamburg, Germany)

Table 2.12 Secondary antibodies.

Secondary antibodies for immunofluorescence were diluted in PBS supplemented with 3% BSA and 0.02% NaN₃ whereas those for Western blots were diluted in blocking solution.

2.1.9 Buffer and Solutions

Buffer/Solution	Composition
Actin polymerization solution	50 mM KCl, 2 mM MgCl ₂ , 1 mM ATP
ATP binding buffer	50 mM Tris-HCl, 5 mM MgCl ₂ , 3 μ M ATP (pH 8.0)
ATP regenerating system	2 mM ATP, 5 mM creatine phosphate and 5 units/ml creatine kinase
Blocking solution	PBS with 8 % low-fat milk powder, 0.1% Tween 20
BSA washing buffer	0.5 M Tris-HCl, 1 % Triton X-100, 1% BSA, 0.02% NaN ₃ (pH 7.0)
Buffer A	100 mM MES, 1 mM EDTA, 0.5 mM MgCl ₂ , and 2 mM CaCl ₂ (pH 6.4)
Buffer G	25 mM HEPES, 125 mM potassium acetate, 5 mM magnesium acetate (pH 7.1)
Buffer W	100 mM sodium phosphate, 50 mM NaCl, 1 mM MgCl ₂ , 1 mM EGTA (pH 7.4)
Cleavage buffer	50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT (pH 7.0)
Coomassie-staining solution	0.2 % Coomassie Brilliant Blue R 250, 10 % acetic acid, 47.5 % ethanol
Destain solution	10 % glacial acetic acid, 10 % ethanol
DMEM	Dulbeccos Modified Eagle's Medium (Gibco BRL, Karlsruhe), 10 % fetal calf serum, 1 % sodium pyruvate, 2 % penicillin / streptomycin
DNA sample buffer	0.025 % bromphenolblue, 0.025 % xylencyanol, 1.5 % ficoll 400 (final concentration)
EDTA washing buffer	0.5 M Tris-HCl, 1 % Triton X-100, 1 mM EDTA, 0.02% NaN ₃ (pH 7.0)
Ethanol/glycerin solution	10 % ethanol, 2 % glycerin
General actin buffer	5 mM Tris-HCl, 0.2 mM CaCl ₂ , 0.5 mM DTT, 0.2 mM ATP (pH 8.0)

GSH-elution buffer	50 mM Tris-HCl, 10 mM glutathione (pH 8.0)
Homogenization buffer	10 mM HEPES, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (pH 7.0)
Imidazol elution buffer	250 mM Imidazol, 300 mM NaCl, 50 mM NaH ₂ PO ₄ (pH 8.0)
Imidazol lysis buffer	10 mM Imidazol, 300 mM NaCl, 50 mM NaH ₂ PO ₄ , 1% Triton X-100 (pH 8.0)
Imidazol wash buffer	20 mM Imidazol, 300 mM NaCl, 50 mM NaH ₂ PO ₄ (pH 8.0)
Kinase buffer	10 mM Tris-HCl, 4 mM MgCl ₂ , 100 µM ATP (pH 7.2)
LB (Luria Bertani) agar	LB media + 1.5 % bacto agar
LB media	0.5 % yeast extract, 1 % trypton, 1 % NaCl (pH 7.5)
Lysis buffer (IP)	50 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100, 0.02% NaN ₃ (pH 7.4) + Complete Proteaseinhibitor Cocktail
Molecular weight marker	0.1 % phosphorylase b, 0.1 % β-galactosidase, 0.1 % bovine serum albumin (BSA), 0.1 ovalbumin, 0.1 % carboanhydrase; 5 mg/ml of each in SDS sample buffer
PBS	2.7 mM KCl, 1.9 mM KH ₂ PO ₄ , 8.2 mM Na ₂ HPO ₄ , 137 mM NaCl (pH 7.4)
Ponceau solution	0.2 % Ponceau S, 1 % acidic acid
Pulse-chase medium	Methionine-free RPMI medium, 20 mM HEPES, 10% dialyzed FCS (pH 7.4)
Running Buffer (Laemmli electrolyte buffer)	50 mM Tris, 370 mM glycine, 0.1 % SDS
SDS lysis buffer	100 µl 10% SDS, 250 µl 4 SDS sample buffer, 650 µl water
SDS sample buffer	25 mM Tris-HCl, 2.5 % SDS, 2.5 % β-mercaptoethanol, 12.5 % glycerin and staining indicator brominephenol blue (pH 8,0)
SL buffer	50 mM Tris-HCl, 5 mM EDTA (pH 8.5)
SOC media	0.5 % yeast extract, 2 % trypton, 10 mM NaCl, 2.5 mM

	KCl, 10 mM MgCl ₂ , 20 mM glucose (pH 7.0)
Stripping buffer	62.5 mM Tris-HCl, 100 mM β-mercaptoethanol, 2% (w/v) SDS (pH 6.7)
TB buffer	100 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1% Triton X-100 (pH 8.0)
TBE buffer	45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA
TBS buffer	20 mM Tris-HCl, 140 mM NaCl (pH 7.6)
Tissue sample buffer	2.5% SDS, 2.5% β-mercaptoethanol, 12.5% glycerol, 25 mM Tris-HCl, 0.5 mM EDTA, 0.0625% brominephenol blue, pH 8.0; herein with additional 2 % SDS
Transfer buffer	50 mM Tris, 39 mM glycine, 0.037 % SDS, 20 % methanol
Ψ-Broth media	LB media + 4 mM MgSO ₄ + 10 mM KCl

Table 2.13 Buffer and solutions.

2.1.10 Laboratory equipment

Tool	Type	Manufacturer
Agarose gel electrophoresis chamber	Sub-Cell GT Mini	BioRad (München, Germany)
Blotting apparatus		MHH- <i>Forschungswerkstätten</i> (Hannover, Germany)
Cell incubator	Hera cell RS 232	Heraeus (Hanau, Germany)
Centrifuge	J2-HS (rotor JA-10 und JA-20)	Beckman Coulter (Krefeld, Germany)

Chromatography Controller Pump Photometer Writer Fraction collector Columns	FPLC-system with LCC-501 Plus P-500 UV-1 REC112 FRAC-100 MonoQ Superdex 200 HR10/30	GE Healthcare (Piscataway, NJ, USA)
Developer machine	Optimax Typ TR	Protec (Oberstenfeld, Germany)
Electroporator	MicroPulser	BioRad (München, Germany)
Epifluorescence microscope	Axiovert 200 M	Carl Zeiss AG (Oberkochen, Germany)
Heating block	DRI-BLOCK DB-2A	Techne (Cambridge, England)
Homogenizer	Potter S	B. Braun (Melsungen, Germany)
Ion exchanger (pure water preparation)	MilliQ	Millipore (Bedford, MA, USA)
Laser scanning microscope	LSM 510 Meta	Carl Zeiss AG (Oberkochen, Germany)
Micro pipettes	Nichipet	Nichiryo (Tokio, Japan)
Microscope incubating chamber	POCmini	Carl Zeiss AG (Oberkochen, Germany)
pH-Meter	pH526	WTW (Weinheim, Germany)
Photometer	DU-640	Beckman Coulter (Krefeld, Germany)
Power supply	PowerPac 300 / 1000	BioRad (München, Germany)
Rotating device	3025	GFL (Burgwedel, Germany)
Scanner	Duoscan	Agfa (Mortsel, Belgium)
SDS-PAGE gel chamber	Mighty Small II	Hoefer (Freiburg, Germany)
Shaking apparatus	KS 15 A control	Edmund Bühler (Hechingen, Germany)
Shaking incubator	Certomat BS-1	B. Braun (Melsungen, Germany)
Sonificator	Sonifier 250 C	Branson (Danbury, CT, USA)

Sterile working bench	HeraSafe HS18	Heraeus (Hanau, Germany)
Syringe	702 N	Hamilton (Bonaduz, Suisse)
Table top centrifuge (I)	5417R (rotor A-8-11 and FA45-24-11)	Eppendorf (Hamburg, Germany)
Table top centrifuge (II)	Megafuge 1.0R	Heraeus (Osterode, Germany)
Thermocycler	UNO-Thermoblock	Biometra (Göttingen, Germany)
Ultracentrifuge	Optima TL100 (rotor TLA45 and 100.4), Optima LE 80 (rotor 70Ti)	Beckman Coulter (Krefeld, Germany)

Table 2.14 Laboratory equipment.

2.1.11 Consumables

Material	Product	Manufacturer
Affinity matrix	GSH-Sepharose	GE Healthcare (Piscataway, NJ, USA)
Affinity matrix	Ni-NTA-Agarose	Qiagen (Hilden, Germany)
Affinity matrix	SulfoLink [®] coupling Gel	Pierce (Rockford, IL, USA)
Autoradiography films	Biomax MR/MS	Eastman Kodak (New York, USA)
Blotting paper	3MM Chr	Whatman (Maidstone, England)
Cell culture dishes and plates	divers	Nunc (Wiesbaden, Germany)
Chemiluminescence film	Hyperfilm ECL	GE Healthcare (Piscataway, NJ, USA)
Coverslips	Round, Ø 12 mm	Menzel-Glaser (Braunschweig, Germany)
Desalting columns	PD-10, NAP-5, NAP-10	GE Healthcare (Piscataway, NJ, USA)
Dialysis tubes	Spectra/Por MWCO:6-	Spectrum (Rancho Dominguez,

	8.000	CA, USA)
Electroporation cuvettes	Gene Pulser Cuvette 0.2 cm	BioRad (München, Germany)
Falcon tubes	15 ml, 50 ml PP	Greiner (Frickenhausen, Germany)
Gaze		Rauscher (Pattensen, Germany)
Gel filtration matrix	Sepharose CL-4B	GE Healthcare (Piscataway, NJ, USA)
Micro reaction tubes	1.5 ml	Sarstedt (Nümbrecht, Germany)
Micro reaction tubes	0.5, 1.5 ml Safe Lock	Eppendorf (Hamburg, Germany)
Nitrocellulose membrane	Protran	Schleicher & Schuell (Dassel, Germany)
Object slides	Mattrand	Menzel-Glaser (Braunschweig, Germany)
Pasteur pipettes		Brand (Wertheim, Germany)
Pipette tips		Brand (Wertheim, Germany)
Sealing film	Parafilm N	American National Can (Chicago, IL, USA)
Ultracentrifuge tubes	divers	Beckman Coulter (Krefeld, Germany)

Table 2.15 Consumables

2.1.12 Software

Software	Manufacturer
AxioVision 4.2	Zeiss AG (Oberkochen, Germany)
C-Primer	Greg Bristol (University of California, Los Angeles, CA, USA)
DNASStar	DNASStar (Madison, WI, USA)
Excel 2002	Microsoft (Redmont, WA, USA)

Illustrator 8.0	Adobe Systems (San Jose, CA, USA)
LSM 510-Meta Software 3.2	Zeiss AG (Oberkochen, Germany)
NIH Image	National Institutes of Health Bethesda, MD, USA)
Photoshop 8.0	Adobe Systems (San Jose, CA, USA)
Word 2002	Microsoft (Redmont, WA, USA)

Table 2.16 Software.

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 RNA isolation and RT-PCR

Cultured cells were harvested by trypsinization and $\sim 5 \times 10^5$ cells were collected by centrifugation. The cell pellet was washed twice with PBS. For RNA isolation from cell pellet an *RNeasy Micro Kit* was used according to the manufacturer's instructions.

Frozen mouse organs from a 16 days old mouse were obtained from J. Mittag (Max-Planck-Institute for Experimental Endocrinology, Hannover, Germany). Organs were homogenized with a plastic Eppendorf tube homogenizer in ice-cold 100 mM HEPES (pH 7.0; 200 μ l / 100 mg tissue) and 10 μ l (~ 5 mg tissue) of the homogenates were processed for RNA isolation according to the manufacturer's instructions. The DNA concentration was determined photometrically at 260 nm.

For reverse transcription (RT) of RNA *RevertAidTM H Minus First Strand cDNA Synthesis Kit* was used according to the manufacturer's instructions. The reaction contained 930 ng of RNA, 10 μ g/ml hexameric random primer or 25 μ g/ml oligo(dT)₁₈ primer, 0.5 mM dNTP mix, 1 U Ribonuclease inhibitor and 10 U reverse transcriptase (RevertAidTM H Minus M-MuLV RT) in a final volume of 20 μ l.

5 μ l of the cDNA as template and oligonucleotides specific for the DNA sequence of CVAK104 were used for PCR reactions. In addition the reaction contained 1 x Taq reaction buffer, 200 μ M dNTPs, 50 μ M of forward and reverse primer and 1 U Taq Polymerase.

Polymerase chain reaction (PCR) was carried out according to the following protocol: Denaturation of the template DNA at 95 °C for 5 min; 30 cycles of denaturation for 1 min at 95 °C, primer annealing for 1 min at 58 °C and polymerization for 1 min at 72 °C. A final round of extension was run at 72 °C for 10 min. PCR products of CVAK104 encoding cDNA or GAPDH cDNA were detected by UV-illumination of ethidium bromide stained agarose gels.

2.2.1.2 PCR amplification of DNA fragments

PCR was used to amplify the full open reading frame and fragments of the CVAK104 cDNA. The template-specific oligonucleotides contained restriction digestion sites in addition at their 5' end to allow the digestion and finally the ligation of the PCR product into a vector that was digested with the same restriction enzymes.

The reaction conditions were as follows: 1x Turbo PfuTM Polymerase reaction buffer, 200 µM dNTPs, 500 µM of forward and reverse primers, 10 ng of template plasmid and 1 U of Turbo PfuTM Polymerase in a final volume of 50 µl. After an initial denaturation step for 2 min at 95°C in the thermocycler the PCR was performed according to the following protocol:

Step	Temperature	Time	Cycles
Denaturation	95°C	30 sec	8 x
Primer annealing	Tm(1)-8°C	30 sec	
Polymerization	72°C	1 min / 1000 bp	
Denaturation	95°C	30 sec	25 x
Primer annealing	Tm(2)-8°C	30 sec	
Polymerization	72°C	1 min / 1000 bp	
Final polymerization	72°C	10 min	

Table 2.17 PCR cycling parameters. Tm(1) is the melting temperature of the oligonucleotide-sequence which is complementary to the template DNA whereas Tm(2) is the melting temperature of the whole oligonucleotide including the flanking sequence with the restriction enzyme site.

PCR products were then loaded on a 1 % agarose gel in TBE buffer with 1.4 µg/ml ethidium bromide and separated until the bromophenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of the DNA fragments (usually after 45 min at 80V). DNA bands were excised from the gel under UV light (366 nm) and purified using the

QIAquick Gel Extraction Kit according to the manufacturer's protocol. The PCR product was digested with the appropriate restriction enzymes (3 U/ μ g DNA) for 4 h at 37°C and ligated in an expression vector as described in 2.2.1.3. All PCR products were checked by automated sequencing (MWG Biotech, Martinsried, Germany).

Product	Template	DNA-Oligodesoxynucleotides	
		Forward	Reverse
CVAK104	BC063798/pCMV-sport6	PAKfullforw	PAKfullrev
CVAK104-(699-929)	CVAK104/pGEX-6P-1	PAK697forw	PAK697rev

Table 2.18 Proteins and protein fragments, which have been generated by PCR. Sequences of corresponding oligodesoxynucleotides are listed in 2.1.6.

2.2.1.3 Subcloning of DNA fragments

For the cloning of DNA fragments from one plasmid into another 2-4 μ g of the donor-plasmid and 2 μ g of the target-vector, respectively, were digested with 4 U/ μ g DNA of the appropriate restriction endonucleases in the recommended buffer for one hour at the temperature optimum of the enzyme (in general, 37°C). The final reaction volume was at least 10 μ l. It was considered that the volume of the restriction endonucleases added was less than 1/10 the volume of the final reaction mixture, because glycerol in the enzyme storage buffer may interfere with the reaction. After DNA cleavage the restriction enzymes were heat inactivated according to the manufacturer's instructions.

For some experiments it was necessary to convert 5' overhanging ends generated by restriction enzymes into blunt ends. This was done with the Klenow fragment of the *E.coli* DNA polymerase I. The Klenow fragment incorporates nucleotides that are complementary to the single-stranded 5' extension of a cleaved DNA fragment. To this end the digested DNA was incubated with 2 U Klenow fragment and 2 mM dNTPs for 30 min at 37°C in the recommended buffer.

CIP (Calf Intestinal Alkaline Phosphatase) was used to remove 5' phosphates from linearized vectors and therefore to prevent self-ligation of the vector. The digested vector was incubated with 1 U CIP at 37°C in the same buffer that was used for the restriction endonuclease.

The purification of linearized vectors and digested DNA fragments was performed as described in 2.2.1.2.

For the ligation of vector and DNA insert 3 μ l of the linearized and gel extracted vector and 12 μ l of the isolated DNA fragment were incubated with 1 U T4 DNA Ligase in a total volume of 20 μ l for 12 hours at 16°C in ligase buffer. As a self-ligation control the same reaction was done with water instead of the DNA fragment. After incubation the ligase was heat inactivated for 10 min at 65°C. Finally, an aliquot of the ligation mixture was transformed into the *E.coli* strain DH5 α . When the ligation mix was designated for electro transformation 10 μ l of it were dialyzed against water for 1-2 hours.

2.2.1.4 Transformation of DNA into thermo- or electro-competent *Escherichia coli*

Plasmids or ligated products were introduced into thermo competent *E.coli* cells. Thermo competent *E.coli* were prepared as follows: cells were first grown in LB media at 37°C until the suspension had reached an OD_{600nm} ~ 0.5. Cells were then cooled on ice and recovered by centrifugation for 15 min at 3000 rpm and 4°C in a Heraeus 1.0R-centrifuge. Cells were first resuspended in 1/5 of the culture volume 100 mM MgCl₂ and after centrifugation in the same volume 100mM CaCl₂. After 30 min on ice cells were recovered again by centrifugation and finally resuspended in 1/20 of the original culture volume 100 mM CaCl₂ + 5 % glycerin. Competent cells were snap-frozen in liquid nitrogen and stored at -80°C.

Prior to transformation competent cells were thawed on ice. 90 μ l of the bacteria suspension were mixed with 10 μ l ligation mix or 1 μ l of purified plasmid DNA and incubated for 30 min on ice. After a heat shock of 3 min at 37°C cells were cooled on ice and mixed with 500 μ l Ψ -Broth media. For the expression of antibiotic resistance markers cultures were shaken for 30 min at 230 rpm and 37°C and plated on LB agar plates with the appropriate antibiotics for selection of the plasmid.

Critical ligations with PCR products generated with the *site-directed mutagenesis kit* were introduced into bacteria by electro transformation. To make *E.coli* competent for electro transformation cells were grown in LB media at 37°C until the suspension had reached an OD_{600nm} = 0.5. Cells were then cooled on ice for 20 min and washed 4 times by centrifugation for 15 min at 4000 rpm and 4°C in a Heraeus 1.0R-centrifuge and resuspending the pellet first in 1/1, then in 1/2, in 1/20 and finally in 1/250 of the original culture volume 10 % glycerin. Competent cells were snap-frozen in liquid nitrogen and stored at -80°C. For transformation

40 µl of the competent bacteria were thawed on ice and mixed with 10 µl dialyzed ligation mix or with a 20 µl aliquot of the site-directed mutagenesis experiment. The suspension was transferred to a 0.2 cm electroporation cuvette and subsequently transformed in the electroporator with the program EC2. Finally the suspension was mixed with 500 µl SOC media and processed as described above.

2.2.1.5 Generation of protein expression plasmids

For the expression of fusion proteins in bacteria or cultivated eukaryotic cell lines the open reading frame of CVAK104 and by PCR generated DNA fragments of CVAK104 were cloned in suitable expression vectors as described in 2.2.1.3 and introduced in cells. The cDNA of CVAK104 in pCMV-sport6 or pGEX-6P were used as donor plasmids. Table 2.19 gives an overview of the cloned protein expression plasmids.

To examine the success of generation of CVAK104 expression constructs, plasmid-DNA was purified from bacteria with the *QIAprep Spin Miniprep Kit* (Qiagen) and cleaved with restriction endonucleases which give a characteristic DNA fragment profile of the desired product. The DNA was digested with the appropriate enzymes as explained in 2.2.1.3 with the modification that ~200 ng plasmid DNA and 0.5 U of the enzymes were used in the experiment. Samples were separated on an agarose gel and analyzed on an UV-light table.

Fusion protein	Donor plasmid	Enzyme	Target plasmid	Enzyme
GST-CVAK104 full length	CVAK104/ pCMV-sport6	<i>EcoRI</i> + <i>NotI</i>	pGEX-6P-1	<i>EcoRI</i> + <i>NotI</i>
GST-CVAK104 (699-929)	CVAK104(1-929)/ pGEX-6P-1	<i>EcoRI</i> + <i>SalI</i>	pGEX-6P-2	<i>EcoRI</i> + <i>SalI</i>
EGFP-CVAK104 full length	CVAK104(1-929)/ pGEX-6P-1	<i>EcoRI</i> + <i>NotI</i> + Klenow	pEGFP-C3	<i>EcoRI</i> + <i>SmaI</i>
EGFP-CVAK104 (699-929)	CVAK104(699-929)/pGEX-6P-1	<i>EcoRI</i> + <i>SalI</i>	pEGFP-C2	<i>EcoRI</i> + <i>SalI</i>
FLAG-Auxilin full length	Auxilin/pGEX4T-1 ¹	<i>BamHI</i> + <i>SalI</i>	pCMV-TAG-2B	<i>BamHI</i> + <i>SalI</i>

FLAG-Auxilin (547-815)	Auxilin (547-813)/ pGEX4T-1 ¹	<i>Bam</i> HI	pCMVTAG- 2B	<i>Bam</i> HI
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Table 2.19 Generated protein expression plasmids.

¹ The auxilin constructs in pGEX4T-1 were provided by Urte Scheele and are described elsewhere (Scheele, 2003).

2.2.1.6 Site-directed mutagenesis

For the generation of C-Terminal deletion mutants of CVAK104, STOP-codons were introduced in the open reading frame of CVAK104 cDNA by site-directed mutagenesis. The mutagenesis was performed with *Quik ChangeTM Site-Directed Mutagenesis* kit (Stratagene) according to the manufacturer's protocol. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by

PfuTurbo DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5'-Gm6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA.⁶ DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue competent cells.

To simplify the identification of positive clones the oligonucleotide primers were designed in that way that restriction endonuclease sites were introduced in the mutated DNA sequence. But it should be considered that the total number of mismatch base pairs of primer and template DNA was not higher than 5. The presence of new restriction endonuclease sites in the mutated vector was analyzed by analytical digestion with the appropriate enzymes as described in 2.2.1.5. Successful mutations were confirmed by automated sequencing (MWG Biotech, Martinsried, Germany).

Mutant	Original plasmid	Oligonucleotid pair
CVAK104-375STOP	CVAK104-(1-929)/pGEX-6P-1	PAK375STOP forw/rev
CVAK104-699STOP	CVAK104-(1-929)/pGEX-6P-1	PAK697STOP forw/rev

Table 2.20 Overview of the CVAK104 mutants generated by site-directed mutagenesis

2.2.2 Protein analysis tools

2.2.2.1 SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis)

SDS-PAGE was used to separate proteins from a mixture according to their molecular weight and to detect them either by Coomassie staining or by Western blot analysis. The protein sample was denaturated with 1/3 volume 4 x SDS-sample buffer for 2 min at 95 °C. 10-20 µl were loaded on a 0.8 mm (6.5 cm x 9 cm) SDS polyacrylamide gel with a linear 9-19 % acrylamide- and 0.09 – 0.38% N,N-methylenbisacrylamide gradient and a migration distance of 5.5 cm. 10 µl of a molecular weight marker with 5 proteins of sizes between 29 and 116 kDa were loaded on another lane. The polyacrylamide gel electrophoresis was performed in a vertical slab gel unit in running buffer (Laemmli buffer) at 80 V for 20 min and at 200 V for 55 min under constant cold water cooling.

