**CRN7 INTERACTS WITH AP-1 AND IS REQUIRED FOR THE MAINTENANCE OF GOLGI MORPHOLOGY AND PROTEIN EXPORT FROM THE GOLGI**

Vasily Rybakin†, Natalia V. Gounko¶, Kira Späte†, Stefan Höning†, Irina V. Majoul§, Rainer Duden§ and Angelika A. Noegel†¤

From the †Institute for Biochemistry I and ¤Center for Molecular Medicine, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, D-50931 Cologne, Germany; ¶Laboratory for Electron Microscopy, Cell Biology Department, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands; §School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom.

Running title: Role of Crn7 in the Golgi

Address correspondence to: Vasily Rybakin, Division of Biological Sciences, University of California San Diego, 9500 Gilman Dr. MC0634, La Jolla CA, USA. E-mail: vrybakin@ucsd.edu

Crn7 is a novel cytosolic mammalian WD-repeat protein of unknown function that associates with Golgi membranes. Here, we demonstrate that Crn7 knockdown (KD) by siRNA results in dramatic changes in the Golgi morphology and function. Firstly, the Golgi ribbon is disorganized in Crn7 KD cells. Secondly, the Golgi export of several marker proteins including VSVG is greatly reduced, but not the retrograde protein import into the Golgi complex. We further establish that Crn7 co-precipitates with clathrin adaptor AP-1, but is not required for AP-1 targeting to Golgi membranes. We identify tyrosine-288-based motif as part of a canonical YxxΦ sorting signal as a major μ1-adaptin binding site in vitro. This study provides the first insight into the function of mammalian Crn7 protein in the Golgi complex.

**INTRODUCTION**

The Golgi complex is a stack of flattened membrane cisternae acting as a central sorting organelle that functionally connects the endoplasmic reticulum with other membrane compartments and the plasma membrane. The total protein output from the ER reaches the Golgi complex at its cis-pole. Protein sorting in the Golgi requires constant cargo progression across the ribbon from its cis- to trans-pole and is accompanied by sequential modification of the cargo. Export from the Golgi complex is based on the interaction of the cytosolic tails of cargo molecules (or cargo receptors for luminal cargo) with elements of the cytosolic sorting machinery such as μ-subunits of adaptor protein complexes. Golgi export in the direction of endosomes and lysosomes requires the binding of cargo to adaptor molecules and adaptor interaction with clathrin (1). In the case of plasma membrane-directed transport, no adaptors have yet been characterized, and the mechanism of cargo selection remains largely unknown.

The coronin family comprises two groups of evolutionally conserved WD-repeat proteins (2,3). Short coronins are actin-binding proteins playing accessory roles in the regulation of the actin cytoskeleton. Several of these protein have been shown to interact with Arp2/3 complex and help regulate the nucleation dynamics of actin filaments (reviewed in 3). Database search reveals up to six short coronins in human genome, and several of them have been characterized previously (4-9). Two of the known long coronin proteins, C. elegans and Drosophila POD-1s, bind to actin as well (10,11). Absence of the worm POD-1 protein leads to accumulation of large cytoplasmic membrane structures along with defects in eggshell formation indirectly suggesting an exocytosis-related function. In Drosophila dpod-1-mutant flies axonal pathfinding is severely impaired (11).

We have previously identified mammalian Crn7 as a novel coronin family member and POD-1 homologue, and demonstrated that it is abundantly expressed in most mouse tissues, is developmentally regulated and distributed between the cytosol and the Golgi (see also Fig. 1A), but is not associated with actin filaments (12). Crn7 sequence analysis revealed two WD-repeat blocks characteristic for longer coronins, two copies of the coronin signature motif and the lack of coiled coil regions. The protein lacks predicted signal sequence or a signal sequence cleavage site, as well as transmembrane domains (12).