For the staining of protein bands with Coomassie the gel was incubated for 60 min in Coomassie-staining solution and then washed in destain-solution until the bands were visible.

2.2.2.2 Western blot analysis

Western blotting allows investigators to determine, with a specific primary antibody, the relative amounts of the protein present in different samples. The semi-dry method was used to transfer the separated proteins from the gel onto a nitrocellulose membrane. To this end the gel and the nitrocellulose membrane were embed between six Whatman blotting papers on each site which were saturated in transfer buffer before. The gel was blotted in the semi-dry blotter for 75 min at 0.8 mA/cm².

The nitrocellulose membrane was stained for 10 sec with Ponceau S staining solution. After marking of lanes and molecular weight standard bands the membrane was blocked in blocking solution for 45 min at room temperature. Primary monoclonal or polyclonal antibodies were applied in PBS; 3 % BSA; 0.02 % sodium azide and incubated shaking for 1 h at room temperature. The blotting membrane was washed with PBS and incubated with secondary horse radish peroxidase conjugated anti-mouse, anti-rabbit or anti-goat 1:2000 antibodies in blocking solution for 1 h at room temperature. After washing with PBS the blot was developed with ECL detection reagent. The signal was detected by exposure of the blot for 1 min to 5 min to Hyperfilm ECL films.

2.2.2.3 Densitometrically analysis

Coomassie-stained SDS-PAGE polyacrylamide gels and Western blot ECL films were scanned with an Agfa Duoscan Instrument, digitized with Adobe Photoshop and converted into TIFF files. The relative ratio of bands was determined densitometrically using the NIH image 1.02 software.

2.2.3 Protein expression and purification

2.2.3.1 Expression of fusion proteins in the *E.coli* strain BL21

For protein expression in *E. coli* 10 ml of an overnight culture of BL21 (DE3) cells were inoculated into 1 l LB medium containing 50 µg /ml ampicillin. The cells were cultured at 37 °C shaking at 200 rpm until an OD_{600 nm} of ~0.5 was reached. Protein expression was induced by adding 0.5 mM isopropylthiogalactosid (IPTG). The cells were incubated for another 3 h at room temperature and 250 rpm. Bacteria were collected by centrifugation for 15 min at 6000 rpm (6.400 x g_{max}) and 4 °C in a Beckman JA-14 rotor, resuspended in ice-cold PBS and centrifuged again. Cells were washed again with ice-cold PBS, transferred in a 50 ml reaction tube and pelleted by centrifugation for 20 min at 4000 rpm (3.300 x g_{max}) and 4 °C in a Heraeus 1.0 R centrifuge. The bacteria pellet was shock-frozen in a mixture of ethanol and dry ice and stored at -80 °C until further use.

2.2.3.2 Purification of fusion proteins from the *E.coli* strain BL21

For the purification of GST fusion proteins the bacteria pellet was thawed and resuspended on ice in 10 ml ice-cold PBS containing 1 % Triton X-100 and protease inhibitors (Complete Proteaseinhibitor Cocktail). The cell suspension was sonificated with a Branson Sonifier 250 at 10 % power for 10 sec for at least 4 times or as long as the suspension is still viscous. Between the sonification steps the lysates were cooled on ice for at least one minute. After sonification bacterial lysates were clarified by ultracentrifugation for 15 min at 40.000 rpm ($164.700 \times g_{\max}$) and 4 °C in a Type 70 Ti rotor. The supernatant was transferred into a 15 ml Falcon tube containing 2 ml of PBS-washed Glutathione (GSH) Sepharose. The beads and the supernatant were incubated for 1 h at 4 °C on a wheel and GST fusion proteins bound to GSH-Sepharose were isolated by centrifugation at 4000 rpm ($3300 \times g_{\max}$) and 4 °C in a Heraeus 1.0 R centrifuge. Beads were washed and centrifuged three times with 10 ml ice-cold PBS. In the second washing step the suspension was underlayered with 2 ml 10 % sucrose in PBS. Fusion proteins produced from pGEX-6P plasmids can be cleaved by PrescissionTM protease between the GST moiety and the cloned fusion partner. To obtain the recombinantly expressed protein of interest without GST the fusion protein was cleaved with PrescissionTM protease directly on the Sepharose beads in cleavage buffer according to the manufacturer's instructions. Alternatively the GST-fusion protein was eluted drop by drop with GSH-elution buffer after transferring the beads into an empty column. The eluate was collected in 0.5 ml fractions of which 1 µl was spotted on a nitrocellulose membrane to check the protein concentration with Ponceau S. Fractions rich in protein were pooled and further purified by gel filtration or transferred into the appropriate buffer using a desalting column (NAP-5 and NAP-10).

The purification of His₆-fusion proteins (expressed on the basis of pQE plasmids) was performed analog to that of GST-fusion proteins. But instead of GSH-Sepharose Ni-NTA-Agarose and instead of PBS imidazol lysis and imidazol wash buffer was used. His₆-fusion proteins were eluted with Imidazol elution buffer.

For some experiments the recombinant proteins were further purified by size exclusion chromatography on a Superdex 200HR 10/30 column, which was connected to an FPLC-System. The column was equilibrated in buffer G.

2.2.4 Preparation of tissues

2.2.4.1 Preparation of rat and pig brain cytosol

To obtain cytosol fresh rat or pig brains were homogenized in homogenization buffer (1 ml of buffer/g of tissue) using a Potter S homogenizer. The homogenate was filtered through a nylon gaze and centrifuged for 45 min at 90.000 rpm in a Beckman TLA 100.1 rotor. The pellet was discarded, and the supernatant was centrifuged for another 45 min as described above. The clarified cytosol was dialyzed against buffer G overnight. When not used immediately, the cytosol was shock-frozen and stored at -80 °C. Frozen cytosol was rapidly thawed in a water bath at 37 °C and then clarified by ultracentrifugation.

2.2.4.2 Preparation of tissue homogenates from mouse

To determine the amount of CVAK104 expressed in different tissues, aliquots of mouse organs were homogenized in six volumes (6 μ l of buffer/mg of tissue) of prewarmed 95°C tissue sample buffer within 30 min after slaughter. The homogenate was first treated with a plastic Eppendorf tube homogenizer and then with an Ultra Turrax. After heating for 5 min at 95 °C samples corresponding to 1.4 mg of tissue were subjected to SDS-PAGE and Western blotting. Blots were reacted with anti-CVAK104 and γ -adaplin (AP1), respectively, followed by horseradish peroxidase-conjugated secondary antibody. The blots were developed using enhanced chemiluminescence.

2.2.4.3 Preparation of clathrin from CCV

Clathrin was extracted from pig brain clathrin-coated vesicles using 0.5 M Tris-HCl (pH 7.0). To this end 1.25 ml CCV suspension was mixed with 250 μ l 3 M Tris-HCl (pH 7.0), incubated on ice for 10 min and centrifuged for 20 min at 70.000 rpm (266.000 x g) and 4°C in a TLA110.4 rotor. The pellet, which contained largely the vesicle membranes, was again extracted with 0.5 M Tris-HCl (pH 7.0) and 1 mM EDTA. After a second centrifugation, the pooled supernatants were loaded onto a preparative Superose 6 gel filtration column (column

size 2.2 x 52 cm) equilibrated in 0.5 M Tris-HCl (pH 7.0) at 4 °C. The eluted 1 ml fractions were analyzed by SDS-PAGE and Coomassie-staining of the gel.

Clathrin fractions without assembly and accessory proteins were concentrated overnight by ammonium sulfate precipitation in the presence of 2 mM EDTA (pH 7.0) on ice and pelleted at 4°C and 20.000 rpm (72.000 x g) in a SW-28 swinging bucket rotor for 20 min. To remove any residual ammonium sulfate, the purified clathrin was resuspended in 700 µl 0.5 M Tris-HCl (pH 7.0) and dialyzed against the same buffer. Assembly into cages was induced by dialysis against buffer A. Clathrin cages were pelleted by centrifugation at 45000 rpm (125.900 x g) for 20 min in a TLA45 rotor. When not used immediately, cages were resuspended in 300 µl buffer A and stored on ice. The separation of clathrin heavy and light chains was performed essentially as described (Winkler and Stanley, 1983)

2.2.5 Affinity purification of CVAK104-specific peptide antibodies

Antisera against three CVAK104 peptides (762-TDNTKRNLTNGLNA-775, 855-QLSQQKPNQWLNQFV-869 and 915-TTMTNSSSASNDLKDLFG-929) were generated by BioGenes (Berlin, Germany) by immunization of rabbits with all 3 different peptides together. The antibodies were purified from the antisera by affinity purification with the respective peptides. The peptides contained a single terminal cysteine which could be coupled with its sulfhydryl group to iodoacetyl groups on SulfoLink[®] coupling Gel. The coupling of peptides was performed essentially according to the manufacturer's protocol. Briefly, 2 mg peptides in SL buffer were incubated with 1 ml SulfoLink[®] Gel (equilibrated in SL buffer) for 45 min at room temperature (the first 15 min on a slow rotating device). After washing 3 x in SL buffer the non-specific binding sites of the matrix were blocked by incubation with 1 ml of 50 mM cysteine in SL buffer for 15 min under rotation and for 30 min without rotation at room temperature. Beads were washed with 16 column volumes of 1 M NaCl and with 16 column volumes of 0.05% sodium azide.

For the affinity purification 2 ml CVAK104 antiserum mixed with 2 ml PBS were centrifuged for 5 min at 14.000 rpm in a FA45-24-11 rotor and then incubated with CVAK104 peptides immobilized on 1 ml SulfoLink[®] Gel and equilibrated in PBS (see above) for 1 h at room temperature. After washing the column with ice-cold PBS the antibodies were eluted with 500 µl 0.1 M glycine pH 2.5 per fraction into Eppendorf tubes which already

contained 50 μ l of 1.5 M Tris-HCl pH 8.8. Protein-rich fractions were pooled, supplemented with 0.1 mg/ml BSA and dialyzed against PBS overnight.

2.2.6 Binding experiments

2.2.6.1 Pulldown and competition experiments with GST fusion proteins

GST-CVAK104 fusion proteins or GST-N-terminal domain of clathrin (GST-TD) immobilized on GSH-Sepharose beads (GE Healthcare) (GST-CVAK104: 38 μ g coupled to 30 μ l GSH-Sepharose beads; GST-TD: 30 μ g on 5 μ l GSH beads mixed with 15 μ l Sepharose CL-4B beads) were incubated with 200 μ l rat or pig brain cytosol (3.7 mg/ml in buffer G) for 1 h on ice. GST immobilized on GSH-Sepharose served as control. The beads were pelleted by centrifugation for 1 min at 10.000 rpm and 4°C in an A-8–11 swinging bucket rotor and resuspended in 1 ml of buffer G and pelleted again. The last step was repeated once. For the third wash the resuspended beads were underlayered with 200 μ l of 10% sucrose in buffer G. After a final washing step aliquots of the supernatant and pellet fractions were analyzed by SDS-PAGE and immunoblotting. For pulldown assays with GST fusion proteins and purified proteins a slightly different protocol was used including the following modifications: the incubation buffer was supplemented with 0.05% BSA and the number of washing steps was reduced to two. GST-CVAK104-, GST-TD- (loading of beads see above) or GST- α -appendage-beads (GST- α -app.; 30 μ g coupled to 5 μ l GSH beads mixed with 15 μ l Sepharose CL-4B beads) were incubated with 5 μ g of isolated adaptor proteins or 9 μ g of recombinant CVAK104 and CVAK104-(699-929), respectively, in buffer G.

For competition experiments a constant amount of rat brain cytosol or recombinant proteins was added to immobilized GST fusion proteins together with increasing concentrations of recombinant proteins or protein fragments that also interact with the GST fusion protein.

2.2.6.2 Cage binding assays

For the binding of recombinant CVAK104 fragments to reconstituted clathrin cages the appropriate amount of cages in buffer A was pelleted by ultracentrifugation as described in 2.2.4.3. The pellet was resuspended in buffer G. To determine the concentration of clathrin an

aliquot of the resuspended cages was diluted 1/10 in 0.5 M Tris-HCl (pH 7.0) and analyzed photometrically. The indicated amounts of clathrin cages were incubated for 1 h on ice with purified recombinant CVAK104 fragments in a total volume of 100 μ l and then centrifuged at $90,000 \times g$ for 20 min in a TLA45 rotor. The pellet was resuspended in 100 μ l 0.5 M Tris-HCl (pH 7.0) and SDS sample buffer and vortexed for 10 min to dissociate the clathrin cages. Resulting supernatant and pellet fractions were analyzed by SDS-PAGE. In this and all subsequent experiments the pellets were resuspended in a final volume that was identical to that of the supernatants.

2.2.6.3 ATP binding assay

The study of the binding of the nucleotide ATP on CVAK104 has been carried out by using the Hummel and Dreyer method (Hummel and Dreyer, 1962). In this gel filtration method, both the gel filtration buffer and the sample had the same ligand concentration, but only the sample contained protein. Elution of a protein through such a column caused an increase in the concentration of ligand where the protein eluted, followed by a trough of ligand concentration representing ligand that had been removed in the binding.

In the experiment 6×10^{-9} mol of the kinase-homology domain of CVAK104 and of GAK as GST fusion proteins (GST-CVAK104-(1-375), GST-KGAK) or GST alone were incubated in ATP binding buffer with 0.053 μ Ci [α^{32}]-ATP in a total volume of 500 μ l for 15 min at room temperature. The sample was then loaded onto a NAP-5 desalting column, which was equilibrated with ATP binding buffer (+ 0.106 μ Ci [α^{32}]-ATP / ml buffer) before. Fractions were eluted with 200 μ l of the same buffer. To determine the eluted radioactivity in each fraction 10 μ l aliquots were spotted onto a piece of filter paper and counts were measured in a Cerenkov counter. The protein content in the eluted fractions was verified by dot-blot analysis. To this end 1 μ l of each fraction was dropped onto a nitrocellulose membrane and the membrane was processed as described in 2.2.2.2 with a primary anti-GST and a secondary horse radish peroxidase conjugated anti-goat antibody. The relative ratio of bands in the ECL film was determined densitometrically using the NIH image 1.02 software.

2.2.6.4 F-actin co-sedimentation assay

F-actin co-sedimentation assays were performed with the *non-muscle actin-binding protein spin-down biochem kit* corresponding to the manufacturer's recommendations. For this, 40 µg G-actin were resuspended in 50 µl general actin buffer, chilled on ice for 30 min and centrifuged at 4°C and $90,000 \times g$ for 20 min in a TLA45 rotor to remove aggregated G-actin. Polymerization to F-actin was induced by adding 5 µl of 10 x actin polymerization buffer to the supernatant and incubation for 1 h at room temperature (RT). Preceding the experiment recombinant GST-CVAK104 or GST alone in general actin buffer was adjusted to 0.2 % Triton X-100 and 0.2 % BSA. The solution was clarified by centrifugation at $90,000 \times g$ for 60 min in a TLA45 rotor. For the sedimentation assay an aliquot of the supernatant was incubated with 0.4 mg/ml F-actin for 30 min at RT. After centrifugation for 1 h at RT and $90,000 \times g$ in a TLA45 rotor, supernatant and pellet fraction fractions were separated and subjected to SDS-PAGE and Western blot analysis.

2.2.7 Kinase-Assays

2.2.7.1 *In vivo* phosphorylation of auxilin

10 cm dishes with HeLa cells were transfected with FLAG-auxilin (either full length auxilin or a fragment comprising the amino acids 547-814) as described in 2.2.8.1. 24 h after transfection cells were washed in phosphate-free DMEM (+ 5% dialyzed FCS) and then incubated with 3 ml/dish of the same medium for 1 h at 37°C and CO₂. The medium was then supplemented with 0.5 mCi [³²P]-orthophosphate and cells were incubated for 3 h under the same conditions. Cells were then lysed and used for immunoprecipitation of FLAG-auxilin constructs with an anti-FLAG antibody as described in 2.2.8.5 with the modification that the lysis buffer contained 2mM sodium pyrophosphate and a phosphatase inhibitor cocktail in addition.

2.2.7.2 *In vitro* kinase assays with recombinant and endogenous CVAK104

For the analysis of the catalytic activity of the kinase-related protein CVAK104, kinase assays with recombinantly expressed or endogenous CVAK104 from eukaryotic cells and [γ^{32}]-labeled ATP as co-substrate were performed. Purified BL21-expressed CVAK104 with or without GST was incubated in kinase buffer with 3 μ Ci [γ^{32}]-ATP in the presence or absence of 50 μ g/ml poly-L-lysine (30–70 kDa) with various potential phosphorylation-substrates like recombinant auxilin fragments, Tris-extract (TE) of CCVs and isolated adaptor proteins for 45 min at room temperature. The kinase reaction was stopped by the addition of SDS sample buffer and boiling for 2 min at 100 °C. Proteins were separated by SDS-PAGE. After detection of protein bands by Coomassie-staining the gel was incubated for 15 min in ethanol/glycerin solution and then dried in a heated vacuum-gel dryer for 1.5 h at 65°C. Incorporation of radiolabeled phosphate in proteins was detected by autoradiography of the dried gel at -80 °C using a Kodak Biomax MS Film.

To determine the catalytic activity of endogenous CVAK104 the protein was immunoprecipitated from HeLa cell lysates as described in 2.2.8.5. Subsequently the protein A Sepharose with the immune complexes was washed in kinase buffer and used for kinase-assays essentially as described above.

2.2.8 Cell biological methods

2.2.8.1 Transient transfection of mammalian cells

The introduction of foreign DNA or RNA into cultured cells is called transfection. In this work this method was used to introduce expression plasmids or siRNA oligonucleotides into HeLa or COS-7 cells. The siRNAs were transfected with the aim to specifically down regulate the expression of certain proteins.

Transient transfection of cultured cells was carried out using the Lipofectamine 2000 reagent according to the manufacturer's instructions. In this lipid- cation based transfection method the positive charged groups of the lipid molecules interact with the negative charged backbone of the DNA or RNA so that the nucleic acid is surrounded by a hydrophobic shell. This complex is able to fuse with the plasma membrane and to release the nucleic acid into the cytosol.

24 h before transfection with plasmids the cells were washed with PBS, trypsin-digested and counted. In general $0.5\text{--}2 \times 10^5$ cells in 500 μl of growth medium were plated so that cells will be 90-95% confluent at the time of transfection. If transfected cells should be analyzed by fluorescence microscopy 3×10^4 (HeLa) or 1.8×10^4 (COS-7) cells were seeded in 500 μl fresh growth medium on sterile glass coverslips coated with 10 μg / ml poly-L-lysine which were placed in 24-well plates. 24 h before transfection with siRNA the cells were resuspended at a density of $0.5\text{--}0.8 \times 10^5$ cells/ml.

For transfection of a 24-well plate vessel with confluent cells 0.8 μg of plasmid DNA or 60 pmol of RNA duplex as well as 1 μl of Lipofectamine 2000 reagent were diluted in 50 μl Optimem each and incubated for 5 min at room temperature. The DNA/RNA and the Lipofectamine 2000 dilutions were mixed and incubated for another 20 min at room temperature. 0.5 ml growth medium containing 10% fetal calf serum but no pyruvate or antibiotics were added to the cells and overlaid with the transfection mixture. The transfection was carried out in a 37 °C cell culture incubator with 5 % CO_2 for 4 h. The transfection mixture was replaced by complete cell culture medium and the cells were assayed 24 h after the transfection. The extent of gene silencing was assessed by Western blotting of cell lysates. To control for any differences in cell numbers the signal of the silenced protein was related to that of actin or tubulin. If cells transfected with CVAK104 or clathrin siRNA were destined for immunofluorescence microscopy cells were trypsin-digested after 24 h and mixed with

approximately the same number of control cells and then replated on glass coverslips. This allowed viewing and photographing control and knockdown cells next to each other within the same field. Immunofluorescence analysis was performed 72 h after transfection of RNA duplexes.

2.2.8.2 Immunofluorescence analysis of fixed cells

To analyze the subcellular distribution of CVAK104 cultured mammalian cells non-transfected or transfected with various expression plasmids were incubated with fluorescently labeled antibodies and investigated by immunofluorescence microscopy.

When not mentioned otherwise all steps were done by room temperature. Cells grown on poly-L-lysine coated glass coverslips were fixed for 10 min in 4% paraformaldehyde dissolved in PBS. After fixation, the cells were washed three times with PBS, incubated for 5 min with TBS and then permeabilized with 0.1% Triton X-100 in TBS (or 0.05% saponin in PBS for detection of endogenous CVAK104) for 5 min. Antibodies were diluted in PBS containing 3% BSA (and 0.05% saponin if cells were permeabilized with saponin). Cells were incubated for 1 h with primary antibodies and 45 min with secondary antibodies at 37°C. Washing after antibody incubations was performed by dipping coverslips into PBS 10 times. Finally, cells were embedded in Prolong Antifade. The effect of nocodazole or brefeldin A on the localization of CVAK104 was tested by incubating HeLa cells with 33 μ M nocodazole for 3 h or 2 μ g/ml BFA for 4 min at 37°C prior to fixation. Labeled cells were viewed with an Axiovert 200 M epi-fluorescence microscope and images were recorded with an AxioCamMRm digital camera controlled by AxioVision software. Confocal light microscopy was performed with a LSM 510 Meta System. Final figures were arranged with Adobe Photoshop version 8.0 and Adobe Illustrator version 11.0. To assay the uptake of transferrin by immuno-fluorescence, transfected cells were serum starved for 30 min in Leibowitz's L15 medium containing 0.1% BSA at 37°C and then incubated with 2.5 μ g/ml Texas-Red labeled transferrin in the same medium for 1 h on ice. Unbound ligand was removed by quick washes with cold serum-free medium, and bound ligand was allowed to be internalized upon incubation in 0.1% BSA/Leibowitz's L15 medium for different times at 37°C. For immunofluorescence the cells were processed as described above.

2.2.8.3 Live-cell immunofluorescence microscopy

8×10^4 COS-7 cells were seeded on 30-mm glass coverslips and transfected the following day with 4 μ g DNA and 10 μ l Lipofectamine 2000 as described in 2.2.8.1. Between 20 and 24 h post transfection, coverslips were placed into a POCmini incubating chamber, overlaid with 1 ml of Leibowitz's L15 medium plus 10 % FCS and transferred to the heating stage of the microscope, which was fitted with an acrylic incubating chamber to maintain a constant 37°C imaging environment. The same setup was used to follow the uptake of Texas Red-labeled transferrin. Briefly, serum-starved cells were pre-incubated for 1 h at 4°C with transferrin. Unbound ligand was removed by quick washes with pre-warmed serum-free medium before cells were overlaid with Leibowitz's L15 medium containing 0.1% BSA and transferred to the microscope for imaging at 37°C.

2.2.8.4 Cell fractionation

A cell fractionation assay was performed to determine the fraction of endogenous CVAK104, which is associated with the plasma membrane and internal membranes, respectively, relative to the cytosolic fraction of CVAK104.

Clathrin siRNA transfected HeLaSS6 cells or control cells were grown in 2-ml dishes for 72 h and then washed first with PBS and then with ice-cold buffer W containing Complete Mini-protease inhibitor mixture. The cells were overlaid with 0.3 ml ice-cold buffer W, freezed by putting the dishes on a dry ice block and then thawed on ice. The procedure of freezing and thawing was repeated once and lysed cells were collected by scraping. The cell lysis was done without detergents like Triton X-100 to prevent a potential influence of the detergent on the interaction of peripheral membrane proteins with membrane lipids. The lysed cell suspension was centrifuged for 45 min at 90,000 $\times g$ in a TLA45 rotor at 4 °C. The pellet was homogenized and resuspended in 0.3 ml buffer W by pipetting the suspension up and down for at least 50 times with a 100 μ l pipette and a yellow tip. Both supernatant and pellet fractions were denatured in SDS sample buffer and subjected to SDS-PAGE and Western blotting. The relative ratio of bands was determined densitometrically using the NIH image 1.02 software.

2.2.8.5 Immunoprecipitation

To examine the catalytic activity of mammalian CVAK104 in an *in vitro* kinase assay the endogenous protein was immunoprecipitated from HeLaSS6 cell lysates.

Cells in an 80-90% confluent 10 cm dish were washed twice with ice-cold PBS and then scraped with a rubber wiper into 800 µl ice-cold lysis buffer. After transferring into an Eppendorf tube the cell suspension was incubated for > 1h on ice to ensure complete lysis. Nuclei were removed from the lysates by centrifugation for 10 min at 1000 x g at 4°C in a FA45-24-11 rotor. Filtered BSA was added at a final concentration of 1% to the supernatant. The lysates was then clarified by ultracentrifugation for 45 min at 4°C and 40.000 rpm in TLA45 rotor. A preclear of the clarified lysates was performed by incubating the lysates for 45 min at 4°C on a slow rotating device with 15 µl protein A Sepharose beads, which have been washed three times in lysis buffer before. The beads are then pelleted by centrifugation (>1000 x g, 10 min, 4°C) and the supernatant was transferred to a new Eppendorf tube. For the immunoprecipitation 5-10 µg purified antibody was added to the precleared supernatant. After inverting the tube 3-4 times the sample is incubated on ice for 1-2 h without any further agitation. Precipitation of the immunocomplexes was carried out by adding 30 µl of a 1+1 suspension of protein A Sepharose beads in lysis buffer to the sample and rotating at 4°C for 45 min. The beads were then pelleted by centrifugation for 4 min at 1200 rpm (330 x g) and 4°C in a FA45-24-11 rotor. The washing steps with pre-chilled washing buffers supplemented with protease inhibitors were as follows: 2 x with BSA washing buffer, 1 x with H₂O_{dd}, 2 x with EDTA washing buffer. For washing 1 ml of the respective wash buffer was applied to the beads and the tube was inverted several times to ensure resuspension of the pellet. After spinning the supernatant was removed by aspiration with an injection needle. Before using the immunoprecipitated protein for kinase assays, the beads were washed three times with kinase buffer as described above.

2.2.8.6 *In vitro* Recruitment of CVAK104 and AP1

For recruitment experiments PtK cells growing on glass coverslips were permeabilized with 40 µg/ml digitonin in buffer G + 1 mM DTT for 5 min on ice and then with 4 µg/ml digitonin in the same buffer for 5 min at room temperature. Permeabilized cells were then incubated with pig brain cytosol either with or without 170 µM GTPγS and/or an ATP-

regenerating system (2 mM ATP, 5 mM creatine phosphate and 5 units/ml creatine kinase) in a total volume of 420 μ l of buffer G supplemented with 4 μ g/ml digitonin and 1 mM dithiothreitol (DTT) for 30 min at 37°C. Cells were washed three times with buffer G and then processed for immunofluorescence analysis.

2.2.8.7 Externalization assay with [¹²⁵I]-labeled transferrin

To examine the rate of transferrin-externalization in CVAK104-depleted HeLa cells, cells in 24-well plates, either transfected with CVAK104-specific siRNAs (oligo I or II) or transfected with LipofectamineTM alone, were starved for one hour in DMEM/0.1% BSA (w/v). Next, cells were incubated with 1 μ g/ml [¹²⁵I]-labeled transferrin (90 ng [¹²⁵I] transferrin (with 0.9 μ Ci/ μ g) and 910 ng “cold” transferrin / μ g total transferrin) in DMEM/0.1% BSA for 30 min at 37°C. Cells were washed 2x with cold PBS and 1x with PBS at room temperature and then incubated with DMEM/0.1% BSA supplemented with 100 μ M desferroxamine at 37°C for the indicated time periods to allow externalization of transferrin. The iron chelator desferroxamine was included in the efflux incubation to block potential iron loading of effluxed apotransferrin and reuptake of transferrin. After externalization cells were washed on ice as follows: 2x with ice-cold PBS, 2x with ice-cold 0.2 N acetic acid, 0.5 N NaCl (pH ~2) for 2 min and 2x with ice-cold PBS. Cells were lysed with prewarmed 95°C 0.1 M Tris-HCl pH 8/ 1% SDS (w/v) (100 μ l/well) and analyzed for associated radioactivity. The measured activity was normalized to the protein concentration of the corresponding sample. To this end a commercial BCA protein assay was used according to manufacturer's recommendations.

2.2.8.8 Cathepsin D sorting assay

To study the role of CVAK104 in the sorting of lysosomal enzymes, metabolic labeling and subsequent immunoprecipitation of cell-associated as well as secreted cathepsin D was performed in COS-7 cells that had been depleted in CVAK104 or AP1.

24 h before transfection 1.7×10^5 COS-7 cells were plated onto a 6 cm dish and transfected the following day as described in 2.2.8.1 with 0.6 nmol of RNA duplexes per dish targeting the mRNA sequence of CVAK104, γ -adaptin (AP1) or GFP. 72 h post transfection cells were washed twice with 37°C warm PBS, placed in 1 ml methionine-free pulse-chase medium and labeled for 2 h at 37 °C with 0.5 mCi/ml Redivue I-(³⁵S)methionine. Excess of unlabeled methionine (10 mM final concentration) was added to initiate a 4 h chase at 37 °C. Cells were then placed on ice. 1100 μ l of medium was transferred to an Eppendorf tube, mixed with 275 μ l 5x TB buffer (supplemented with 2.5% BSA) and centrifuged for 10 min at 4°C and 16.000 rpm in a FA45-24-11 rotor. For the immunoprecipitation of secreted cathepsin D, the supernatant was incubated overnight at 4°C on a slow rotating device with 2.5 μ l of a polyclonal anti-cathepsin D antiserum, which was coupled to 60 μ l protein A Sepharose beads before. In parallel cells were harvested and lysed with 2 x 500 μ l TB buffer (supplemented with 0.5% BSA) and processed as described above for the 1100 μ l medium aliquot. Next day beads with immunoprecipitated cathepsin D from media and cell lysates, respectively, were washed as follows: 1 x with TB buffer, 4 x with TB buffer containing 1 M NaCl, 1 x with TB Buffer, 1 x with TB buffer containing 1 M NaCl and 2 x with PBS. All washing steps were done on ice with 1 ml ice-cold buffers in each washing step. Immunoprecipitated proteins were eluted with 2 x 25 μ l 2 x non-reducing sample buffer (SDS sample buffer without β -mercaptoethanol) and separated by SDS-PAGE under non-reducing conditions on 11% gels. After Coomassie-staining gels were treated with NAMP 100 corresponding to the manufacturer's recommendations, dried and exposed to Kodak Biomax MR Film at -80 °C.

3. Results

3.1 *In vivo* phosphorylation of auxilin

In addition to the coat-protein clathrin, adaptors and accessory proteins there are several protein kinases known to be associated with purified clathrin-coated vesicles (CCVs). The CCV-associated kinases GAK, CK2 and AAK1 phosphorylate endocytic proteins *in vitro* and therefore are candidates for regulators of the clathrin-dependent trafficking machinery (Korolchuk and Banting, 2003). During my diploma thesis we discovered that recombinant auxilin fragments purified from *E.coli* are phosphorylated *in vitro* by kinase-activities that co-purify with CCVs (Düwel, 2003). This prompted us to test if auxilin is also phosphorylated *in situ*. To this end HeLa cells were transfected with two FLAG-tagged auxilin fragments: full-length auxilin (auxilin 1-910) and a fragment that exhibits several clathrin- as well as AP2- interaction motifs such as two DLL and two DPF (auxilin 547-813) (Scheele *et al.*, 2003). After incubation of the transfected cells with radioactive phosphate [^{32}P], auxilin fragments were immunoprecipitated with an anti-FLAG antibody. The incorporation of [^{32}P]-labeled phosphate was analyzed by SDS-PAGE, Western blotting and autoradiography. As illustrated in figure 3.1 the experiment revealed that both, full-length auxilin and auxilin (547-813) are subjected to phosphorylation *in situ*.

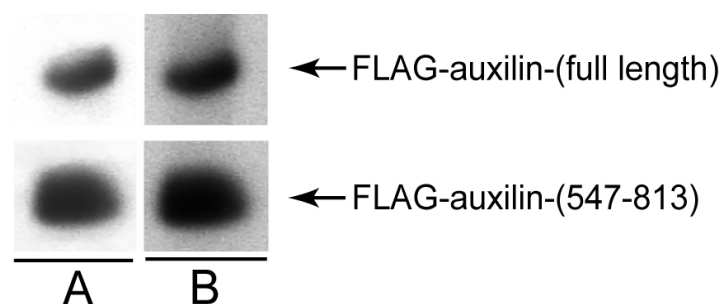


Figure 3.1: *In vivo* phosphorylation of auxilin. HeLa cells either transfected with FLAG-auxilin-(full length) or with FLAG-auxilin-(547-813) were incubated with radioactive phosphate [^{32}P] for 3 h at 37°C and then lysed. Auxilin fragments were immunoprecipitated from lysates with an anti-FLAG antibody and separated by SDS-PAGE. Western blot analysis with an anti-auxilin antibody (A) and autoradiography of the blot (B) revealed that both auxilin fragments were phosphorylated *in situ*.

This result raised the question which protein kinase(s) is/are responsible for the phosphorylation of auxilin. The findings of the diploma thesis indicate that the auxilin-kinase is associated with CCVs. However, *in vitro* kinase assays revealed that the enzymes GAK, CK2 and AAK1 are not capable of phosphorylating recombinant auxilin (Düwel, 2003).

At that time of investigation, McPherson and co-workers performed a tandem MS analysis of rat brain CCVs and discovered several so far undescribed proteins (Blondeau *et al.*, 2004). Moreover, a closer look at the ORF of one of those novel proteins (KIAA1360) uncovered that this protein exhibits a serine/threonine kinase homology domain. Given the fact that this potential kinase is associated with CCVs we considered KIAA1360 to be a candidate for the auxilin-kinase and ordered the corresponding cDNA for further investigations. Later on this novel protein was termed CVAK104 (coated yesicle-associated kinase of 104 kDa) (Conner and Schmid, 2005).

3.2 Cloning and expression of CVAK104

The cDNA of CVAK104 encodes a 104 kDa protein with 929 amino acids. The N-terminal part of CVAK104 harbors a serine/threonine kinase homology domain. Besides two potential DLL clathrin-interaction motifs and an NPF motif shown to support interaction with Eps15 homology (EH) domain-containing proteins, such as Eps15 and intersectin, there are no other obvious binding motifs present in the amino acid sequence. Figure 3.2 shows a schematic overview of the protein CVAK104. Based on the amino acid sequence bioinformatic tools predict a modular structure for CVAK104 with at least three domains (tools accessible at www.expasy.ch and www.pondr.com). The first is the kinase homology domain (32-327), which is followed by a coiled-coil structure with similarity to armadillo/HEAT repeats (330-630). Armadillo/HEAT repeats comprise a special arrangement of α -helices and are supposed to mediate protein-protein interactions. These repeats are also found in the core domain of the adaptor protein complexes and in the protein huntingtin (Andrade *et al.*, 2001; Heldwein *et al.*, 2004; Owen *et al.*, 2004). The carboxy-terminal end of CVAK104 (630-929) is predicted to be only poorly structured. Interestingly, unstructured and therefore flexible domains seem to be a common feature of many endocytic proteins. In proteins like epsin, AP180 and auxilin the linear peptide motifs, which are necessary for the interaction with clathrin and AP2, are located within unfolded regions of the protein (Kalthoff *et al.*, 2002).

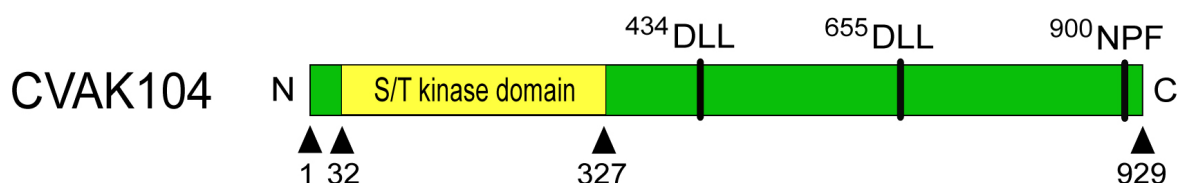


Figure 3.2: Schematic overview of CVAK104. The protein is 929 amino acids long and exhibits a serine/threonine kinase homology domain at the N-terminal region. CVAK104 also contains two conserved interaction motifs that are thought to support binding to clathrin (DLL) and EH domain-containing proteins (NPF).

With the aim to express CVAK104 as a GST-fusion protein the cDNA of CVAK104 was amplified by PCR and ligated into a pGEX-6P vector. The plasmid was transformed into BL21 bacteria and expression of GST-CVAK104 was verified by SDS-PAGE and Western blot analysis of bacterial lysates with an anti-GST antibody. The Western blot analysis showed an IPTG-dependent expression of a GST-CVAK104 fusion protein with the predicted molecular weight of ~130 kDa (Figure 3.3). Furthermore, it became obvious that GST-CVAK104 is not very stable in bacteria, which resulted in an accumulation of degradation products. The degradation products were still detectable with the anti-GST antibody, thus indicating that these fragments were degraded starting from the C-terminus of CVAK104.

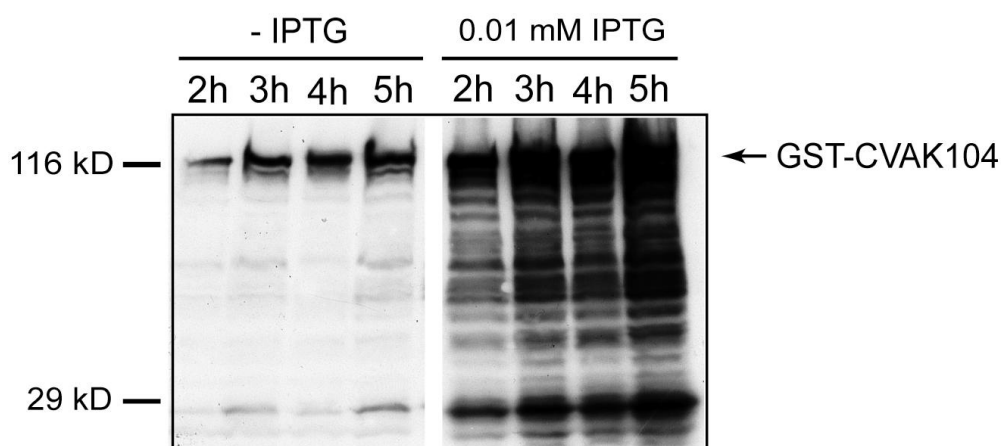


Figure 3.3: Expression of recombinant GST-CVAK104. BL21 bacteria - transformed with a GST-CVAK104 expression plasmid - were grown for the indicated time periods at room temperature in the presence or absence of IPTG and then lysed in SDS sample buffer. Lysates were analyzed by SDS-PAGE and Western blot with an anti-GST antibody. Note that in addition to full length GST-CVAK104 the lysates contained many degradation products of the protein.

3.3 Analysis of the kinase-activity of CVAK104

3.3.1 ATP-binding of recombinant CVAK104

A kinase catalyzes the transfer of a phosphate group from ATP (or GTP) to the OH-group of a serine-, threonine- or tyrosine-residue. Thus, it is essential for kinases to bind the appropriate protein substrate and the co-substrate ATP in the catalytic domain. To determine the ATP-binding capacity of CVAK104 we carried out the chromatographic method of Hummel and Dreyer as described in chapter 2.2.6.3 and in (Hummel and Dreyer, 1962). Three proteins were used in the assay: GST-CVAK104-(1-375), which harbors the kinase homology domain of CVAK104, GST-KGAK (the kinase domain of the kinase GAK as positive control) or GST alone (as negative control). The respective proteins were incubated with [α^{32} -P]-ATP and then loaded on a gel filtration column that has been equilibrated with the same concentration of radioactively labeled ATP. The eluted fractions were tested for protein concentration as well as for radioactivity and the data were illustrated in the diagrams shown in Figure 3.4. The elution profile of GST-KGAK and the nucleotide concentration in each fraction is typical for an ATP-binding protein (Figure 3.4B). The interaction of the GAK-kinase domain with ATP causes an increase in the concentration of ligand where the GAK-kinase domain elutes, followed by a trough of radioactive ATP concentration, which represents ligand that has been removed by binding to the kinase. The kinase homology domain of CVAK104 showed a similar elution profile. Again the ATP peak coincided with the protein peak (Figure 3.4C). In the case of GST, which is not an ATP-binding protein, the eluted nucleotide-concentration did not vary significantly above the average background (Figure 3.4A). Taken together, this data suggests that CVAK104 is an ATP-binding protein.

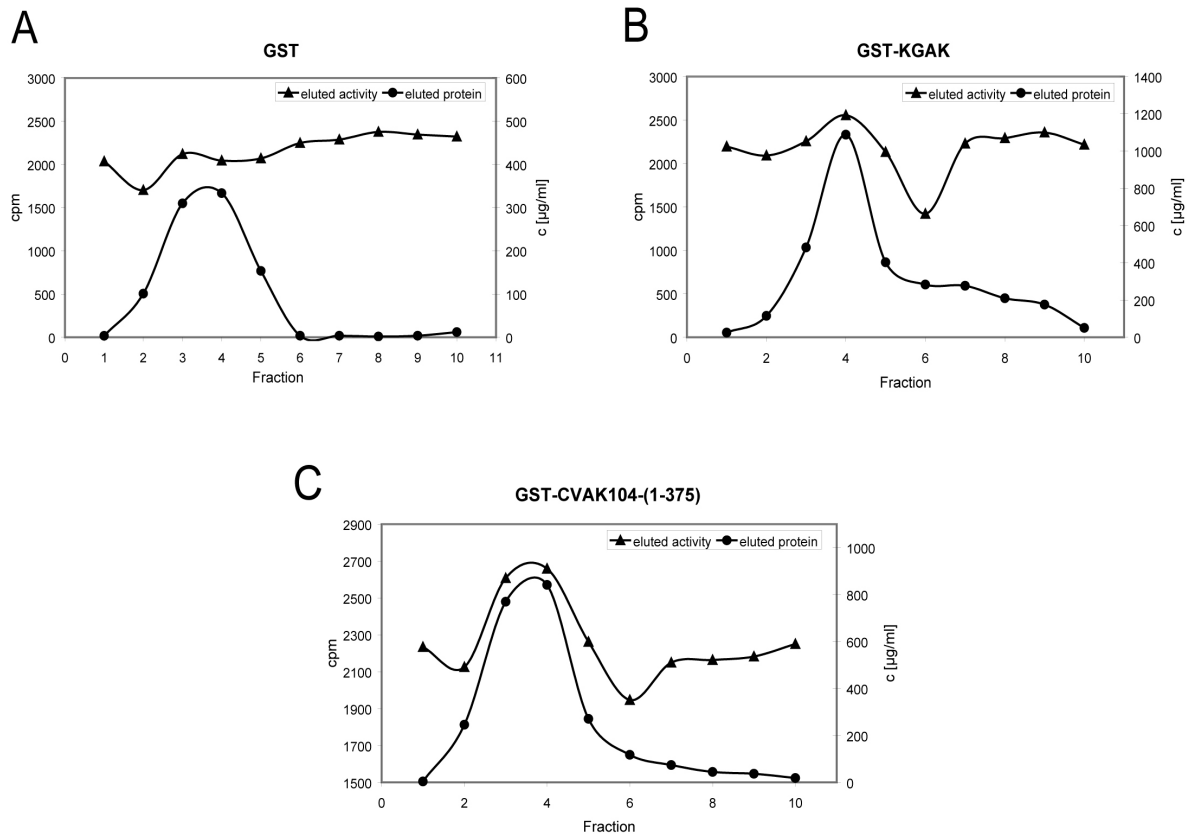


Figure 3.4: ATP-binding to the kinase homology domain of CVAK104. GST (A), GST-KGAK (kinase domain of GAK) (B) and GST-CVAK104-(1-375) (C) were incubated with [α^{32} P]-ATP for 15 min at room temperature and loaded on a NAP-5 desalting column, which was equilibrated in ATP-binding buffer. Eluted fractions were analyzed regarding protein concentration as well as radioactivity and results were plotted in a diagram. In the case of GST-KGAK and GST-CVAK104-(1-375) the protein peak coincided with an increased elution of radioactive ATP, whereas incubation with GST had no significant effect on the ATP elution profile.

3.3.2 *In vitro* kinase assays with CVAK104

Next, we addressed the question whether CVAK104 has any catalytic activity and is capable of phosphorylating endocytic proteins like auxilin or itself. To answer this question, *in vitro* kinase assays were performed with the recombinant protein purified from bacteria and [γ - ^{32} P]-labeled ATP. The GST-moiety of the fusion protein was removed by prescission protease cleavage prior to the experiment. As potential substrates for CVAK104 the reactions contained recombinant auxilin fragments, histone H1, myelin basic protein (MBP) and proteins from purified CCVs like clathrin and adaptor proteins. Surprisingly, none of the proteins showed an incorporation of radioactive phosphate after incubation with CVAK104 (Figure 3.5). In agreement with earlier data the protein pools derived from CCVs contained endogenous phosphotransferase activity resulting in the phosphorylation of several endocytic proteins such as clathrin light chain, μ 2-adaptin and α -adaptin (Düwel, 2003; Korolchuk and

Banting, 2003). However, there was no protein with an increased phosphorylation and no protein that was phosphorylated in addition when CVAK104 was present in the reaction.

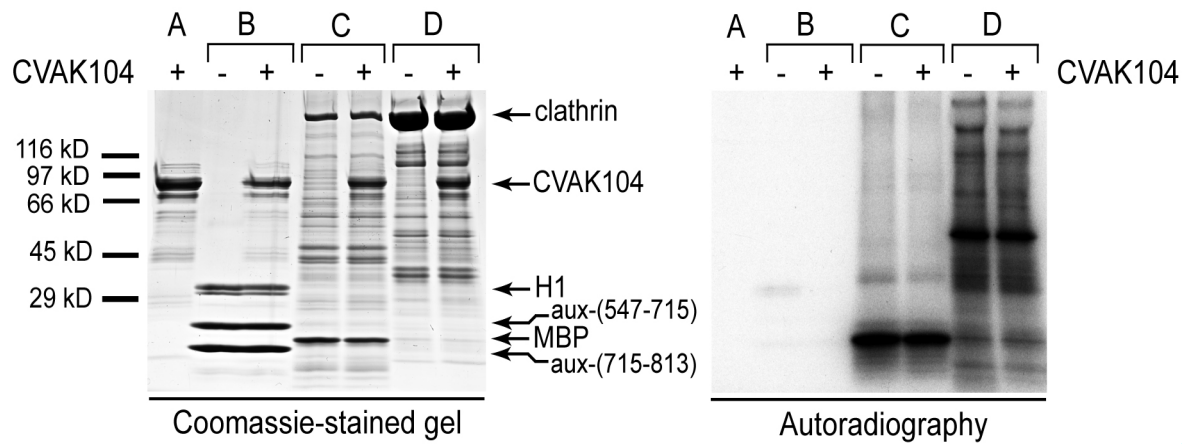


Figure 3.5: *In vitro* kinase assays with recombinant CVAK104. Different protein mixtures were incubated with [γ - ^{32} P]-labeled ATP and with or without bacterially expressed CVAK104. Proteins were then separated by SDS-PAGE and phosphorylation was detected by autoradiography of the dried gel. In (A) only the purified CVAK104 was present in the reaction. The potential kinase substrates were as follows: B) Histone H1, recombinant auxilin-(547-715) and (715-813), respectively. C) MBP as well as CCV proteins purified by hydroxyapatite chromatography. D) Tris-extract of CCVs. Neither autophosphorylation of CVAK104 nor the phosphorylation of other proteins was detected when reactions contained recombinant CVAK104.