**MATERIALS AND METHODS**

Reagents

Rabbit polyclonal antibodies against TGN38, GM130 and LAMP2 were generously provided by
Dr. Mark McNiven, Martin Lowe and Anna Shestakova. Antibodies against Crn7 and Crn3 (monoclonal), MPR46 and Erd2p (polyclonal) were reported previously (9,12-14). Monoclonal antibodies against γ-adaptin were from Sigma, against Src from Calbiochem, polyclonal antibodies against α-adaptin and against AP-3 were from Santa Cruz Biotechnology. GFP-VSVG(ts-O45) plasmid was from Dr. Jennifer Lippincott-Schwartz. Alexa633-labelled CTxB was from Molecular Probes (Utrecht, the Netherlands). Fine chemicals were purchased from Sigma, unless indicated otherwise. Short interfering 21-mer RNA oligonucleotides targeted against human Crn7 (designed and supplied by Qiagen): siRNA(1)123, siRNA(2)+520, siRNA(3)1452, siRNA(4)+63, siRNA(5)+2639, siRNA(6)+2055, siRNA(7)+60, siRNA(8)+2454. Lower indices indicate the starting positions of corresponding sequences in the Crn7 mRNA; oligonucleotides 2 and 7 targeted fragments of the 3’ region of Crn7 mRNA starting 520 and 60 bases after the last coding base, respectively. COG3 siRNA was from Dr. Anna Shestakova. “Fast forward” transfection of HeLa using HiPerFect reagent (Qiagen) was performed according to the manufacturer’s protocol. For plasmid transfections, we used the Lipofectamin Plus system (Invitrogen) or FuGENE6 reagent (Roche) according to the manufacturer’s guidelines. Synthetic peptides, HPLC-purified: CON: KVEKIGEGTYGVVYK (Jena Biosciences, Germany), YEVV: GKGERQLYCEVVPQ, AEVA: GKGERQLYCAEVAPQ, YELL: GKGDTRVFLYELPE, AELA: GKGDTDVFLOELAPE (all from JPT, Germany).

**Cell culture, transfection and immunofluorescence**

HeLa and Vero cells were from ATCC. Cells were grown according to standard procedures (12). For immunofluorescence, cells were grown on coverslips to approx. 50% confluency, fixed with 3.7% paraformaldehyde, rinsed twice with 20 mM glycine, blocked with 0.045% fish gelatine in the presence of 0.2% saponin and incubated with primary antibodies diluted in blocking buffer. 0.02% saponin was present at all washing steps. Primary antibodies were detected using Cy3-, Alexa-568- or Alexa-488-conjugated secondary antibodies (Sigma, Molecular Probes). For immunofluorescence, siRNA was used to transfect 50%-confluent cells on 12-mm coverslips (5 nM RNA) or 25-mm (30 nM RNA) coverslips in all experiments. For electron microscopy, 3 nM siRNA were used to transfect 90%-confluent HeLa cells on 12-mm coverslips. For Western blotting, fresh cells were plated onto 6-well plates at 50% confluency, and analyzed 48 and 72 h after transfection with siRNA. As controls, mock-transfected and scrambled RNA-transfected cells were used.

**Electron microscopy**

Mock- and siRNA-transfected HeLa cells were fixed for 10 min at room temperature in 100 mM PB containing 4% paraformaldehyde and stored at 4°C in PB containing 1% paraformaldehyde until further use. Cells were osmicated, dehydrated and embedded as described above. Ultra-thin sections were cut and counterstained with uranyl acetate and lead citrate.

**Immunoprecipitation and western blotting**

Immunoblotting was performed according to standard procedures. Anti-Crn7 antibody (hybridoma supernatant) was used at 1:5, mouse monoclonal anti-actin antibody (Sigma) at 1:20,000. Immunoprecipitation using protein A-Sepharose (Amersham) was performed essentially as described (15). Where indicated, cells were incubated with protein crosslinker DSS (2 mM, Pierce) for 30 min at 20°C prior to lysis.

**Cloning, expression and purification of AP µ-chains**

The coding sequences of truncated versions of µ1 (residues 158-423), µ2 (residues 158-435) and µ3 (residues 166-418) containing 5´NdeI and 3´EcoRI restriction sites were generated by PCR and cloned in frame into the vector pET28b to create a C-terminal fusion with a hexa-histidine (6xHis) tag. The recombinant plasmids were used to transform E. coli strain BL21, and protein production was induced by addition of IPTG for 3h at 30°C. The purification of µ-chain proteins was performed according to a standard protocol (Qiagen) using Ni-NTA agarose as an affinity matrix. The purity of the µ-chain proteins which were stored in 10 mM HEPES-KOH pH 7.4, 500 mM NaCl, 10 mM β-mercaptoethanol was controlled by SDS-PAGE, analytical gel filtration chromatography and CD spectroscopy. Before any BIAcore experiment, the proteins were centrifuged for 30 min at 100,000g to ensure the removal of any possible protein aggregates.