To rule out the possibility that recombinantly expressed CVAK104 is not folded correctly in bacteria or needs posttranslational modifications for a catalytic activity, the protein was also expressed using a baculovirus expression system in SF9 insect cells. But again the purified protein exhibited no catalytic activity regarding the chosen substrates (data not shown). The same holds true for endogenous CVAK104, which was immunoprecipitated from HeLa cells and then used for kinase assays.

Although it can not be ruled out the possibility that the experimental conditions or the substrates chosen in the kinase assays were not appropriate, this data suggests that CVAK104 is not an active kinase.

3.3.3 Sequence analysis of the kinase homology domain of CVAK104

The negative results regarding the catalytic activity of CVAK104 prompted us to have a closer look on the serine/threonine kinase homology domain of the protein, which covers the amino acids 32 to 327. Surprisingly, CVAK104 lacks highly conserved catalytic residues known to be important for the catalytic mechanism of active kinases. For instance, the highly

conserved aspartic acid residue in position 135, which is used for nucleotide catalysis in other kinases, is mutated to an asparagine residue in CVAK104. Thus, CVAK104 belongs to the SCY1-like family of protein kinases (referred to as SCYL2) (Manning *et al.*, 2002) that are thought to be catalytically inactive. Moreover, CVAK104 exhibits several other specific mutations of residues involved in the catalytic mechanism. Figure 3.6 shows an alignment of the catalytic loop of active phosphotransferases and the predicted catalytic loop of CVAK104. Highly conserved residues in the catalytic loop of kinases are marked by asterisks.

Catalytic loop	**	*****
1JNK	GIIHR 189	DLKPSNIVVK.[2].CTLKILDFGLARTAGT
CVAK104	KMVHG 158	NITPENIILN.[2].GAWKIMGFDFCVSSTN
1DAW_A	GIMHR 150	DVKPHNVMID.[3].RKLRLIDWGLAEFYHP
1F3M_C	QVIHR 141	DIKSDNILLG.[2].GSVKLTDFGFCAQITP
1G3N_A	RVVHR 145	DLKPQNILVT.[2].GQIKLADFGLARIYSF
1QMZ_A	RVLHR 128	DLKPQNLLIN.[2].GAIKLADFGLARAFGV
1TKI_A	NIGHF 127	DIRPENIIYQ.[4].STIKIIEFGQARQLKP
2PHK_A	NIVHR 136	DLKPENILLD.[2].MNIKLTDFGFSCQLDP

Figure 3.6: Alignment of the catalytic loop of kinases with the predicted catalytic loop of CVAK104. Highly conserved residues known to be essential in the catalytic loop of active kinase are marked by asterisks. Note that some of these residues are mutated in CVAK104.

Sequence analysis with bioinformatic tools (data not shown) and the alignment illustrated above clearly show that there are indeed kinase signature motifs present in CVAK104 but residues important for nucleotide catalysis are mutated.

Based on the data of the *in vitro* kinase assays and on the sequence analysis it is likely that CVAK104 is only a kinase-related protein and not an active phosphotransferase.

3.4 Protein-protein interaction studies with CVAK104

3.4.1 Identification of CVAK104 interaction partners

CVAK104 was discovered in a mass-spectroscopic analysis of CCVs. Thus, it is likely that CVAK104 has at least one interaction partner present in these vesicles by which it is recruited. This might be membrane lipids or other vesicle coat components.

With the aim to identify potential CVAK104 binding partners, bacterially expressed GST-CVAK104 or GST alone as control were immobilized on GSH-Sepharose beads and incubated with rat brain cytosol as a source of soluble proteins. The Sepharose-beads were pelleted and pellet as well as supernatant fractions were analyzed by SDS-PAGE and Western blot for the major coat-, adaptor- and accessory proteins. The experiment clearly showed that immobilized GST-CVAK104 was able to pull down clathrin out of the cytosol (Figure 3.7A), whereas other proteins like AP1, AP2, auxilin, AP180, dynamin, Eps15 and GGA3 remained in the supernatant (data not shown).

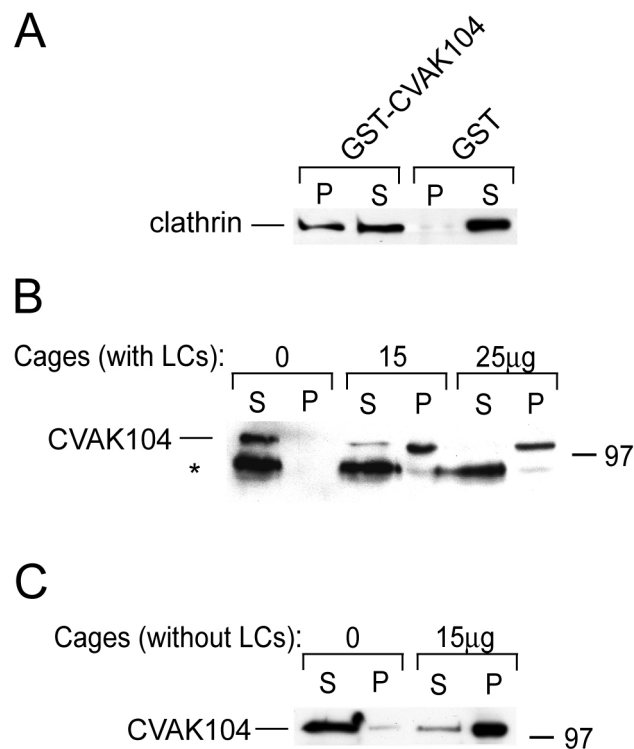


Figure 3.7: Interaction of CVAK104 with clathrin. A) GST-CVAK104 or GST alone were immobilized on glutathione Sepharose and incubated with rat brain cytosol for 60 min on ice. After sedimentation of beads the supernatant (S) as well as the bead (P) fractions were subjected to SDS-PAGE and Western blotting with a clathrin antibody. B and C) Purified recombinant CVAK104 was incubated with the indicated amounts of clathrin cages with (B) or without light chains (C). Samples were ultracentrifuged and the resulting supernatants (S) and pellets (P) were analyzed by SDS-PAGE and Western blotting with a CVAK104 antibody. Note that an occasionally visible degradation product of CVAK104 fails to bind to the clathrin cages (asterisk in B). In (A) the pellet fractions were fourfold concentrated compared to the supernatants. In the cage binding assays (B and C) the loadings are directly comparable.

The association of cytosolic clathrin with the bait CVAK104 does not necessarily mean that clathrin directly interacts with CVAK104. It is also feasible that the interaction is mediated by another cytosolic protein. To answer the questions if the binding of CVAK104 to clathrin is direct and if the interaction of both proteins is mediated by the light chains of clathrin, a sedimentation assay with reconstituted clathrin cages was performed. To this end clathrin

triskelions with light chains or clathrin triskelions, from which the light chains had been removed with thiocyanate (Winkler and Stanley, 1983), were assembled into cages that are readily sedimented by ultracentrifugation under conditions where CVAK104 remains in the supernatant. When recombinant CVAK104 was mixed with the cages most of it co-sedimented with the clathrin irrespective of the presence of their light chains (Figure 3.7B and 3.7C). Thus, CVAK104 interacts directly with the clathrin heavy chain.

The adaptor proteins AP1 and AP2 are known to serve as the major binding partners for accessory proteins involved in the generation of CCVs at the TGN (AP1) and at the plasma membrane (AP2), respectively. To rule out the possibility that the lack of interaction between CVAK104 and the adaptors is due to the competition with other cytosolic proteins, the pulldown assay with immobilized GST-CVAK104 was repeated with a highly enriched adaptor protein fraction from pig brain clathrin-coated vesicles. Surprisingly, with this alternative approach a weak interaction of CVAK104 with the adaptor protein AP2 was detected (Figure 3.8). However, no association of CVAK104 with the adaptor protein AP1 was observed.

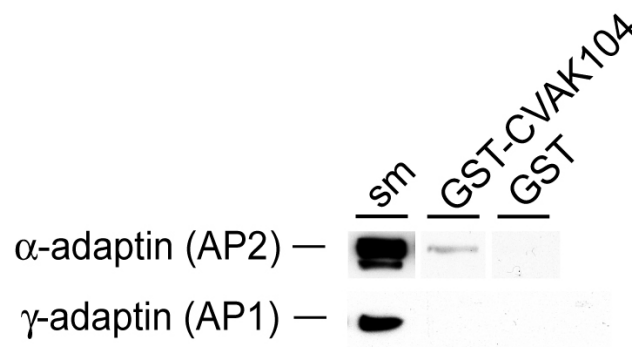


Figure 3.8: Association of CVAK104 with AP2. Fractions that were highly enriched in AP1 and AP2 from pig brain were incubated with immobilized GST-CVAK104. Note that only AP2 but not AP1 associated with CVAK104. One-tenth of starting material (sm) used in the binding reaction was run in parallel. The monoclonal antibodies AP.6 and 20D6 that specifically recognize α - and γ -adaptin, respectively, were used for Western blot analysis.

Taken together, CVAK104 specifically binds to the coat protein clathrin and to the adaptor protein AP2. These data suggest that CVAK104 is predominantly associated with AP2-containing CCVs derived from the plasma membrane.

3.4.2 Localization of binding domains of CVAK104 for clathrin and AP2

CVAK104 possesses two DLL motifs (⁴³⁴DLL and ⁶⁵⁵DLL), which are known to support the binding of the neuronal adaptor protein AP180 to clathrin (Morgan *et al.*, 2000). However, whereas intact CVAK104 was largely associated with assembled clathrin cages, a proteolytic fragment of about 90 kDa failed to bind (Figure 3.7B, asterisk). The size of the fragment indicated that the loss of about 150 residues from the N- or C-terminal end of CVAK104 abolished clathrin binding. This suggested that the clathrin binding site is most likely located close to either end of the CVAK104 polypeptide chain and that the DLL-sequence motifs at positions 434 and 655, respectively, are not sufficient for clathrin binding. To determine the region of CVAK104 that is responsible for the interaction with clathrin and to clarify if the two DLL motifs are important for the interaction, C-terminal deletion mutants of CVAK104 were generated by introducing STOP-codons within the sequence of CVAK104. In addition to full-length CVAK104 two GST-CVAK104 fusion protein fragments were expressed: the first covered the N-terminal segment from the N-terminus to ³⁷⁵Cys (GST-CVAK104-(1-375)) that includes the kinase homology domain; the second, GST-CVAK104-(1-697) extends to ⁶⁹⁷Lys with the two DLL motifs included (Figure 3.9A). For pulldown binding assays the aforementioned GST-fusion proteins were attached to GSH-Sepharose beads and incubated with rat brain cytosol. Whereas full length CVAK104 associated with clathrin, neither of the two N-terminal fragments did (Figure 3.9B). This result suggests that the interaction of CVAK104 with clathrin is mediated by the C-terminal region of CVAK104 and not by the two DLL motifs. To test this hypothesis a pulldown binding assay was carried out with a fragment that covers the C-terminal region of CVAK104 (GST-CVAK104-(699-929)) (Figure 3.9A). As expected clathrin readily associated with the GST-CVAK104-(699-929) (Figure 3.9C). Moreover, repeating the pulldown assays with the generated CVAK fragments and purified adaptor proteins revealed that AP2 like clathrin only associates with full-length CVAK104 and with the C-terminal region (Figure 3.9D).

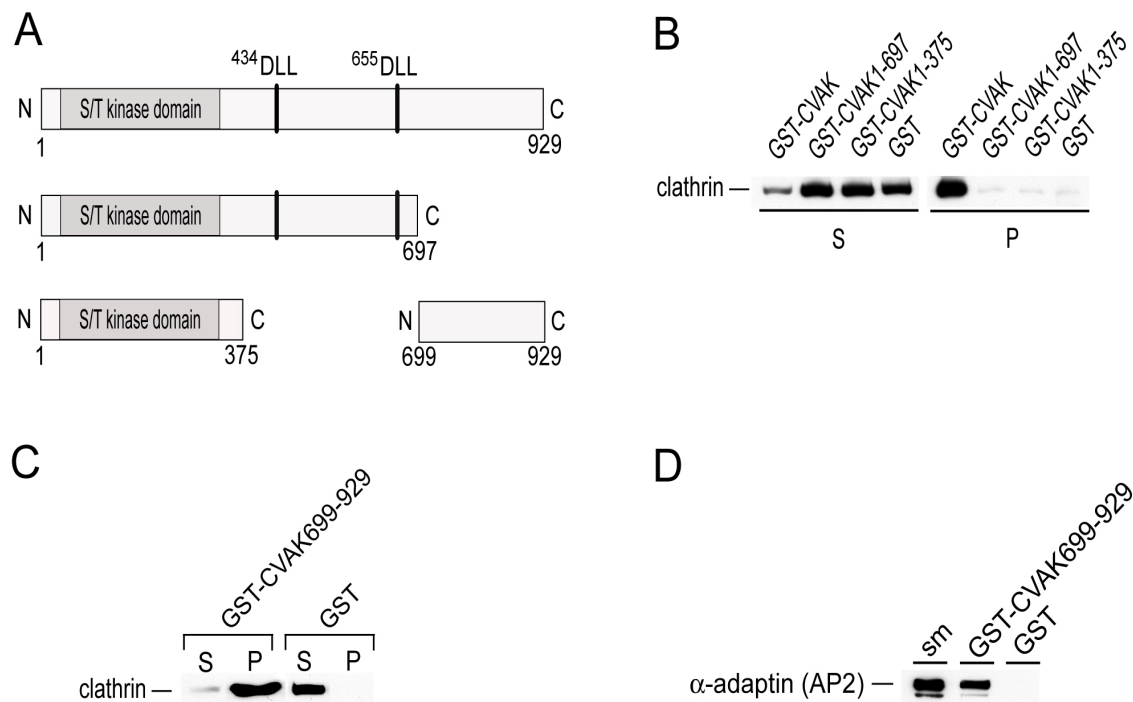


Figure 3.9: Localization of binding domains within CVAK104. A) Schematic representation of the recombinantly expressed (GST-fusion)-fragments of CVAK104 used in this work. Putative clathrin interaction motifs are indicated. B + C) Incubation of rat brain cytosol with immobilized GST-CVAK104 fusion proteins. Note that only full-length (B) and the C-terminal segment 699-929 (C) of CVAK104 pulled clathrin out of the cytosol. Pellet fractions are threefold concentrated compared to the supernatants. D) Fractions that were highly enriched in adaptor proteins from pig brain were incubated with immobilized GST-CVAK104-(699-929). One-tenth of starting material (sm) used in the binding reaction was run in parallel. Both, clathrin and AP2 interact with the C-terminal region of CVAK104. The two DLL motifs are not responsible for the binding of CVAK104 to clathrin.

Surprisingly, we detected a somewhat better binding of AP2 to the C-terminal segment 699-929 than to the full-length protein (Figure 3.8 and 3.9D). The apparent stronger binding is readily explained by the fact that CVAK104 is very sensitive to proteolytic attack, which results preferentially in the loss of C-terminal portions of the immobilized GST-tagged CVAK104 thereby lowering in effect the concentration of the bait, whereas the GST-CVAK104-(699-929) proved to be more stable. Another explanation could be that the AP2 binding site in the C-terminal segment of CVAK104 is not readily accessible in the intact protein.

3.4.3 Localization of binding domains of clathrin and AP2 for CVAK104

The cage binding assays already revealed that the heavy chain of clathrin is sufficient to support the interaction with CVAK104. Given that a number of interaction partners of clathrin bind to its globular amino-terminal domain (TD) a GST-TD fusion protein was used to test the interaction with CVAK104 in a pulldown experiment. We observed that CVAK104 does indeed interact directly and specifically with the clathrin TD and that the C-terminal region of CVAK104 is responsible for the interaction (Figure 3.10A).

CVAK104 does not contain typical binding motifs for the clathrin TD in its C-terminal region. Hence it is an open question to which site(s) CVAK104 binds on the clathrin TD. For further information we examined if the interaction of CVAK104 and clathrin TD can be competed by protein fragments encompassing known binding motifs for the TD. Pulldown experiments with immobilized GST-TD revealed that the interaction of the C-terminal CVAK104 fragment with the clathrin TD was efficiently competed by adding an excess of a polyhistidine-tagged recombinant fragment of AP180 (segment 329-746) that contains 5 DLL motifs and is known to associate with the TD of clathrin (Lafer, 2002) (Figure 3.10B). We also observed that the β 2-hinge + ear domain (β 2-residues 592-951) of AP2, which contains in its flexible hinge region the ^{631} LLNLD clathrin box motif, inhibited binding of CVAK104 to TD (Figure 3.10C). Given that we did not detect any association of AP180 and the β 2-subunit of AP2 with CVAK104 in our pulldown assays (data not shown) we can rule out the possibility that the decreased binding of CVAK104 to clathrin in the competition experiment is caused by sequestering CVAK104 through direct interactions with the AP180- and the AP2/ β 2-fragments. Thus we can conclude that CVAK104, AP180 and the β 2-subunit of AP2 bind to the same or overlapping sites on the clathrin TD.

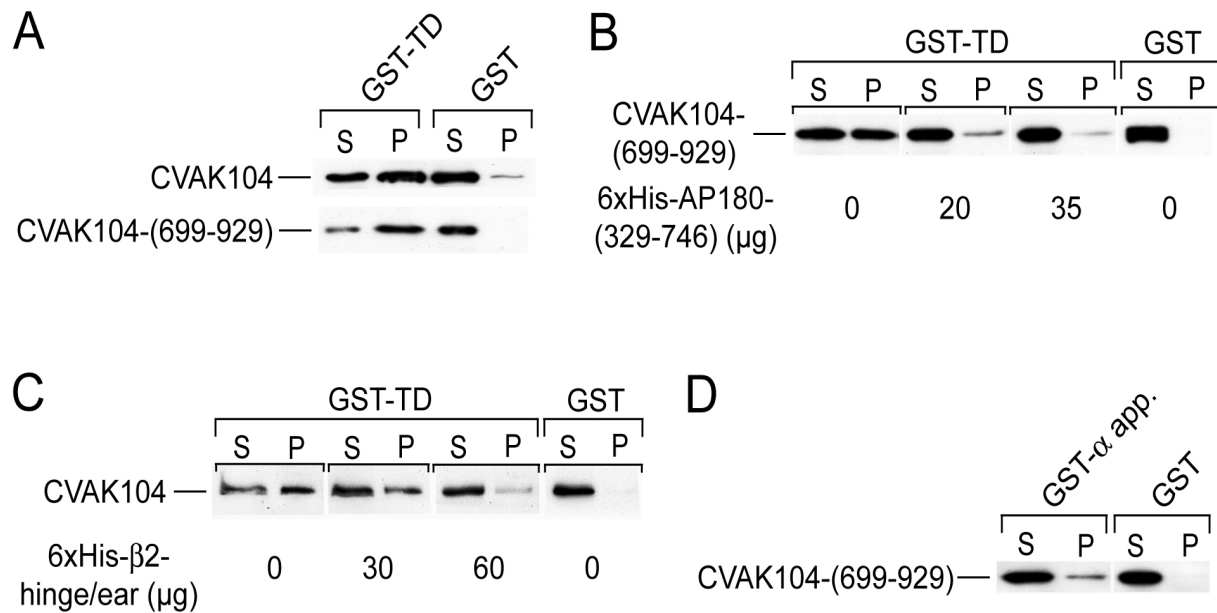


Figure 3.10: CVAK104 binding sites on clathrin and AP2. A) Pulldown of recombinant CVAK104 full-length and CVAK104-(699-929) by recombinant clathrin N-terminal domain (TD) fused to GST and immobilized to glutathione-Sepharose. Immobilized GST served as a control. Supernatant- and pellet fractions (S and P) were analyzed by SDS-PAGE and Western blotting. The results demonstrate that CVAK104 interacts directly with the TD of clathrin. B) Competition between CVAK104-(699-929) and His₆-AP180-(329-746) for GST-TD. A constant amount of CVAK104-(699-929) was added to immobilized GST-TD together with increasing concentrations of His₆-AP180-(329-746). Note, that the recombinant AP180 fragment displaces the CVAK104 fragment. Signals of supernatant- and pellet fractions are directly comparable. C) Competition between cytosolic CVAK104 and His₆-β2-hinge/ear-(592-951) for the TD. A constant amount of rat brain cytosol was added to immobilized GST-TD together with increasing concentrations of His₆-β2-hinge/ear-(592-951). Note, that the recombinant His₆-β2 fragment displaces the CVAK104 fragment. In (A) the pellet fractions were twofold and in (B+C) threefold concentrated compared to the supernatants. D) Direct association of the immobilized GST-α-appendage domain of AP2 with recombinant CVAK104-(699-929). Loadings are directly comparable.

The α-appendage domain of AP2 is known to constitute a major hub for protein-protein interactions with diverse binding partners. A GST-pulldown experiment showed that the C-terminal CVAK104 segment 699-929 directly associates with the α-appendage domain of AP2 (Figure 3.10D) although this fragment of CVAK104 does not contain any obvious binding motif for this AP2-subdomain.

Taken together, we have demonstrated that the clathrin N-terminal domain and the α-appendage domain of AP2 associate *in vitro* with a C-terminal segment of CVAK104 that starts with ⁶⁹⁹Gln.

3.5 Tissue distribution of CVAK104

So far there is only evidence that CVAK104 is expressed in brain (Blondeau *et al.*, 2004). To assess the distribution of CVAK104 in other tissues, we first evaluated the gene expression levels by RT-PCR by using total RNA isolated from cultured cells and mouse organs. As shown in figure 3.11, the CVAK104 transcript is expressed throughout all tested cell lines and mouse tissues.

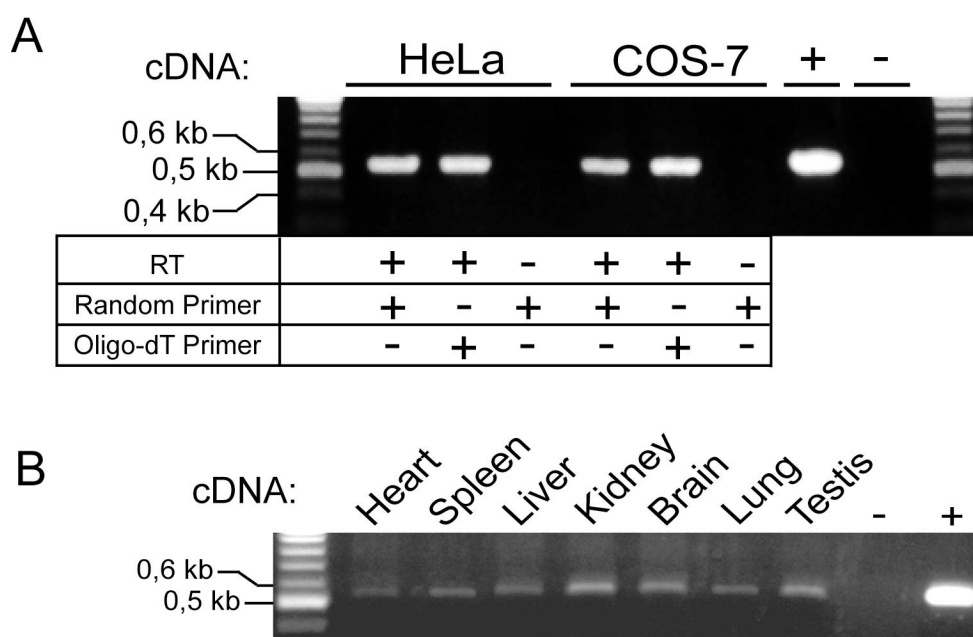


Figure 3.11: Gene expression of CVAK104 in different tissues analyzed by RT-PCR. Total RNA was isolated from different cell lines (A) and mouse organs (B). RT was performed with oligo-dT (A) or random (A+B) primer. Total cDNA was used as template for PCR with specific CVAK104 primer. A plasmid containing the CVAK104 served as positive control for the PCR. The CVAK104 transcript was detected in all tested cell lines and mouse organs.

The RT-PCR data provide no information about the expression level of the protein CVAK104. Therefore we analyzed several mouse organs as well as HeLa- and COS-7 cultured cells for the expression of CVAK104 by immunoblotting. We observed that CVAK104 is ubiquitously expressed and that its expression relative to that of the AP1 γ subunit appeared to be constant in all organs and cell lines that were examined (Figure 3.12).

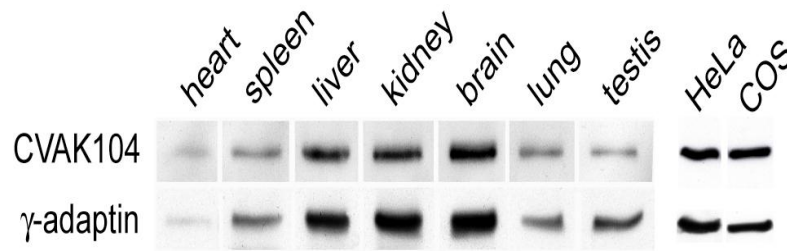


Figure 3.12: Tissue distribution of CVAK104. Comparable amounts of lysed mouse organs and lysates of cultured cells were analyzed by SDS-PAGE and Western blotting with antibodies to CVAK104 and AP1.

3.6 Subcellular localization of CVAK104

3.6.1 Colocalization studies with CVAK104 in HeLa and COS-7 cells

3.6.1.1 Colocalization of CVAK104 with clathrin and adaptor proteins

CVAK104 associates *in vitro* with the coat protein clathrin and the adaptor protein AP2. Next we wished to determine if the *in vitro* interaction of CVAK104 with clathrin and AP2 manifests itself in a colocalization of CVAK104 with those proteins *in vivo*. The subcellular distribution of endogenous mammalian CVAK104 was analyzed by indirect immunofluorescence microscopy in HeLa and COS-7 cells. CVAK104 localizes predominantly to the perinuclear region but is also present on peripheral vesicular structures (Figure 3.13). The CVAK104 staining clearly overlapped with that of clathrin. An unexpected observation, however, was that endogenous CVAK104 did not colocalize with its *in vitro* binding partner AP2 (Figure 3.13). In contrast, double staining of AP1 and CVAK104 in HeLa and COS-7 cells demonstrated almost perfect colocalization of the two proteins in the perinuclear region. Moreover, many AP1-positive more peripherally localized endosomes contained also CVAK104 (Figure 3.13). Although contradictory to the *in vitro* binding data these results suggest that CVAK104 is located preferentially at the Golgi and on endosomes rather than at the plasma membrane.

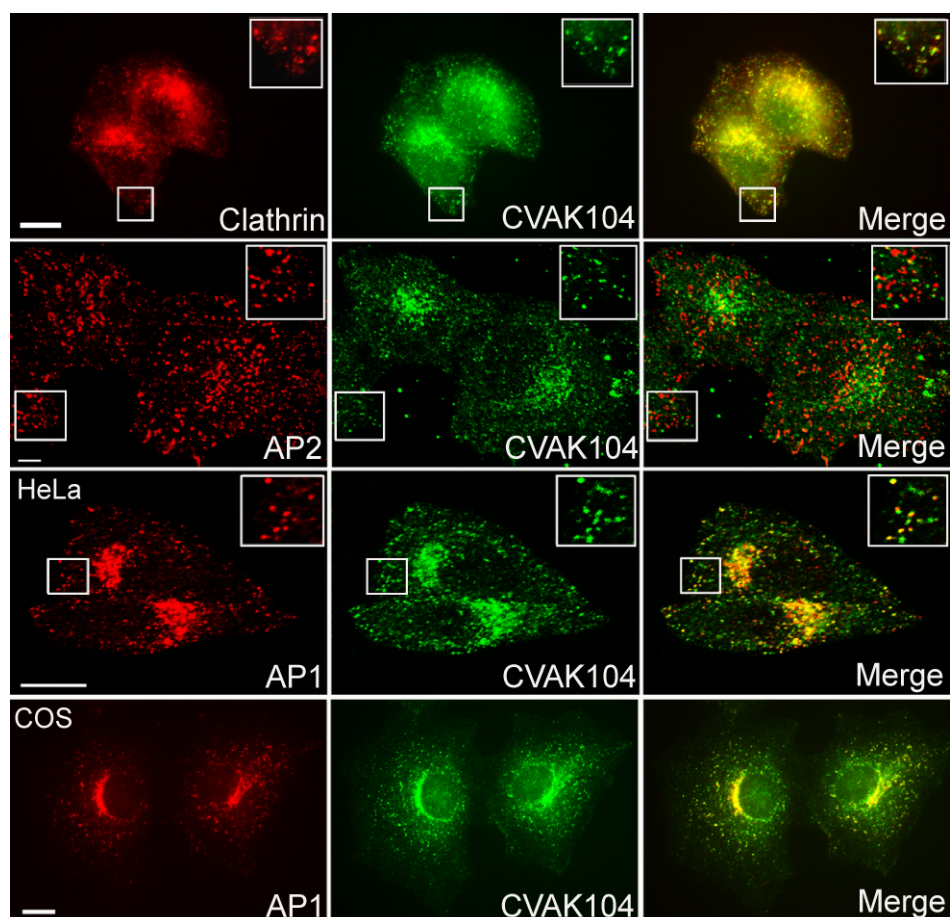


Figure 3.13: Subcellular localization of CVAK104 in HeLa and COS-7 cells. Endogenous CVAK104 localization in cultured cells, determined by indirect immunofluorescence, is compared to that of clathrin heavy chain (HeLa), the α -adaptin subunit of AP2 (HeLa), and the γ -adaptin subunit of AP1 (HeLa and COS-7). CVAK104 is predominantly localized to the perinuclear region and on peripheral vesicular structures. The localization overlaps with that of clathrin and AP1 but not with that of AP2. Rows 2 and 3 are confocal images. Insets show magnified views of the boxed regions. Bars, 10 μ m.

3.6.1.2 Colocalization studies with CVAK104 and Golgi-, endosomal- and lysosomal markers

For further characterization of the subcellular distribution of CVAK104, the colocalization studies were extended to include Golgi-, endosomal- and lysosomal markers. β -1,4-galactosyltransferase (GT) is located in the *trans*-Golgi and in the TGN. Endogenous CVAK104 overlapped well with this marker but less so with the *cis*-Golgi matrix protein GM130 (Figure 3.14A). The absence of CVAK104 from the *cis*-Golgi was more clearly seen when the Golgi stacks were disrupted by treatment with nocodazole. Figure 3.14A clearly shows that in contrast to GM130 many of the GT spots coincide with CVAK104 puncta in nocodazole-treated cells, indicating that perinuclear CVAK104 is also part of the TGN.

At steady state the cation-independent mannose 6-phosphate receptor (CI-M6PR) is mainly found in the endosomal system and in the TGN (Kornfeld, 1992). To relate its subcellular distribution to that of CVAK104, GFP-CVAK104 was expressed in COS-7 cells and stained after fixation with a polyclonal serum against the CI-M6PR. The transiently expressed GFP-CVAK104 co-localized well with CI-M6PR (Figure 3.14B). Furthermore, in COS-7 cells that expressed the FLAG-tagged phosphatidyl inositol 5-phosphatase OCRL and GFP-CVAK104 we noted extensive colocalization of the two proteins in the perinuclear area (Figure 3.14B). Previous work had shown that OCRL localizes to endosomes and the TGN (Ungewickell *et al.*, 2004; Lowe, 2005). CVAK104 overlapped also significantly with the adaptor protein complex AP3, which is involved in the sorting of lysosomal membrane proteins (Le Borgne *et al.*, 1998; Rous *et al.*, 2002) (Figure 3.14B). In contrast, very little colocalization of CVAK104 with early endosomal markers such as EEA1 (Figure 3.14B) and rab5 (data not shown) was seen. CVAK104 did not colocalize with the late endosomal/lysosomal component CD63 (Figure 3.14B).

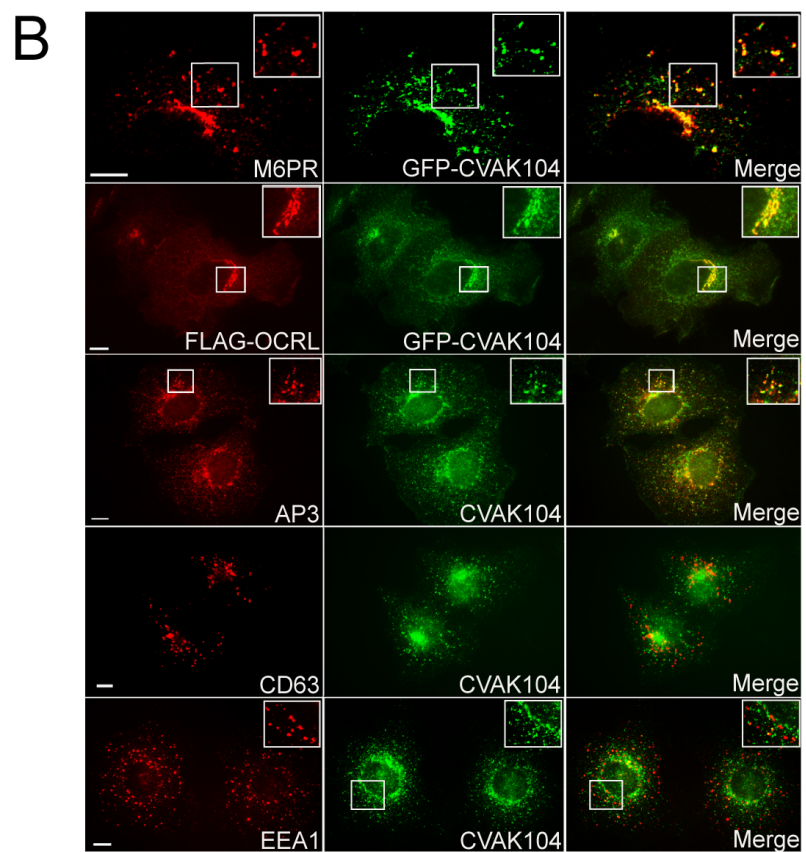
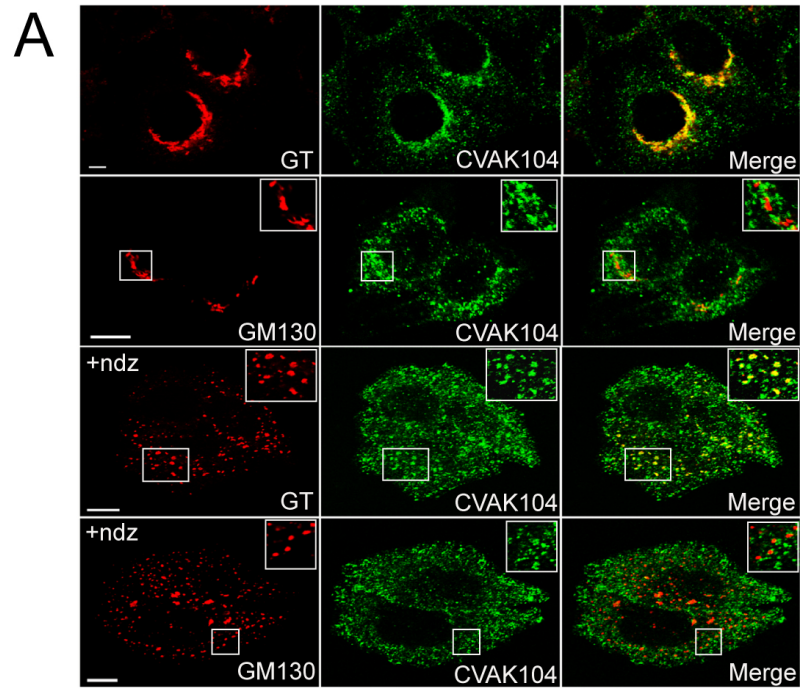


Figure 3.14: Colocalization studies of CVAK104 with Golgi-, endosomal- and lysosomal markers. A) Confocal images of HeLa-cells double-labeled for CVAK104 and the *trans*-Golgi marker galactosyltransferase (GT) and the *cis*-Golgi marker GM130, respectively. The disruption of the Golgi-stacks with nocodazole (+ndz) confirmed the close spatial relationship between GT and CVAK104, whereas GM130 positive vesicles did not contain CVAK104. B) Spatial relationship of CVAK104 with the mannose 6-phosphate receptor (M6PR), OCRL, AP3, CD63 (lamp II) and the early endosomal antigen 1 (EEA1) in COS-7 cells. GFP-CVAK104 expressing cells were used for the staining of the cation-independent mannose 6-phosphate receptor (M6PR). To obtain information on the spatial relationship between OCRL and CVAK104, COS-7 cells were transfected with FLAG-tagged OCRL and GFP-CVAK104. The first panel in B (M6PR and GFP-CVAK104) shows confocal images. Insets show magnified views of the boxed regions. Bars, 10 μ m.

3.6.1.3 Colocalization studies with CVAK104 and endocytosed transferrin

Iron-laden transferrin binds to cell-surface transferrin-receptors (TfR) and is then internalized with the receptor via clathrin-mediated endocytosis. During the transport through the endosomal system (early and recycling endosomes) transferrin loses the iron and is then - still bound to the TfR - recycled back to the plasma membrane to be released into the extracellular space. To confirm the endosomal localization of CVAK104, Texas Red-labeled transferrin was internalized in COS-7 cells for different times and localization of transferrin was compared to that of CVAK104. Transferrin bound to cell surface transferrin receptors at 4°C appeared within 2 minutes in CVAK104-positive structures (Figure 3.15A) where it remained for at least 3 minutes (Figure 3.15B). These observations provide evidence for the endosomal localization of CVAK104.

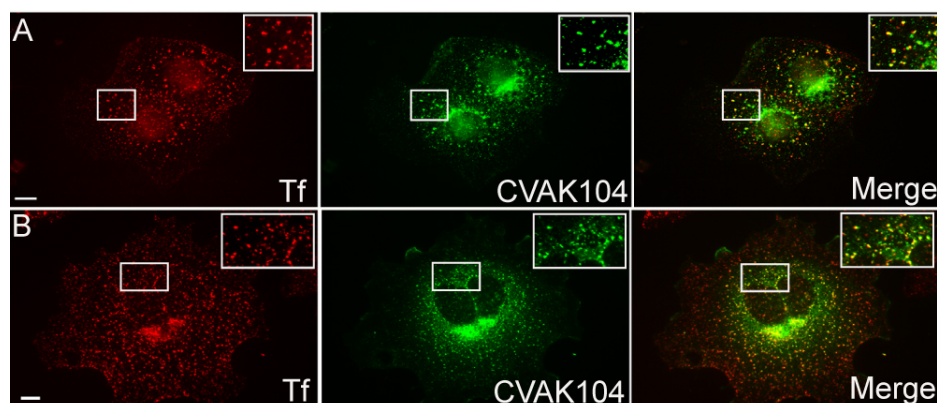


Figure 3.15: Colocalization of CVAK104 and endocytosed transferrin. COS-7 cells were allowed to take up pre-bound Texas Red-labeled transferrin for 2 min (A) and 5 min (B), fixed and stained for endogenous CVAK104. At both time points transferrin-positive structures overlapped well with the localization of CVAK104. Bars, 10 μ m.

The very rare colocalization of CVAK104 with EEA1 suggests that the organelles that contain transferrin and CVAK104 do not correspond to early endosomes. Triple labeling of COS-7 cells that were allowed to internalize pre-bound transferrin for 2 minutes demonstrated directly that transferrin-containing endosomes, which stain positive for EEA1, lack CVAK104, whereas those that stain positive for CVAK104 do not usually contain EEA1 (Figure 3.16A). It was noted for COS-7 cells that transferrin is taken up and recycled to the cell surface very rapidly, since most of the transferrin-staining was gone within 8 minutes after warming the cells to 37°C (data not shown).

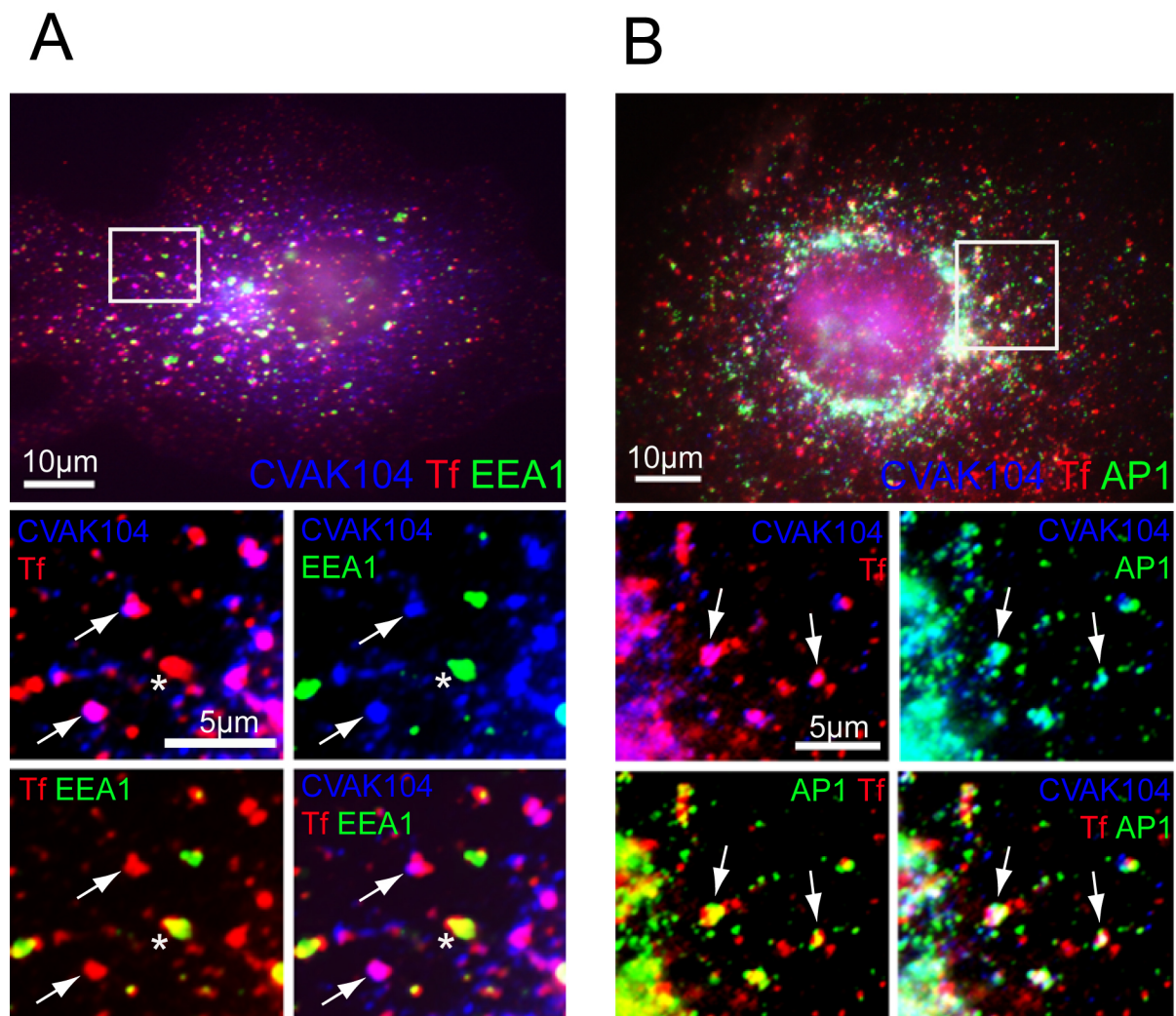


Figure 3.16: Relationship between CVAK104, internalized transferrin, EEA1 and AP1. Pre-bound Texas Red-labeled transferrin was internalized for 2 min (A) and 5 min (B), respectively. After fixation cells were stained for CVAK104 (blue) as well as for EEA1 (green in A) or AP1 (green in B). The last two rows in A and B show enlarged regions of the cell marked with a box. Arrows in (A) indicate structures that are positive for transferrin and CVAK104 but not for EEA1. The asterisk marks a vesicular structure that contains transferrin and EEA1 but no CVAK104. Arrows in (B) indicate structures that are positive for CVAK104, Tf and AP1.

In addition to its presence in the TGN, the AP1 adaptor is also associated with early and recycling endosomes and it functions in both anterograde and retrograde trafficking

between the TGN and endosomes (Futter *et al.*, 1998; Mallard *et al.*, 1998; Hanners and Tooze, 2003). We therefore asked whether CVAK104 and AP1 would be found together in transferrin-laden endosomes. Although many transferrin-positive structures in the periphery of the cell were devoid of AP1 and CVAK104, we observed that a number of the perinuclear spots that contained CVAK104 and transferrin also stained positive for AP1 (Figure 3.16B). Taken together, these colocalization studies demonstrate that CVAK104 is located predominantly at the TGN and on endosomal structures that contain AP1, AP3 and transferrin and likely correspond to recycling endosomes.

3.6.2 Determinants for subcellular localization of CVAK104

3.6.2.1 Clathrin-dependent association of CVAK104 with membranes

The association of CVAK104 with membranes was examined by subcellular fractionation. To this end HeLa cell homogenates were separated by ultracentrifugation into a particulate (membrane bound) and soluble (cytoplasm) fraction. SDS-PAGE and Western blot analysis of both fractions showed that about 33% of the endogenous CVAK104 in HeLa cells is associated with membranes.

CVAK104 binds and partially colocalizes with clathrin in mammalian cells. For this reason we wished to know if CVAK104 requires clathrin for its association with membranes. Therefore the distribution of CVAK104 was investigated in HeLa cells after depletion of the clathrin heavy chain by RNAi (Figure 3.17A). For the adaptor protein AP1 it could already be shown that its association with membranes is increased upon clathrin depletion (Hinrichsen *et al.*, 2003). Indeed, cell fractionation assays with mock- and clathrin-siRNA transfected cells confirmed this phenotype for AP1 (Figure 3.17B). Interestingly, in case of CVAK104 the proportion of membrane-associated protein dropped from 33% to 23% (Figure 3.17B). This result suggests that clathrin is an important factor for the membrane association of CVAK104.