**Surface plasmon resonance-based in vitro binding assays**

The binding of recombinant His-tagged AP complex medium chains (µ1, µ2, µ3) to the putative Crn7 sorting signal-containing peptides and mutants thereof (see above) was measured by surface plasmon resonance using a BIAcore 3000 biosensor. All peptides were synthesized as 15-
mers harboring a typical YXXΦ motif close to their carboxy-terminus. In the mutant peptides, the critical tyrosine residue and the hydrophobic residue in the +3 position were substituted for alanine (for sequences see “Reagents”). Throughout the manuscript the peptides are named by their original or mutated tyrosine sorting motif. The peptides were immobilized via amino coupling onto carboxy-methylated dextran (CM5) sensor surfaces according to the manufacturers instructions. In order to minimize mass-transfer effects, the amount of immobilized peptide was kept low (∼200 RU). After the peptide immobilization, the surface was regenerated with a pulse injection of 50 mM NaOH to remove non-covalently bound peptide. The AP complex µ-chains were injected in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Polysorbate 20; from BIAcore) followed by regeneration. The obtained sensorgrams were used to calculate the kinetic rate constants for association (Kₐ), dissociation (Kₕ) and the equilibrium rate constants (KD). µ-chain binding to Crn7 peptides could be best modeled assuming a 1:1 interaction which is consistent with the structural information on peptide binding to AP µ-chains (16,17) and several other published reports using the same technology (18,19).

**VSVG and CTxB trafficking assays**

 Trafficking assays were performed essentially as described (20,21). In brief, for the VSVG trafficking assay, HeLa or Vero cells were co-transfected with GFP-VSVG-tsO45 and CFP-GalT plasmids, kept at 37 °C for 2 h, and at 40 °C for 16 h. Cells were then shifted to 32 °C in the presence of 150 μg ml⁻¹ cycloheximide in phenol red-free DMEM high-glucose medium supplemented with 10% serum and 20 mM HEPES to allow the synchronous export of VSVG from the ER. Where indicated, cells were transfected with siRNA 6 h prior to transfection with VSVG. For the CTxB trafficking assay, Alexa633-labelled CTxB (500 ng ml⁻¹) was added to the cell culture medium for 3 min at room temperature, followed by washing, internalisation at 37°C, fixation and immunolabeling.

**RESULTS**

**Crn7 RNAi affects the Golgi architecture**

In mammalian cells, Crn7 is present on Golgi membranes and in the cytosol (Ref. 12, see also Fig. 1A). To reveal the in vivo function of Crn7 in the Golgi, we used the small interfering RNA (siRNA) methodology using eight different siRNA oligonucleotides (Fig. 1B, see Materials and Methods for details). 48 and 72 h after transfection, cells were harvested and analyzed by western blotting. Although all tested siRNA constructs were capable of downregulating Crn7 protein (Fig. 1B), the construct siRNA(8)2454 consistently showed the highest degree of downregulation and was used in further experiments. None of the tested siRNAs affected the level of coronin 3 protein (Fig. 1B), which is an important proof of the specificity of siRNA as Crn7 and Crn3 have several highly similar sequence features (see Ref. 3). Western blot with actin antibody was used as loading control.

The effect of Crn7 knockdown on the architecture of the Golgi complex was evaluated using electron microscopy studies on Crn7 knockdown (KD) cells. Mock-transfected cells displayed a characteristic Golgi morphology with several flat cisternae surrounded by transport intermediates (Fig. 1C). In contrast, Crn7 siRNA HeLa cells were characterized by the loss of Golgi integrity and exhibited significant scattering of Golgi membranes. In most cells, the Golgi was present as a dense accumulation of vesicles still containing one or several cisternae-like structures. In the most extreme cases, no cisternae were observed, and the density of Golgi remnant compartments decreased (Fig. 1C). To better understand the effect of Crn7 knockdown on Golgi architecture, we studied the distribution of cis- and trans-Golgi markers in fixed mock- and Crn7 siRNA-transfected HeLa cells (Fig. 1D). In Crn7 KD cells, the Golgi ribbon disappeared, and smaller Golgi fragments were scattered in the perinuclear area. The trans-Golgi protein TGN38 and the cis-Golgi marker GM130 were both still detectable in predominantly non-overlapping domains localized close to each other, some of them being organized in ministacks (Fig. 1D).

**Defects in anterograde transport in Crn7 KD cells**

The dramatic defects we observed in Crn7 knockdown cells prompted us to address the question whether these defects correlate with impairment of Golgi transport. We monitored the Golgi export of the VSV envelope G glycoprotein (VSVG), known to hijack the anterograde transport system to reach the cell surface after proceeding through the ER and the Golgi. The experiments were performed with a mutant VSVG (VSVG-tsO45) carrying a single point mutation F204S (22), which renders it temperature-sensitive with regard to its intracellular trafficking. The mutant protein accumulates in the ER at 39.5°C,
and is released to the Golgi complex upon a temperature switch to 32°C (see Methods). This mutant is widely used to study the dynamics of protein trafficking along the biosynthetic pathway (20,23).

In mock-transfected HeLa cells, VSVG-tsO45-GFP gradually accumulated in the Golgi complex upon shifting the temperature to 32°C (not shown). Starting at 10 minutes after the shift, formation of characteristic tubular compartments originating from the Golgi was observed. These intermediates, morphologically similar to Golgi-to-plasma membrane carrier (GPC) precursors described previously (24), released vesicles targeted to the plasma membrane (Fig. 2A). Apart from vesicles derived from GPC precursor-like tubules, we observed direct formation of vesicular transport intermediates leaving the Golgi in the direction towards the plasma membrane.

Cells treated with Crn7 RNAi and recognized as Crn7 knockdown by their scattered CFP-GalT (Golgi galactosyltransferase) pattern were imaged under the same conditions and demonstrated a similar rate of accumulation of VSVG in the Golgi apparatus (data not shown). However, we did not observe any release of VSVG in GPC precursor-like tubules or vesicles from the Golgi even 120 minutes after the temperature shift. At all times, the VSVG GFP signal was present in the scattered Golgi remnants (Fig. 2B). We further quantified the VSVG GFP signal intensity at the plasma membrane in 100 images spanning 30 minutes of time in mock-transfected and Crn7 RNAi cells using MetaMorph image analysis software. In control cells, the GFP fluorescence intensity gradually increased to nearly double the initial value, reflecting the delivery of VSVG from the Golgi complex, whereas in RNAi cells, the GFP fluorescence signal at the plasma membrane remains at basic threshold level, which approx. 50% of that of mock-transfected cells (Fig. 2E).

In order to confirm our data on VSVG export defect, we assessed VSVG trafficking in Crn7 KD Vero cells, a primate kidney cell line. We did not observe any GFP-like structures in either control or KD Vero cells (Fig. 2C, D). However, vesicular transport of VSVG from the Golgi to cell surface was greatly reduced in Crn7 KD cells as compared to the control (Fig. 2D, see also Supplementary Movies 1 and 2). Note that in Vero cells, Crn7 knockdown led to the formation of significantly larger, compacted Golgi remnants than in HeLa cells (compare Fig. 2B and D). Quantification of cell surface VSVG further confirmed the defect in VSVG delivery to cell surface comparable with that in HeLa cells (data not shown).

The Golgi does not only export trafficking intermediates, but also receives material by retrograde trafficking. To test whether the retrograde transport to the Golgi requires Crn7, we studied the intracellular dynamics of CTxB, the non-toxic B-subunit of the AB5 toxin, cholera toxin (25). In control cells, Alexa633-labeled CTxB (CTxB-633) was internalised by cells, and reached the Golgi complex approx. 30 minutes after its addition to the cells (Fig. 2G), which is in good agreement with data from the literature (13,21). In Crn7 RNAi cells (asterisk in Fig. 2F, G), CTxB-633 reached the scattered perinuclear Golgi remnants present in these cells with kinetics indistinguishable from those observed for its arrival in the Golgi complex in control cells (Fig. 2G). Thus, the protein import into the TGN is Crn7-independent.

The Golgi export block that we detected in Crn7 siRNA cells can be due to numerous reasons, such as defects in proper cargo protein modification and/or machinery for protein sorting and recruitment. To experimentally assess the possibility of interplay of Crn7 with cargo glycosylation, we used LAMP2, a cargo protein destined to late endosomes and lysosomes, in Crn7 siRNA and control cells. In Crn7 KD cells, the fully glycosylated form of LAMP2 appeared at approx. 110 kDa, and a band of similar size was detected in control siRNA-transfected cells (Fig. 2H). As a positive control, we used siRNA directed against the COG3 protein, a part of the multisubunit Golgi protein complex acting at several stages of glycoprotein and glycolipid metabolism (Ref. 26, and references therein). COG3 knockdown is correlates with a massive defect in Golgi glycosylation (26). In accordance with literature data, immature LAMP2 migrated as a protein of lower molecular weight at approx. 80 kDa in COG3 RNAi, but not Crn7 RNAi cells. Thus, Crn7 knockdown influences the Golgi export of cargo, but not cargo glycosylation.