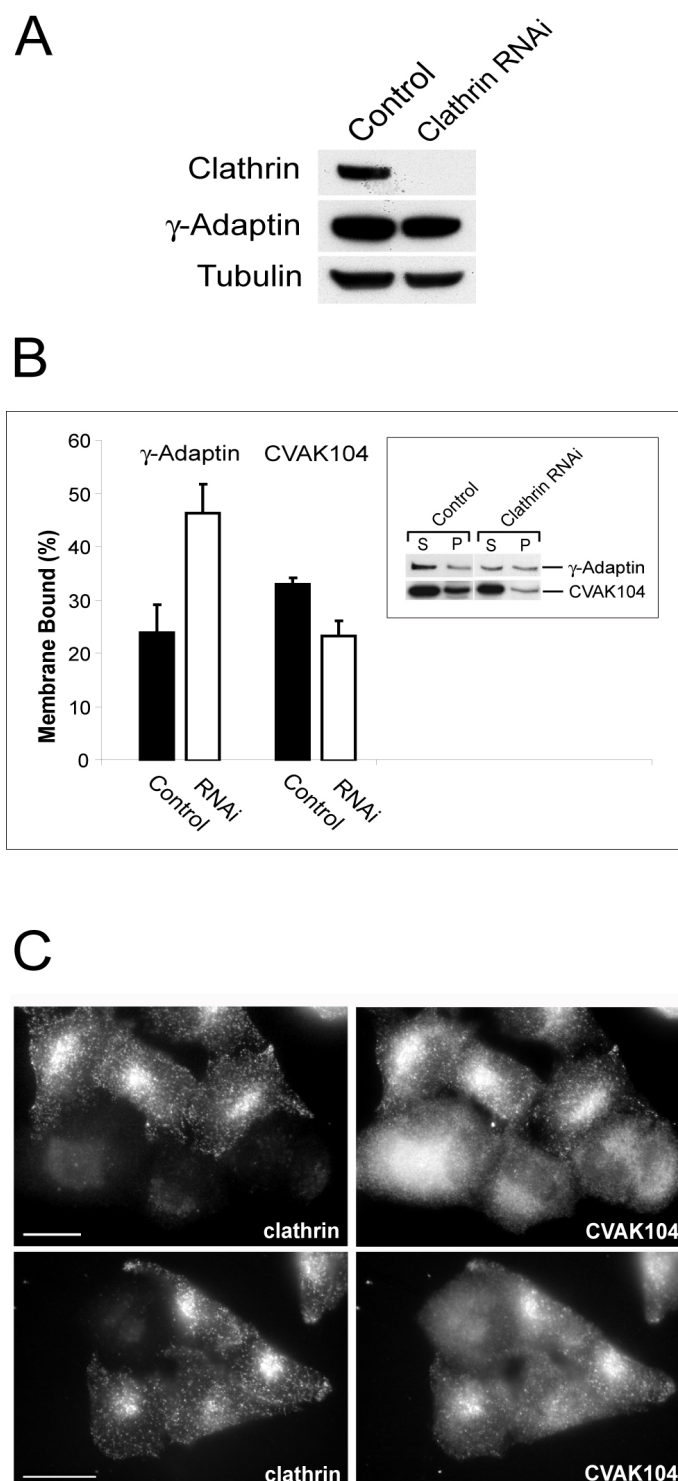


Figure 3.17: Requirement of clathrin for the subcellular localization of CVAK104. A) Western blot analysis of lysates from clathrin siRNA- and mock-transfected HeLa cells, respectively. Blots are stained for clathrin heavy chain, the γ -adaptin subunit of AP1 and for tubulin. B) Membrane associations of AP1 and CVAK104 upon clathrin depletion. Cells were scraped off the tissue culture plates in lyses buffer and fractionated by ultracentrifugation into membrane (P) and cytosol (S) fraction. Both fractions were analyzed by SDS-PAGE and Western blotting. The membrane-bound fraction of CVAK104 is reduced in clathrin-knockdown cells. C) Distribution of CVAK104 in clathrin-depleted HeLa cells. To view knockdown and control cells within the same field, control and knockdown cells were trypsinized, mixed and replated 24 hours prior immunostaining with antibodies to CVAK104 and clathrin HC. In clathrin-depleted cells CVAK104 shows a more diffuse staining. Bar, 10 μ m.

To further examine the effect of clathrin depletion on the membrane association of CVAK104, we performed immunofluorescence microscopy in clathrin knockdown cells. For this approach siRNA-treated cells were co-plated with control cells. This procedure allowed the microscopic observation of control and knockdown cells within the same field. Immunofluorescence studies clearly showed that in clathrin-depleted cells CVAK104-staining became very diffuse which suggested that the protein had dissociated from the TGN and endosomes (Figure 3.17C). In AP1-depleted cells we only noted a minor phenotype which we believe is caused indirectly by a concomitant reduction in TGN-associated clathrin (data not shown). These data confirmed the results that were obtained by cell fractionation and clearly demonstrate that clathrin is not only a binding partner *in vitro* but that in addition it is essential for targeting CVAK104 to the TGN and a subpopulation of endosomes.

The *in vitro* binding studies clearly showed that the association with clathrin is mediated by the C-terminal segment 699-929 of CVAK104. The clathrin-dependent perinuclear localization of CVAK104 thus strongly suggests that the correct localization of CVAK104 is supported by its C-terminal segment that starts at ⁶⁹⁹Gln. To test this hypothesis we transiently expressed the fragment CVAK104-(699-929) as a GFP-fusion protein in COS-7 cells (Figure 3.18A). Fluorescence microscopy analysis revealed that like full-length GFP-CVAK104 (Figure 3.14B) also the C-terminal segment of CVAK104 was recruited to the perinuclear area where it colocalizes with clathrin and AP1 (Figure 3.18B). In addition, substantial amounts of the fusion protein showed a nuclear localization which is probably due to the small size of the fusion protein and the net basic charge of the CVAK104 C-terminal segment (Figure 3.18B). Furthermore we observed the tendency of GFP-CVAK104-(699-929) to form aggregates which showed no colocalization with clathrin or AP1. In sum, these data support the conclusion that the C-terminal part of CVAK104, which interacts with the clathrin heavy chain *in vitro*, mediates the recruitment of CVAK104 to clathrin-positive structures in the perinuclear area.

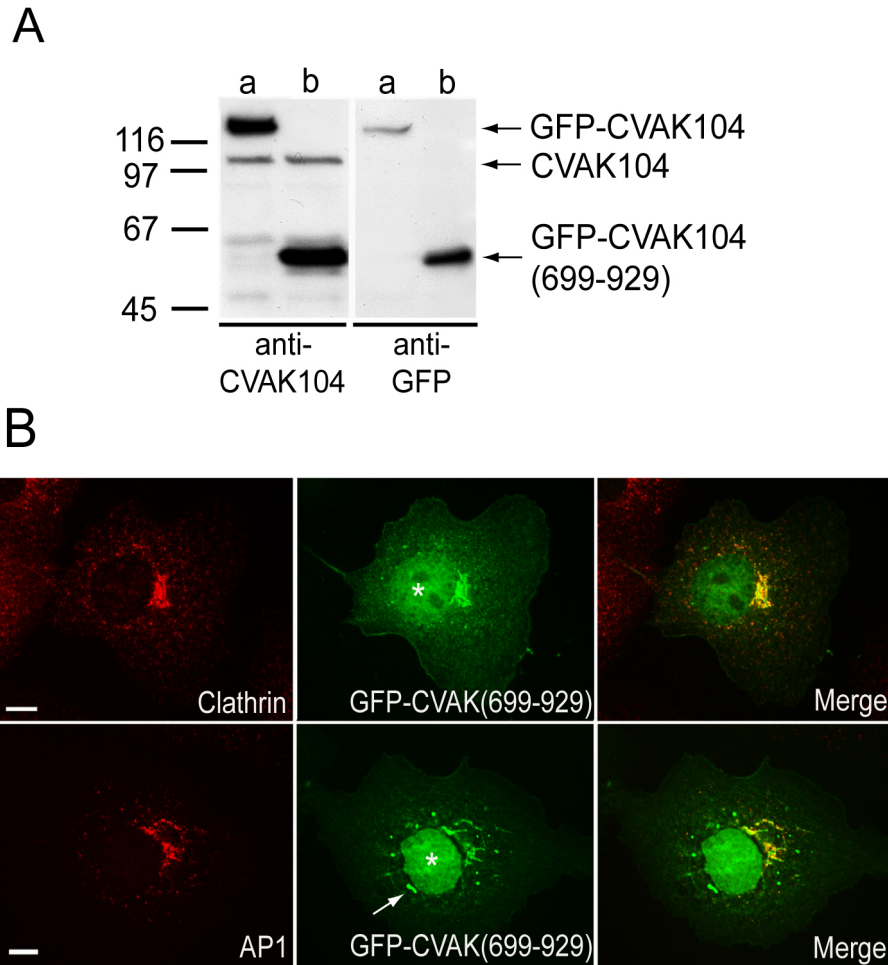


Figure 3.18: Subcellular localization GFP-CVAK104-(699-929) in COS-7 cells. A) Western blot analysis of lysates from COS-7 cells either expressing GFP-CVAK104 full-length (a) or GFP-CVAK104-(699-929) (b). Blots are stained for CVAK104 and for GFP. B) GFP-CVAK104-(699-929) expressing cells were stained for clathrin HC and AP1, respectively. A blob-like structure positive for GFP-CVAK104-(699-929) that does not colocalize with AP1 is marked by an arrow. Note, that the transiently expressed GFP-CVAK104-(699-929) fusion protein is also localized to the nucleus (denoted by asterisks). Bars, 10 μ m.

3.6.2.2 Interaction of CVAK104-(699-929) with actin

When GFP-CVAK104-(699-929) was transiently expressed in COS-7 cells, we occasionally observed some kind of bundles throughout the cytoplasm of the cells. Intriguingly, actin staining with phalloidin revealed that the actin filaments were partly decorated with the GFP fusion protein of CVAK104-(699-929) (Figure 3.19A+B). Moreover, both proteins seem to coincide rarely at the plasma membrane and at cell-cell contacts. However, we could not confirm these observations when GFP-CVAK104 full length was expressed in COS-7 cells. Nevertheless this result prompted us to test if CVAK104 is capable in actin-binding *in vitro*. For this aim recombinant GST-CVAK104 was incubated with or without polymerized F-actin

for 45 min at room temperature and then ultracentrifuged. The analysis of pellet- and supernatant-fractions by SDS-PAGE and Western blot revealed that substantial amounts of GST-CVAK104 associated and co-pelleted with F-actin (Figure 3.19C).

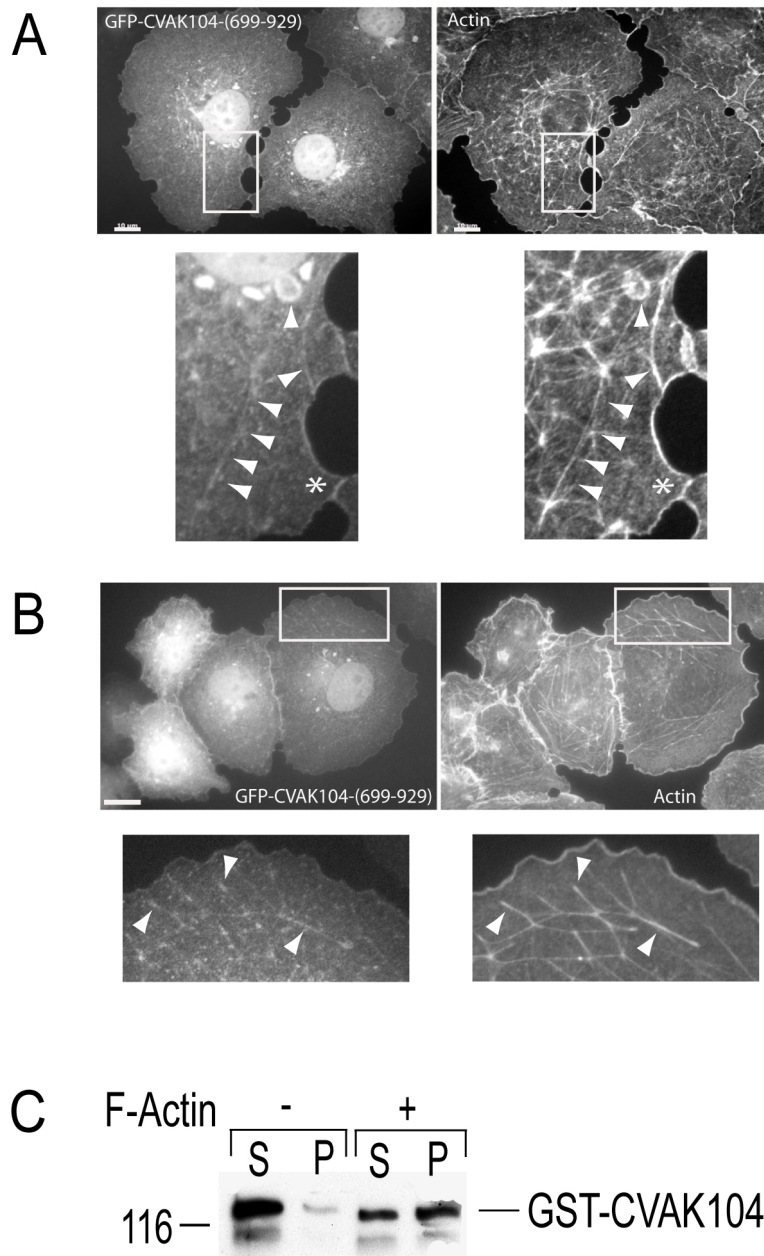


Figure 3.19: Relationship between CVAK104 and actin. A + B) GFP-CVAK104-(699-929) expressing cells were stained for actin. The second row in A and B, respectively, shows enlarged regions of the cell marked with a box. GFP-CVAK104-(699-929) decorated actin filaments are indicated by arrowheads. The asterisk marks a cell-cell contact that is positive for both, GFP-CVAK104-(699-929) and actin. Bars, 10 μ m. C) Pulldown of CVAK104 with F-actin. Recombinant GST-CVAK104 was incubated with or without F-actin. After ultracentrifugation pellet (P) and supernatant (S) fractions were analyzed by SDS-PAGE and Western blot with an anti-CVAK104 antibody. Note that GST-CVAK104 co-sedimented with F-actin.

The experiment was also performed with GST instead of GST-CVAK104 and showed that the GST-tag is not responsible for the association of the fusion protein with F-actin (data not shown).

Taken together, the fluorescence microscopy and the binding-assay data suggest that CVAK104 might be an actin interacting protein.

3.6.2.3 Determination of other recruiting factors for CVAK104

The *in vitro* binding studies and the clathrin RNAi experiments clearly demonstrated that CVAK104 interacts directly with the clathrin heavy chain and that clathrin is required for the correct perinuclear localization of CVAK104. Interestingly, CVAK104 is recruited by clathrin to perinuclear membranes but not to the plasma membrane. This suggests that there is at least one further recruiting factor, which promotes in addition to clathrin the recruitment of CVAK104 to the TGN and to endosomes. The small GTPase ADP-ribosylation factor1 (ARF1) is responsible for the localization of proteins such as the adaptor protein AP1 to the TGN (Stamnes and Rothman, 1993; Traub *et al.*, 1993; Ooi *et al.*, 1998). The fungal metabolite brefeldin A (BFA) inhibits binding of ARF1 to Golgi membranes and thus disrupts the ARF-dependent TGN localization of AP1 for instance. To determine whether the association of CVAK104 with perinuclear membranes is also an ARF-dependent process, HeLa cells were treated with BFA for 4 min and then stained for AP1 and CVAK104. Both, AP1 and CVAK104 changed from a predominantly perinuclear localization to a broad cytoplasmic distribution (Figure 3.20). In contrast, the brief BFA treatment did not disrupt significantly the perinuclear location of the TGN marker Syntaxin 6 (Figure 3.20). This ensures that the redistribution of AP1 and CVAK104 is not due to the disruption of the TGN.

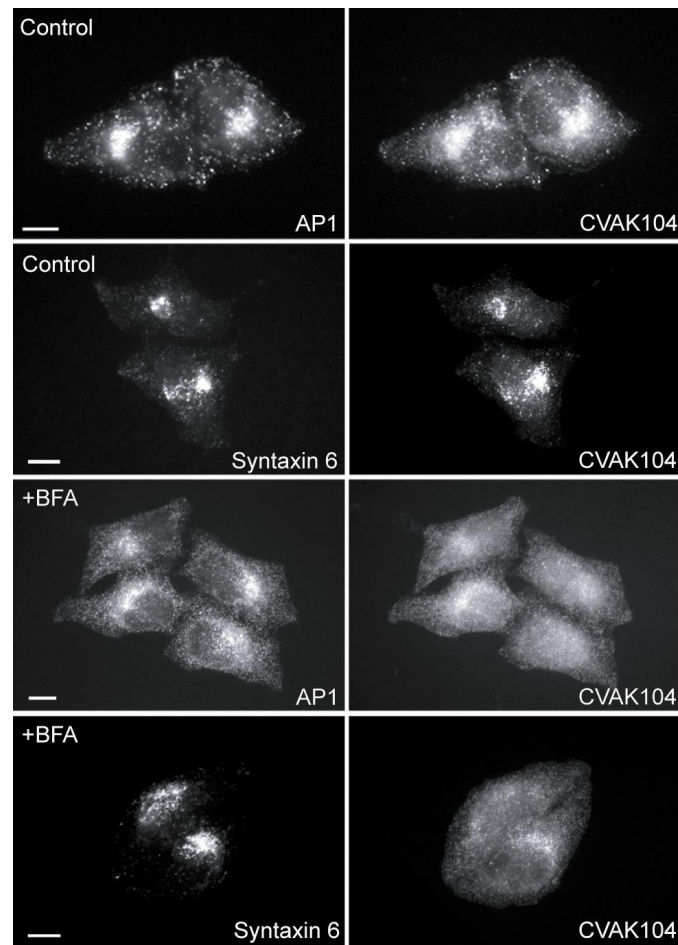


Figure 3.20: Effect of brefeldin A (BFA) on CVAK104 localization. HeLa cells were incubated with BFA-containing media (+BFA) or with medium alone (control) for 4 min at 37°C, fixed and double stained for either CVAK104 and AP1 or for CVAK104 and the TGN-marker Syntaxin 6. Both AP1 and CVAK104 disperse in response to BFA whereas the localization of syntaxin 6 remains unaffected. Bars, 10 μ m.

Next we analyzed the biochemical requirements for the association of CVAK104 with Golgi- and endosomal membranes using an *in vitro* recruitment assay. In this assay digitonin-permeabilized PtK2-cells were incubated with pig brain cytosol for 30 min at 30°C in the absence or presence of ATP and/or GTP γ S. Recruitment of CVAK104 and AP1 was detected by immunofluorescence (Figure 3.21). Upon inclusion of an ATP-regenerating system to the cytosol no significant binding above background level of CVAK104 and AP1 was observed (Figure 3.21A and B). However, in the presence of GTP γ S, a poorly hydrolyzable GTP analogue that locks GTPases such as ARF in their active membrane bound state, substantial amounts of both proteins were found on Golgi and/or endosomal membranes of the permeabilized PtK cells (Figure 3.21C). The GTP γ S induced recruitment of CVAK104 and AP1 was not significantly altered when the ATP-regenerating system was additionally present in the assay (Figure 3.21D). These results strongly suggest that the perinuclear localization of

CVAK104 is at least indirectly dependent on a GTP-binding protein, probably on ARF1. ARF1 is also known to stimulate the synthesis of phosphatidylinositol-4,5-bisphosphate on the Golgi-complex by recruiting phosphatidylinositol-4-OH kinase-beta (Godi *et al.*, 1999). Therefore, we considered the possibility that CVAK104 might be recruited onto membranes *via* PIP₂. However, a binding assay with phosphoinositides spotted onto a nitrocellulose filter and recombinant GST-CVAK104 revealed no significant association of CVAK104 with phosphoinositides (data not shown).

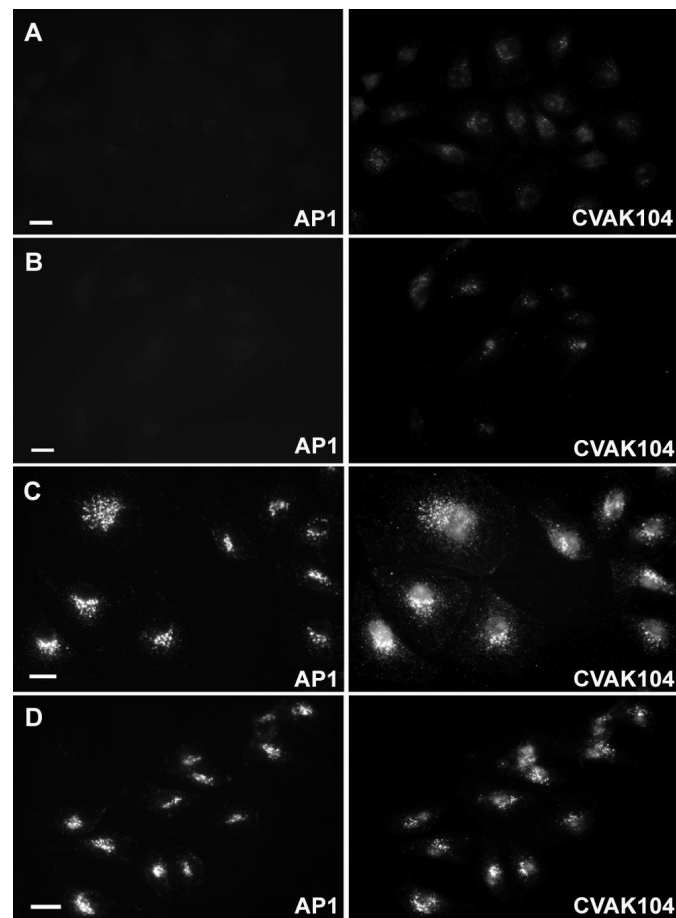


Figure 3.21: Recruitment of CVAK104 and AP1 by permeabilized cells. PtK2 cells were permeabilized by treatment with 40 µg/ml digitonin for 5 min on ice. The cells were then incubated at 30°C for 30 min with rat brain cytosol either alone (A) or in the presence of an ATP-regenerating system (B), GTP γ S (C) or in the presence of both, an ATP-regenerating system and GTP γ S (D). Cells were fixed and immunostained for AP1 and CVAK104. Recruitment of CVAK104 only occurs in the presence of GTP γ S, indicating that it requires a GTP binding protein. Bars, 10 µm.

3.7 Live-cell imaging of GFP-CVAK104

To further characterize the subcellular localization of CVAK104 and to examine its dynamic behavior we decided to image GFP-CVAK104 *in vivo*. COS-7 cells were transiently transfected with GFP-CVAK104 and imaged the following day. Most strikingly, GFP-CVAK104 labeled organelles or vesicles were highly motile and moved throughout the cell periphery (Figure 3.22A and supplementary movie 1).

The colocalization of clathrin with CVAK104 observed in fixed cells prompted us to co-transfect COS-7 cells with GFP-CVAK104 and RFP-tagged clathrin light chain A (RFP-CLC) and to image both proteins in living cells. The series of frames from supplementary movie 2 shown in Figure 3.21B illustrate that GFP-CVAK104 and RFP-CLC are often localized to the same rapidly moving structures. Furthermore, when GFP-CVAK104 expressing COS-7 cells were allowed to take up Texas-Red-labeled transferrin for 5 min it was observed that GFP-CVAK104 and transferrin often moved together (suppl. movie 3 and Figure 3.22C). Overall, the live-cell imaging data confirm the colocalization of CVAK104 with clathrin and internalized transferrin and implicate a role of CVAK104 in clathrin-mediated endosomal trafficking.