Interaction of Crn7 with the clathrin adaptor AP-1

We further wanted to test whether Crn7 knockdown affects cargo selection and export vesicle formation. Adaptor proteins such as monomeric GGAs and heterotetrameric adaptor complexes (AP) are the key factors for the incorporation of cargo membrane proteins into nascent coated vesicles (1,27). AP complex


subunits are called adaptins. Binding of the membrane proteins to adaptor proteins is mediated by small peptide sequences (sorting signals), localized in the cytosolic portions of the proteins, such as tyrosine-based signals of the YXXΦ type (X, any residue; Φ, a bulky hydrophobic residue). The μ-subunits of AP complex tetramers interact with YXXΦ motifs of cargo proteins, while β-subunits interact with clathrin (1). There are four AP complexes in mammalian cells (AP-1 – AP-4), all of them functioning in the same way but at distinct cellular sites. While AP-2 is restricted to the plasma membrane and is involved in clathrin-coated vesicle formation during endocytosis, AP-1 has the same function in the TGN and most likely in endosomes. The functions of AP-3 and AP-4 are less well understood (1).

Crn7 harbours two sequences matching a classical YXXΦ motif localized directly downstream of each coronin core domain (Fig. 3A). We tested whether these motifs of Crn7 can bind to purified μ subunits of the AP-1, -2 and -3 adaptor complexes. 15-mer synthetic peptides harbouring each of the YXXΦ motifs close to their carboxy-terminus were used along with mutant peptides where the tyrosines and the hydrophobic (Φ) amino acids in the +3 positions were replaced by alanine residues. The peptides were covalently coupled via their amino-terminus to a CM5 surface of a BIAcore 3000 biosensor. Subsequently, purified recombinant μ1, μ2 or μ3 subunits were injected at different concentrations. The peptide comprising Y288 bound with high affinity to μ1 and μ2, but not to μ3 (Fig. 3B, YEVV). Binding was specific as indicated by the low degree of background binding observed for the mutant peptide. The second more distal Crn7 peptide containing Y758 also bound to μ1 and μ2, but only at a very low level (Fig. 3B, YELL). Although the evaluation of the data for the Y288 peptide binding to μ1 and μ2 did not perfectly match the expected model for a one-to-one type of interaction, we could estimate the in vitro equilibrium binding rate constant K_D at ~150 nM which fits well to published data on signal binding to μ-chains (28). Binding of both μ1 and μ2 subunits was concentration-dependent and reversible, with minimal re-binding during the dissociation phase (Fig. 3C, D). Fig. 3E shows the purity of μ subunit preparations.

In order to ascertain that the Crn7 binding to AP-1 and AP-2 is significant in vivo, we tested whether Crn7 can be co-immunoprecipitated with AP complexes. As the -YEVV- motif was shown to interact with μ1 and μ2 in vitro, we performed immunoprecipitation experiments using antibodies recognizing the complex-specific subunits of AP-1, -2 and -3. Crn7 was detectable in AP-1 pull-down, but not AP-2 or AP-3 pull-downs (Fig. 4A), inferring an in vivo interaction of Crn7 with AP-1, but not with AP-2 or -3. This observation was confirmed by reverse immunoprecipitation of AP-1 together with Crn7 from cell lysate derived from HeLa cells treated with protein crosslinker DSS (Fig. 4A, lower panel).

Crn7 colocalizes with AP-1 on the Golgi but not on AP-1-positive endosomes (Fig. 4B), suggesting that the interaction of Crn7 and AP-1 takes place at Golgi membranes. Taken together, our in vitro binding and immunoprecipitation data suggest that Crn7 interacts with AP-1.

While the AP-2 interaction appears biochemically possible (see Fig. 3B), the Crn7 and AP-2 proteins are localized in very different cellular compartments and are unlikely to interact in vivo due to spatial restrictions. AP-3 like AP-1 is present at the Golgi, but does not interact with any YXXΦ motif of Crn7 as demonstrated by surface plasmon resonance experiments, and it does not co-immunoprecipitate with Crn7 (Fig. 4A). This important observation strongly infers that the presence of a putative sorting signal in the protein sequence is not sufficient for the interaction with any given sorting machinery. Specificity of such interaction may be regulated by amino acid composition in the vicinity of the sorting signal, or by additional protein factors.