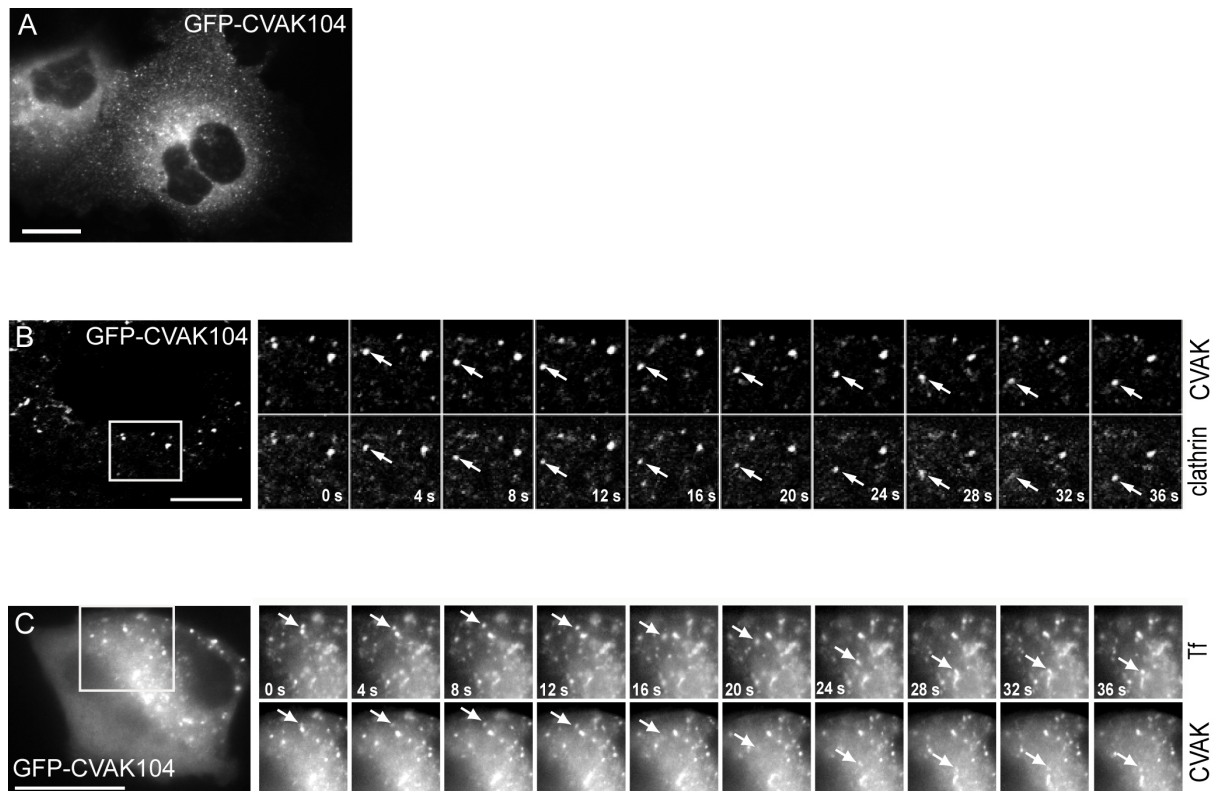


Figure 3.22: Live fluorescence microscopy of GFP-CVAK104. A) Frame of a video (see supplementary movie 1) showing COS-7 cells transfected with GFP-CVAK104. B and C) COS-7 cells were transfected with GFP-CVAK104 and either cotransfected with RFP-tagged clathrin light chains (B) or incubated with Texas-Red labeled transferrin (C) and then analyzed by live cell imaging (see supplementary movie 2 and 3). Frames were acquired every 4 sec. The series of frames are taken from the movies and show an enlarged region of the cells. The arrows indicate moving structures positive for both GFP-CVAK104 and either RFP-CLC or transferrin. The frames of the movie illustrated in B are confocal images. Bars, 10 μ m.

3.8 Depletion of CVAK104 by RNAi

3.8.1 Effect of CVAK104 depletion on subcellular localization of clathrin, AP1 and AP2

CVAK104 is a novel kinase-related protein that is found in CCV preparations. The lack of conserved catalytic residues in the kinase-like domain and the negative results in the *in vitro* kinase-assays with recombinant and endogenous CVAK104 let us favor the notion that CVAK104 has other accessory functions in clathrin-mediated trafficking processes rather than a function as an active phosphotransferase. To better understand the cellular role of CVAK104 we started to characterize cells in which CVAK104 was depleted by post-transcriptional gene silencing. With a siRNA that targets the mRNA sequence of CVAK104

we obtained an efficient knockdown of the endogenous protein in HeLa and COS-7 cells (Figure 3.23A). Upon establishing the importance of clathrin for the targeting of CVAK104 to membranes we first wanted to know whether CVAK104 is of similar importance for the association of clathrin and its adaptor protein AP1 with the TGN and/or endosomes. However, by immunofluorescence microscopy no obvious effects on the distribution of clathrin, AP2 and AP1 were observed (Figure 3.23B). This indicates that clathrin and the adaptor proteins AP1 and AP2 do not require CVAK104 for their correct localization in HeLa cells.

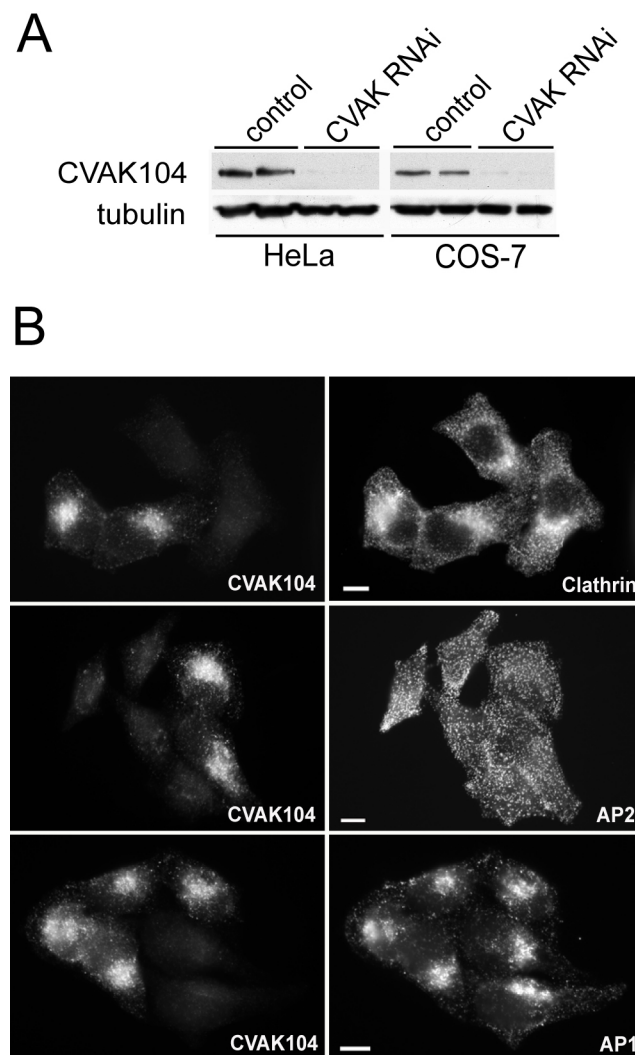
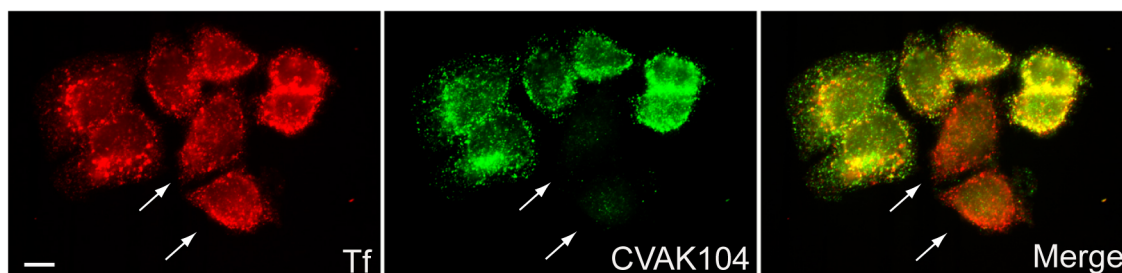


Figure 3.23: Subcellular localization of clathrin, AP1 and AP2 in CVAK104-depleted cells. A) Western blot analysis of lysates from HeLa and COS-7 cells either mock-transfected or transfected with a CVAK104-specific siRNA. Tubulin staining served as loading control. B) 48h after transfection HeLa cells transfected with CVAK104-specific siRNAs and mock-transfected cells were trypsinized, mixed and grown for another 24 h on coverslips. This made it possible to view knockdown and control cells within the same field. The cells were double stained for CVAK104 and either clathrin heavy chain, the α -adaptin subunit of AP2 or the γ -adaptin subunit of AP1. Bars, 10 μ m.

3.8.2 Uptake and recycling of transferrin in CVAK104-depleted cells

Next we wanted to explore the functional relevance of CVAK104 in endocytosis and in the recycling of internalized ligands. The iron-transporter transferrin is endocytosed via clathrin-coated vesicles and recycled back to the plasma membrane through the endosomal system. It therefore provides a classical sensor for the analysis of these pathways. In clathrin- and AP2-depleted cells, for instance, the uptake of transferrin is strongly reduced (Hinrichsen *et al.*, 2003). However, in CVAK104 knockdown cells the uptake of Texas Red-labeled transferrin revealed no significant alterations relative to the transferrin uptake in control cells (Figure 3.24A). The endosomal localization of CVAK104 prompted us to test if CVAK104 is involved in the recycling of transferrin receptors. To answer this question CVAK104 knockdown and control cells were incubated with [^{125} I]-labeled transferrin for 30 min at 37°C. Afterwards cells were allowed to externalize the ligand into the media for various times. Finally, cells were lysed and the radioactively labeled transferrin that was not released from the cells was measured in the lysates. The rate at which internalized transferrin is released from cells can be used as a measure of the rate at which internalized transferrin receptors return to the cell surface. The knockdown of CVAK104 either by siRNA oligo I or II caused only a slight delayed externalization of endocytosed transferrin (Figure 3.24B). This minor effect could not be reproduced when the same assay was performed with Texas Red-labeled transferrin and analyzed by fluorescence microscopy (data not shown). In sum, it can be concluded that CVAK104 has no significant influence on the endocytosis and the recycling of transferrin.

A



B

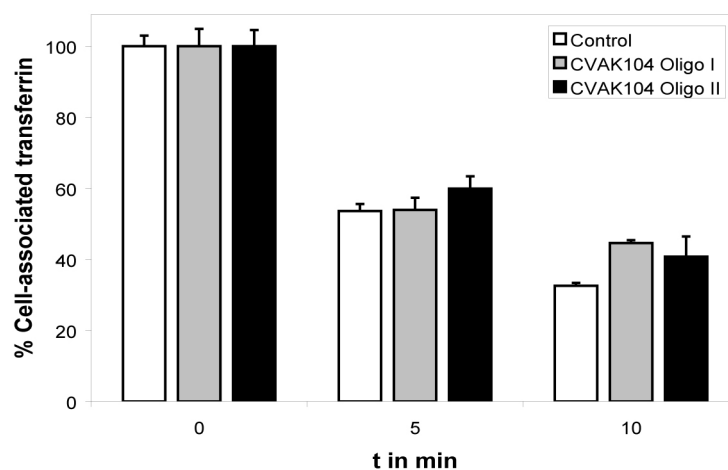


Figure 3.24: Uptake and recycling of transferrin in CVAK104 knockdown cells. A) 48h after transfection HeLa cells transfected with CVAK104-specific siRNAs and mock-transfected cells were trypsinized, mixed and grown for another 24 h on coverslips. This made it possible to view knockdown and control cells within the same field. Cells were allowed to internalize surface-bound Texas Red-labeled transferrin for 5 min at 37°C. Cells were then stained for CVAK104. CVAK104 knockdown cells - indicated by an arrow - exhibit no impaired transferrin internalization. Bars, 10 μ m. B) HeLa cells either mock transfected or transfected with two different CVAK104 siRNAs (oligo I and II) were allowed to endocytose [^{125} I]-labeled transferrin for 30 min at 37°C. After removal of unbound ligands cells were incubated for the indicated time points at 37°C. Cells were then lysed and analyzed for cell-associated transferrin/radioactivity. In CVAK104-depleted cells the externalization rate of transferrin is only slightly delayed relative to the rate in control cells.

3.8.3 Cathepsin D sorting assay

CVAK104 shows very convincing colocalization with the adaptor protein AP1 and its localization also partly coincides with that of the cation-independent mannose 6-phosphate receptor (CI-M6PR). AP1 and the M6PR are involved in the sorting of lysosomal enzymes into clathrin-coated vesicles and the transport from the TGN *via* endosomes to the lysosomes (Rouille *et al.*, 2000). As demonstrated before, depletion of AP1 leads to an increased secretion of lysosomal hydrolases due to an impaired M6PR dependent sorting of these enzymes at the TGN (Meyer *et al.*, 2000; Hirst *et al.*, 2003). In considering a role for CVAK104 in the sorting of lysosomal enzymes we next investigated whether the sorting of the lysosomal hydrolase cathepsin D is affected in CVAK104-depleted cells. Cathepsin D is a proteolytic enzyme involved in protein catabolism. The enzyme is synthesized in the rough Endoplasmic Reticulum as a larger pre-proenzyme, which is subsequently converted to its mature form during its transport to lysosomes (Erickson, 1989). The cleavage of a pre-peptide results in a 52- to 53-kDa catalytically inactive pro-enzyme. Next, the pro-enzyme is converted to a single-chain cathepsin D (47- to 48-kDa) by removal of a 44-amino acid pro-peptide. The single-chain cathepsin D undergoes further cleavage into a two-chain enzyme formed by a light chain (14 kDa) and a heavy chain (31–34 kDa). In the experiment siRNA-transfected COS-7 cells were labeled with [³⁵S] methionine for 2 h and were then chased with unlabeled amino acids for 4 h to allow the newly synthesized cathepsin D to follow the secretory pathway from the Endoplasmic Reticulum, to the Golgi and from there *via* the TGN either to the endosomal/lysosomal compartment or to the cell surface from where it is released into the culture medium. Intracellular and secreted cathepsin D was immunoprecipitated and the precipitates were analyzed by nonreducing SDS-PAGE and autoradiography. Each experiment was performed in duplicate and a representative result is shown in Figure 3.25. We observed a three-fold decrease in cell-associated cathepsin D precursor in cells depleted of either γ -adaptin or CVAK104 compared to cells transfected with a control siRNA. Moreover, the percentage of secreted cathepsin D relative to cell-associated mature plus secreted cathepsin D was 21% in control cells, 25% in AP1-depleted cells and 33% in CVAK104-depleted cells. Interestingly, the CVAK104 knockdown seemed to have a more severe effect on cathepsin D sorting than the AP1 depletion. But this phenotype can readily be explained by the different efficiencies of the knockdowns. CVAK104 expression in the siRNA-treated COS-7 cells was found to be 15% of control whereas the AP1 expression was only reduced to 38% of control (data not shown). Thus, the loss of CVAK104 causes

missorting of a significant amount of cathepsin D from the TGN/endosomal pathway to the cell surface. Taken together these data provide evidence that CVAK104 functions chiefly in clathrin-dependent trafficking processes at the level of the TGN and the endosomes.

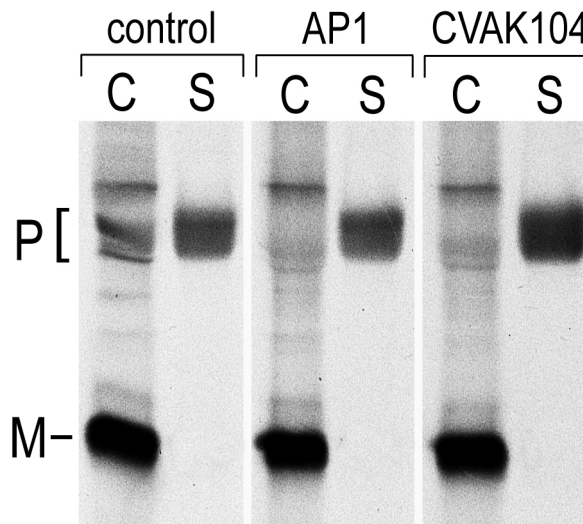


Figure 3.25: Sorting of cathepsin D in CVAK104-depleted cells. COS-7 cells transfected with GFP (control), γ -adaptin (AP1) or CVAK104 siRNA were pulsed for 2 h with [^{35} S]-labeled methionine followed by a 4 h chase with unlabeled methionine. Cell-associated Cathepsin D was immunoprecipitated from cell lysates (C) and secreted cathepsin D from culture media (S). Samples were analyzed by SDS-PAGE and autoradiography. The positions of the precursor (P) and mature (M) forms of the enzyme are indicated. AP1 and CVAK104 knockdown cells exhibit decreased levels of cell-associated and increased levels of secreted cathepsin D precursor compared to the levels in control cells.

4. Discussion

4.1 Catalytic activity of CVAK104

Phosphorylation is a fundamental mechanism by which interactions of proteins and thereby the function of these proteins can be regulated. The clathrin-coated vesicle (CCVs) machinery seems not to be an exception, since many of the involved constituents are subjected to cycles of phosphorylation and dephosphorylation *in vitro* and *in vivo*. Some of the responsible kinases such as AAK1 and GAK have been shown to copurify with isolated CCVs (Korolchuk and Banting, 2003). Recently a potential new member of CCV-associated kinases was identified in a mass spectroscopic analysis of CCVs and later on termed CVAK104 (coated-vesicle-associated kinase of 104 kDa) (Blondeau *et al.*, 2004). CVAK104 contains a putative serine/threonine kinase domain and by using the gel chromatographic procedure of Hummel and Dreyer we observed ATP binding to CVAK104 (Figure 3.4). However, in the course of our characterization accumulating evidences suggested that CVAK104 is lacking catalytic activity. First, in *in vitro* kinase assays using either recombinantly expressed CVAK104 or the immunoprecipitated protein from eukaryotic cells we were not able to demonstrate that CVAK104 can phosphorylate any substrate tested, including clathrin, auxilin, AP2 or itself. Second, sequence analysis revealed that CVAK104 has indeed a kinase-like domain but it lacks several conserved residues that are required for catalysis. Therefore CVAK104 belongs to the group of ‘pseudokinases’. Genome-wide analysis revealed that ~10% out of 518 protein kinases encoded by the human genome have been classified as pseudokinases (Manning *et al.*, 2002). The common feature of these pseudokinases is that they lack at least one of three conserved kinase motifs in their catalytic domain. CVAK104 belongs to the SCY1-like family of protein kinases (referred to as SCYL2) that actually lack invariant residues in all three motifs of their pseudokinase domain (Manning *et al.*, 2002).

While this study was in progress, S. Conner and S. Schmid demonstrated that recombinant CVAK104 purified from insect cells is able to phosphorylate itself and the adaptor protein complex AP2 *in vitro* (Conner and Schmid, 2005). In fact, the lack of essential catalytic residues does not necessarily mean that the respective kinase is inactive. For example, the kinase WNK1, which plays an important role in the regulation of electrolyte homeostasis, lacks the invariant catalytic lysine residue in one of the conserved kinase motifs

(Xu *et al.*, 2000; Kahle *et al.*, 2004). However, WNK1 has been shown to be catalytically active. Structural analysis of the WNK1 kinase domain suggested that a lysine residue in another subdomain substitutes for the missing lysine residue in the conserved kinase motif (Min *et al.*, 2004). The idea that other residues substitute for the lack of invariant residues has also been used to explain the activity of other pseudokinases (Boudeau *et al.*, 2006), but in these cases the active ‘pseudokinases’ exhibit only one degenerated conserved kinase motif. SCYL2 (CVAK104) on the other hand lacks essential residues in three kinase motifs. Together with our unsuccessful attempts to confirm the *in vitro* kinase activity of CVAK104 demonstrated by Conner *et al.*, this data strongly suggests CVAK104 to be an inactive pseudokinase. Conner and Schmid could have corroborated their data by introducing additional disabling mutations in the kinase-like domain of CVAK104. The loss of phosphotransferase-activity would support the conclusion that CVAK104 is responsible for the measured activity and not a contaminating insect cell kinase. Indeed, several pseudokinases have been shown to form complexes with active protein kinases in mammalian cells, which thereby might simulate a kinase-activity of the purified pseudokinase (Boudeau *et al.*, 2006). Moreover, the conditions in an *in vitro* kinase assay do not reflect the situation in the cell. Given that AP2 is definitely a phosphoprotein (Wilde and Brodsky, 1996) it would have been interesting to see whether AP2-phosphorylation *in vivo* is affected in CVAK104-deficient cells.

The detailed sequence analysis of the CVAK104 pseudokinase domain has shown that it is highly conserved between species ranging from mammals to fish and is predicted to lack activity in most of the species. This indicates that the mutated residues and the pseudokinase-domain of CVAK104 in general are likely to have a crucial role. In this context it is noteworthy that pseudokinases are well known to regulate diverse cellular processes despite lacking the ability to phosphorylate substrates (an overview gives (Boudeau *et al.*, 2006). One function of pseudokinases is the regulation of the activity of functional kinases. For instance, the EGF receptor protein-tyrosine kinase (RTK) HER3 is classified as a pseudokinase but is nevertheless crucial in the stimulation of the autophosphorylation and activation of other members of this receptor family, namely HER1, HER2 and HER4 (Zhang *et al.*, 2006). Another possibility that emerges from the ongoing characterization of pseudokinases is that these proteins participate in the formation of multi-protein complexes as a scaffolding factor. For this role these proteins feature protein-protein interaction domains or motifs in addition to their pseudokinase domain. Taken together the properties of known pseudokinases suggest

that CVAK104 features similar pseudokinase-functions. Before discussing this in detail in chapter 4.3 it is necessary to review the specific properties of CVAK104.

4.2 Binding capacities and determinants for subcellular localization of CVAK104

Clathrin and adaptor protein complexes are now considered to be part of a complex network of interacting accessory proteins that contribute to CCV formation. The identification of CVAK104 in a CCV preparation indicated that this protein is a *bona fide* component of the CCV machinery. As a first step towards elucidating the role of CVAK104 in this machinery the binding partners of this novel CCV-associated protein were identified.

The interaction of CVAK104 with other constituents of CCVs was examined using pulldown assays with recombinantly expressed GST-CVAK104 as bait. Like Conner *et al.* we could demonstrate that CVAK104 strongly interacts with the clathrin heavy chain and with somewhat lower affinity with the AP2 adaptor *in vitro* (Conner and Schmid, 2005). Clathrin-cage binding assays further confirmed that the association with triskelions is direct and does not involve the clathrin light chains (Figure 3.7). The interactions with both, clathrin and AP2, are mediated through binding sites within a C-terminal CVAK104 domain, starting with residue 699. Surprisingly, we did not detect any of the well characterized short protein-protein interaction motifs for clathrin and AP2, respectively, within this region. Moreover, two potential clathrin-binding motifs (DLL) at positions 434 and 655 are not functional, probably because they are not exposed at the surface of the CVAK104. The linear peptide motifs by which accessory proteins interact with clathrin and adaptor protein complexes are located within unstructured regions of the protein thereby allowing the flexible and cooperative binding of multiple copies of these motifs to the corresponding interaction partners (Kalthoff *et al.*, 2002). Likewise the C-terminal domain of CVAK104 is also predicted to be only poorly structured.

Pulldown assays with recombinant clathrin fragments revealed that the C-terminal domain of CVAK104 interacts directly with the N-terminal domain of the clathrin heavy chain (TD) (Figure 3.10). The TD has emerged as the major protein-interacting domain for clathrin binding partners. Structural analysis of clathrin coats by cryo electron microscopy showed that three TDs from different clathrin triskelions are in close proximity underneath the vertex. This constellation is perfect for contacting membrane-associated adaptors and

accessory proteins (Heuser and Kirchhausen, 1985; Smith *et al.*, 1998; Musacchio *et al.*, 1999; Kirchhausen, 2000). Due to the fact that some accessory proteins like AP180 and epsin bear multiple binding motifs for the clathrin TD that are tandemly arranged in an unstructured region they are able to cross-link different clathrin molecules during polymerization, thereby promoting the assembly of a clathrin-coat. However, a clathrin assembly experiment demonstrated that CVAK104 was not able to support the polymerization of soluble triskelions into cages (data not shown). This result suggests that CVAK104 harbors only one clathrin-interaction motif or multiple copies that can not be arranged in a manner to allow cross-linking of different triskelions.

X-ray crystallography analysis of the 330 residue β -propeller N-terminal domain of clathrin complexed with peptide ligands revealed that the clathrin-box motif binds to a site between blade 1 and 2 of the seven-bladed β -propeller, whereas the W-box motif PWDLW was shown to interact with the top face of the β -propeller (Owen *et al.*, 2004). The interaction of CVAK104 with clathrin is efficiently competed by a fragment of AP180 (³²⁹Asp-⁷⁴⁶Leu) that contains five DLL motifs and by the classic clathrin box motif of the β 2-subunit of AP2 (Figure 3.10). This suggests that, for its association with clathrin, CVAK104 requires a site located in between blade 1 and 2 of the seven-bladed propeller structure of the clathrin N-terminal domain. Since both, the DLL motifs and the clathrin box motifs interfered efficiently with CVAK104 binding to clathrin, it can also be concluded that AP180 and AP2 bind to the same or at least overlapping sites on the clathrin N-terminal domain. In contrast, the interaction of the C-terminal segment of CVAK104 with AP2 appears to involve an unconventional site on the α -appendage domain of AP2. This is supported by two lines of reasoning: first, there are neither DPF/W- or FXDXF type nor WXX(F/W)X(D/E) type binding motifs present in CVAK104. The former are known to engage the α -appendage platform domain and the latter the α -appendage sandwich subdomain. Second, mutations in the subdomains that are known to prevent binding to the subdomains failed to completely abolish CVAK104 binding (data not shown).

To answer the question where exactly the binding motifs for clathrin and AP2 are located within the C-terminal region of CVAK104, and whether these motifs are degenerated versions of existing binding sites or novel motifs, further investigations are needed. One possibility is to use an array of short and partly overlapping CVAK104 peptides synthesized *in situ* on cellulose membranes. The incubation of the membrane with recombinantly expressed clathrin TD or the α -appendage subdomain of AP2 could help to identify the binding-sites of CVAK104 for these protein domains. Such a peptide array was already

successfully used to locate clathrin- and AP2-binding motifs within the sequence of auxilin (Scheele *et al.*, 2003), and also to determine auxilin-residues that are phosphorylated by CCV-associated kinases *in vitro* (Düwel, 2003). As an alternative approach we are currently generating smaller fragments of the C-terminal region of CVAK104 by site-directed mutagenesis and PCR for further pulldown assays. After defining the clathrin-binding peptide motif of CVAK104, a co-crystallization experiment of the clathrin TD with this peptide followed by X-ray crystallography analysis could reveal the exact binding-site in the β -propeller N-terminal domain of clathrin for CVAK104.

Taken together these data suggest that CVAK104 is a component of AP2-containing clathrin coated structures at the plasma membrane or a component of endocytic coated vesicles. Unexpectedly, however, the subcellular localization of CVAK104 only partly reflects the *in vitro* binding data. We observed that endogenous as well as transiently expressed GFP-tagged CVAK104 was not found at the plasma membrane, but was instead localized to the TGN and to endosomal membranes of HeLa- and COS-7 cells. CVAK104 staining overlaps significantly with that of perinuclear clathrin, AP1, AP3 and endocytosed transferrin in the area of the TGN and throughout the cytoplasm (Figure 3.13, 3.14 and 3.15). In contrast there is little co-localization of CVAK104 with the early endosomal marker EEA1 (see Figure 3.14B and 3.16A). Outside the juxtanuclear area the number of CVAK104 positive structures exceeds those of AP1 probably because CVAK104 is also present on AP3 structures. AP1 is known to be located at the TGN and on tubulo-vesicular structures that also contain GGA1 and the mannose-6-phosphate receptor (Puertollano *et al.*, 2001; Waguri *et al.*, 2003). These structures are very dynamic and they undergo transient fusions with peripheral endosomes which allows for the mixing of their contents, e.g. transferrin (Waguri *et al.*, 2003; Polishchuk *et al.*, 2006), and explains the partial colocalization of AP1 with transferrin (Figure 3.16B and (Waguri *et al.*, 2003)). The triple labeling experiments demonstrating colocalization of transferrin with AP1 and CVAK104 (Figure 3.16B) strongly suggest CVAK104 to be a component of the tubulo-vesicular structures and the peripheral endosomes. However, the absence of EEA1 from the peripheral CVAK104 or AP1-positive structures suggests that they do not correspond to classical early endosomes but are more closely related to recycling endosomes.

Why is CVAK104 found together with clathrin and AP1 on internal membranes rather than together with AP2 at the plasma membrane? It is possible that the exclusion of CVAK104 from AP2-containing clathrin-coated pits results from the competition with other accessory proteins - such as AP180 - that bind to AP2 with higher affinity. This conjecture is

supported by the observation that CVAK104 was able to pull-down AP2 only from highly enriched adaptor protein preparations ((Conner and Schmid, 2005) and this study) but not from rat brain cytosol, where the AP2 concentration is much lower than in the enriched fractions. Our *in vitro* binding studies also indicated that the interaction of intact CVAK104 with AP2 is very weak in contrast to its isolated C-terminal segment. One explanation might be that the AP2 binding site is only poorly accessible in the intact protein. It is possible that CVAK104 requires post-translational modifications to unmask the AP2-binding module or, alternatively, CVAK104 may need the disengagement of high-affinity AP2-binding partners from AP2 as a precursor for targeting AP2-containing CCPs. Such a scenario has been proposed for explaining the subcellular localization of the endocytic protein Numb. Numb interacts with the α -subunit of AP2 and is proposed to be a component of the endocytic machinery (Santolini *et al.*, 2000). In ‘untreated’ cultured cells Numb is dispersed throughout the cytoplasm and partially concentrated in the perinuclear Golgi region. However, upon stimulation of cells with EGF, Numb undergoes dramatic relocalization to the plasma membrane where it coincides with AP2 in CCPs. It is believed that Numb competes with Eps15 (EGF receptor pathway substrate clone no. 15) that has a higher affinity for the α -subunit than Numb. The disruption of Eps15-AP2 upon coat assembly could allow Numb to associate with AP2 and to relocate to CCPs (Cupers *et al.*, 1998; Santolini *et al.*, 2000). In any case, whether CVAK104 needs a ligand triggered stimulation before its function can be observed at the plasma membrane and whether CVAK104 is a biologically relevant AP2 binding partner in general, are interesting questions that require further investigations.

Importantly, the interaction of CVAK104 with clathrin is necessary for targeting the protein to the TGN- and to endosomal membranes, since upon clathrin depletion by RNAi CVAK104 was partially redistributed to the cytosol. On the other hand, the absence of CVAK104 from AP2-containing clathrin-coated pits indicates that the association with clathrin is not sufficient for the recruitment of CVAK104 to any clathrin-coated structure. It is thus likely that the recruitment of CVAK104 to specific membranes requires at least one more factor in addition to clathrin. The localization GFP-CVAK104-(699-929) to clathrin- and AP1-positive structures in the perinuclear region suggests that these additional recruiting factors also interact with the C-terminal domain of CVAK104. Although CVAK104 and AP1 show a very convincing colocalization, we can nevertheless rule out the possibility that both proteins may recruit each other. First, like Conner and Schmid (Conner and Schmid, 2005), we could not observe any interaction between AP1 and CVAK104 *in vitro* and, second, depletion of AP1 or CVAK104 by RNAi had little effect on the distribution of the respective

other protein. Possible recruiting factors for CVAK104 are Eps15 homology (EH) domain-containing proteins, since this domain is known to interact with the tripeptide NPF that is also located in the C-terminal region of CVAK104 (Figure 3.2). Although we could not identify an EH domain-containing binding partner for CVAK104 so far, our plans are to explore the importance of the NPF-motif for the subcellular localization of CVAK104 by a mutagenesis approach.

The GTP γ S-dependent association of CVAK104 with Golgi/and or endosomal membranes in permeabilized PtK2 cells and the effect of Brefeldin A suggest that small GTPases of the Rab or ARF families might be involved in the correct targeting of CVAK104. These GTPases can alternate between an active GTP-bound form, which is associated with an individual subset of internal membranes, and an inactive cytosolic GDP-bound form (Behnia and Munro, 2005; Jordens *et al.*, 2005). Thus, members of the Rab and ARF family would be ideally suited for the organelle-specific recruitment of CVAK104.

The identification of proteins that - in addition to clathrin - interact with CVAK104 could be the key for defining the exact mechanism by which CVAK104 is targeted to the TGN and to endosomes. Furthermore, the knowledge of CVAK104 binding partners would also help to determine the physiological function of this novel protein, which is discussed in the following chapter.

4.3 Function of CVAK104

As already discussed in chapter 4.1 the possible role of CVAK104 as a kinase ought to be viewed with caution until the original claim has been substantiated by specific mutations of residues involved in the catalytic mechanism or ATP binding. Furthermore, the absence of CVAK104 in AP2-containing clathrin-coated pits (CCPs) argues against a function in assisting CCV formation at the plasma membrane. Moreover, the internalization of transferrin is not affected in CVAK104-depleted cells. In contrast, the localization of CVAK104 at the TGN and at endosomes, which coincides almost perfectly with that of AP1 and partly with that of perinuclear clathrin, rather suggests that CVAK104 might be involved in vesicular trafficking processes at the Golgi and within the endosomal system. This conjecture was bolstered by the observation that a GFP-tagged fusion protein of CVAK104 was associated with highly dynamic vesicular structures in living cells that also contained clathrin and transferrin (Figure 3.22 and supplemental movies). To provide further evidence for a role of

CVAK104 in membrane trafficking events between the TGN and/or the endosomal system we investigated several pathways that are linked to these organelles and known to depend upon AP1. It was already shown that after depleting AP1, internalized transferrin is recycled more slowly, and that the number of transferrin-receptors on the plasma membrane is decreased (Hirst *et al.*, 2005). In contrast, RNAi-mediated CVAK104-depletion has only negligible effects on the recycling of internalized transferrin. This result does not necessarily rule out a function of CVAK104 in receptor recycling due to the fact that residual CVAK104 or other proteins might compensate or substitute for CVAK104 loss. However, the most striking phenotype observed so far in CVAK104-depleted cells is that the M6PR-dependent sorting of the lysosomal hydrolase cathepsin D, either at the TGN or in the peripheral endosomes was effected to the end that a significant amount of the newly synthesized enzyme was diverted from the lysosomal route to the cell surface and secreted from there into the culture medium (Figure 3.25). A phenotype that was also observed in a μ 1-deficient cell line (Meyer *et al.*, 2000) indicates that CVAK104 functions along with AP1 in the sorting of M6PR at the TGN. Recently, the results of Borner and co-workers have indicated a crucial role of CVAK104 in the sorting of membrane proteins to lysosomes and lysosome-related organelles (Borner, 2006). They demonstrated that *Xenopus tropicalis* embryos, in which CVAK104-expression has been diminished by antisense oligonucleotides, exhibit a lack in body and eye pigmentation. Surprisingly, perturbation of the AP1 expression revealed only a minor phenotype that was characterized by a hypopigmentation of the body and a somewhat reduced pigmentation of the eyes. Since pigment granules are related to the lysosomes in their origin, these data strongly suggest that CVAK104 mediates the transport of lysosomal membrane proteins. Additionally, the *Xenopus* studies imply a functional redundancy of CVAK104 and the adaptor protein complex AP3 since AP3 has also been proposed to be involved in the sorting pathway to pigment granules (Ooi *et al.*, 1997) and, moreover, both proteins colocalize in COS-7 cells (Figure 3.14B).

Although these data indicate important roles for CVAK104 in the context of a cell or an organism in general, the question remains how CVAK104 contributes to these pathways at the molecular level. So far, it can only be speculated, but the most likely possibility is that CVAK104 functions as a scaffolding protein for clathrin and contributes to CCV formation at the level of the TGN and endosomes. In this accessory function CVAK104 might connect clathrin with other factors and thereby stabilize protein complexes essential for the assembly of CCPs. Most of the accessory proteins of the CCV machinery that act specifically at the Golgi share the common feature that they harbor interaction motifs for both clathrin and the

adaptor AP1. Examples are the proteins epsinR, aftiphilin and the GGAs. CVAK104 differs from this group in that it fails - at least *in vitro* - to interact with AP1. Nevertheless, CVAK104 is predicted to interact with other proteins in addition to clathrin and thus has the potential to participate as a scaffold in the complex protein-protein interaction network of a CCP. This is supported by several lines of reasoning: first, as discussed in chapter 4.2, the fact that the interaction with clathrin is necessary but not sufficient for the specific recruitment of CVAK104 to perinuclear membranes argues for an additional binding factor located at these organelles. The GTP γ S-dependent and BFA-sensitive recruitment of CVAK104 suggest small GTPases like ARF1 as suitable candidates. Second, the C-terminal region of CVAK104 bears a NPF-tripeptide motif that is proposed to interact with EH-domain containing proteins such as γ -synergisin and Eps15. Moreover, both proteins (γ -synergisin and Eps15) have been localized to the TGN, where they interact with the γ -adaptin appendage domain and therefore could provide a physical link between CVAK104 and AP1 (Kent *et al.*, 2002; Hirst *et al.*, 2005). Third, the pseudokinase domain could function as a protein-protein interaction module. This module might be regulated by nucleotides since CVAK104 is an ATP binding protein ((Conner and Schmid, 2005) and this study). It has already been shown for other inactive kinases that they have important scaffolding roles and mediate the assembly of multi-protein complexes (reviewed in (Boudeau *et al.*, 2006)). The integrin-linked (pseudo)kinase (ILK), for instance, is - despite lacking a phosphotransferase-activity - crucial for the formation of a protein complex called IPP that has been implicated in the control of actin-cytoskeleton dynamics (Zamir and Geiger, 2001). The loss of ILK in mice results in early embryonic lethality, further strengthening the importance of this pseudokinase (Sakai *et al.*, 2003).

An even more speculative and so far unsubstantiated model, which includes the potential scaffolding feature of CVAK104, is that CVAK104 might recognize and interact with transmembrane proteins, either with the kinase-like domain or with other so far undefined domains. The simultaneous binding of clathrin could then promote the selective sequestration and packaging of protein cargo into CCPs. Such a model would classify CVAK104 as monomeric clathrin-associated sorting protein (CLASP) (Brett and Traub, 2006). Actually, it has become evident that the classical adaptor proteins such as AP2, AP1 and GGA do not physically recognize all of the sorting motifs known to promote clathrin-dependent internalization. The endocytosis of G protein-coupled receptors (GPCRs) for example is dependent on the endocytic CLASP β -arrestin. β -arrestin engages phosphorylated and therefore activated GPCRs and targets them to pre-existing endocytic clathrin structures by simultaneous interaction with clathrin and AP2 (Marchese *et al.*, 2003; Lefkowitz and

Whalen, 2004). CVAK104 may exercise such an alternate adaptor function at the TGN and/or endosomes thus promoting the selective trafficking of specific cargo molecules. To support this hypothesis it is absolutely necessary to identify the corresponding binding partner(s) of CVAK104. A powerful tool for this goal would be the usage of either full-length or fragments of CVAK104 as a bait in a yeast or mammalian two-hybrid system. This method has the advantage that it can detect interactions that may occur only transiently in the cell. Once identified, the importance of CVAK104 for the internalization of this cargo molecule could be tested by an RNAi approach: the depletion of CVAK should alter the intracellular distribution of the cargo molecule and furthermore, it should cause a diminution of this molecule within CCVs isolated from the CVAK104-RNAi-treated cells. The specific recruitment of CVAK104 to Golgi membranes and its putative function as scaffolding protein and CLASP, respectively, are illustrated in figure 4.1.

Finally, the *in vitro* interaction of CVAK104 with F-actin as well as the colocalization of GFP-CVAK104-(699-929) with actin bundles in COS-7 cells (Figure 3.19) point to an intriguing alternative accessory function for CVAK104. CVAK104 could provide a link between the clathrin-dependent trafficking machinery and the actin cytoskeleton. In this context it is important to know that the actin cytoskeleton is suggested to support the invagination as well as the scission of nascent CCVs. Another role for actin could be to propel CCVs away from the membrane and to move endosomes through the cytoplasm by the formation of actin tails (possible roles for actin in clathrin-mediated endocytosis are reviewed in (Kaksonen *et al.*, 2006)). Several other accessory proteins are known to link CCV formation and actin cytoskeleton dynamics either by interacting directly with actin and endocytic proteins or by recruiting actin polymerization nucleators to endocytic hotspots (Qualmann *et al.*, 2000; Engqvist-Goldstein and Drubin, 2003). For example, the clathrin-binding protein Hip1R (huntingtin-interacting protein 1-related) interacts directly with actin filaments and the knockdown of the protein causes an uncoupling of CCV formation from actin dynamics (Carreno *et al.*, 2004). Whether CVAK104 features a similar role remains to be investigated.

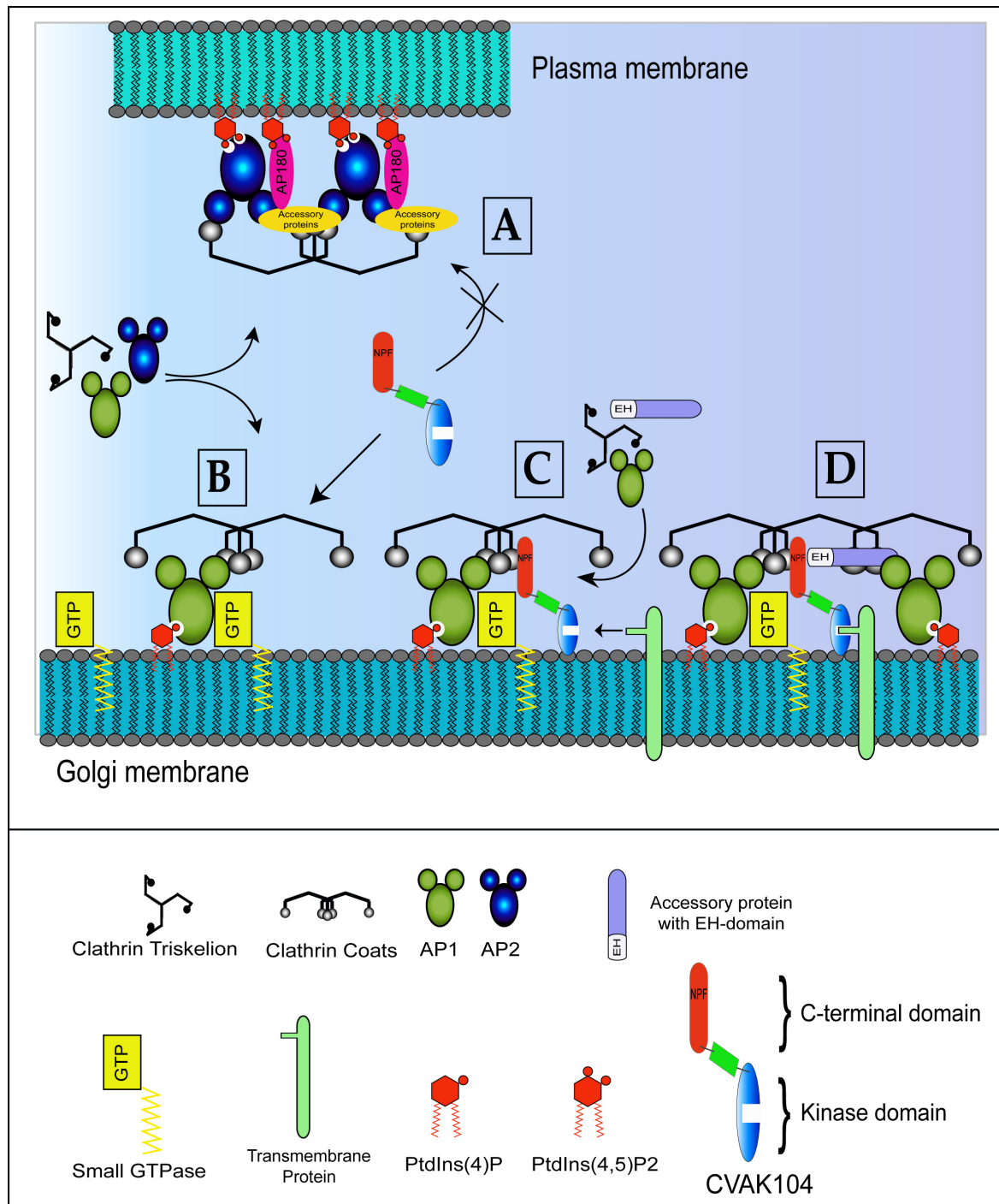


Figure 4.1: Schematic overview of the presumed recruitment of CVA104 to Golgi membranes and the accessory function of CVA104 during CCV formation.

- A) Despite interacting with clathrin and AP2 *in vitro* CVA104 is not recruited to plasma membrane localized clathrin-coated pits. This phenomenon is possibly due to the competition of CVA104 with other accessory proteins for binding to clathrin and AP2.
- B) Small GTPases, phosphatidylinositol 4-monophosphate and cargo molecules (the latter are not depicted in the picture) initiate the specific recruitment of AP1 to Golgi membranes and the formation of clathrin-coated pits (CCPs). Due to the concerted interaction with the clathrin N-terminal domain (TD) and small GTPases such as ARF1 CVA104 is targeted to Golgi localized CCPs.
- C) The association of CVA104 with other accessory proteins, which is probably mediated by the engagement of the NPF-tripeptide motif by EH-domains, supports the formation of a stable clathrin/adaptor protein scaffold at the membrane.
- D) The interaction of the pseudokinase-domain of CVA104 with transmembrane proteins and the simultaneous binding of clathrin might promote the selective sequestration and packaging of protein cargo into CCPs.

4.4 Closing comments and outlook

In this dissertation, I presented an initial but important characterization of the novel CCV-associated protein CVAK104. The data have implicated that CVAK104 is a kinase-related ubiquitously expressed protein involved in clathrin-mediated trafficking pathways at the level of the TGN and the endosomes such as the sorting of lysosomal enzymes. We currently possess only a few pieces of a puzzle but further characterization of CVAK104 that includes the identification of additional binding partners is a challenging task that will help to further dissect the molecular mechanisms by which CVAK104 contributes to CCV formation and/or vesicular trafficking. Furthermore, the knockout of CVAK104 genes in other organisms like *S. cerevisiae* or in mice and the following determination of phenotypes could shed light on the physiological significance of CVAK104 in particular and on the affected intracellular pathways in general.

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Zhang, X., Gureasko, J., Shen, K., Cole, P.A., and Kuriyan, J. (2006). An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 125, 1137-1149.

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Education

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Publications

- 10.2003 Scheele U., Alves J., Frank R., **Düwel M.**, Kalthoff C., Ungewickell E.J.: “Molecular and functional characterization of clathrin- and AP-2-binding determinants within a disordered domain of auxilin.”; JBC, 278(28):25357-68
- 10.2006 **Düwel M.** and Ungewickell E.J.: “Clathrin-dependent association of CVAK104 with endosomes and the trans-Golgi network”; Mol Biol Cell. 2006 Oct;17(10):4513-25.

Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently, and that I have not submitted it at any other university worldwide.

Hannover, 27th November 2006

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