**Localization of AP-1 and AP-1-dependent cargo in Crn7 KD cells**

Next, we were interested to know whether Crn7 acts upstream or downstream of AP-1 in the Golgi export pathway, and whether Crn7 is required for the targeting of AP-1 to the Golgi. Subcellular distribution of AP-1 in control and Crn7 KD cells was studied by immunofluorescence microscopy using an antibody specific to γ-adaptin (Fig. 4C, upper panel). In both control and RNAi cells, AP-1 was detectable on Golgi membranes and endosomes. Crn7 KD cells, however, displayed marked increase of γ-adaptin on the scattered Golgi. Thus, Crn7 knockdown appears not to interfere with the ability of AP-1 to be recruited to the cargo, and Crn7 possibly acts downstream of AP-1 recruitment.

VSVG export from the Golgi has never been demonstrated to depend on AP-1. As we have established the interaction of Crn7 with AP-1 in vivo and in vitro, we were interested whether bona
fide AP-1 cargoes require Crn7 to be exported from the Golgi complex. To this end, we studied the distribution of 46-kDa mannose-6-phosphate receptor MPR46 in HeLa cells treated with Crn7 siRNA. In Crn7 KD cells, MPR46 was retained and markedly accumulated in the scattered Golgi (Fig. 4C, lower panel). A lysosomal marker protein LAMP2 showed similar behaviour (data not shown).

DISCUSSION

Coronins constitute a phylogenetically conserved group of proteins (reviewed in Ref. 3). According to their structure and function, coronin proteins can be subdivided into two subfamilies. The first group comprises shorter, approx. 45-50 kDa coronins are abundant actin-binding proteins implicated, among other function, in nucleation of actin filaments. The second group consists of just two highly homologous POD-1 proteins from C. elegans and Drosophila, along with their mammalian counterpart coronin 7. A fourth subfamily member has been recently discovered in the social amoeba Dictyostelium discoideum (AN, unpublished data). POD-1 proteins and Crn7 are characterized by a high degree of homology in their core WD repeat-containing domains, and a far lesser similarity in flanking sequences.

Crn7 is the first coronin found to be localized to the Golgi and to directly execute a membrane trafficking-related function. Although mutant phenotypes of both worm and fly homologues (see Introduction) may be explained by defects in Golgi function and anterograde trafficking, we do not envision the same function for Crn7 and either of the two POD-1 proteins. We have no data implying any interaction of Crn7 with the cytoskeleton, while there is no indication for the Golgi localization of any of the POD-1 proteins. We anticipate the Golgi localization and Golgi-related function to be unique for vertebrate or mammalian POD-1 homologues. This consideration is in line with the fact that Y288 and Y758, parts of the two putative tyrosine-based sorting signals in Crn7, are not present in the C. elegans POD-1 protein, while in Drosophila, a tyrosine residue is present in a vicinity of Y288 position, but its amino acid environment is very different, and so is the predicted tertiary structure in this area (VR, unpublished data). We suggest that the specificity of functions of POD-1/Crn7 subfamily of proteins is specified by unique non-core subdomains rather than conserved WD-repeat containing core regions.

Here, we provide detailed characterization of the function of Crn7, a novel mammalian Golgi protein belonging to the coronin family. We establish that Crn7 is a protein indispensable for the maintenance of both Golgi architecture and function. Crn7 knockdown leads to a breakdown of the Golgi structure and to a disruption of anterograde Golgi-to-plasma membrane transport. Crn7 binds to AP-1 in vivo and in vitro via the interaction of its Y288-based sorting signal with µ1-adaptin, but does not bind to AP-2 or AP-3. The protein is not required for targeting AP-1 to Golgi membranes, as the amount of AP-1 on Golgi in Crn7 KD cells is enriched rather than downregulated. Most probably, the accumulation of AP-1 on Golgi membranes reflects the fact that the cargo stuck in the compartment is already primed for export, e.g. the complex between cargo and AP-1 has already formed, but the formation of transport intermediates is impaired.

The marked accumulation of AP-1 on the Golgi together with accumulated cargo would suggest that the role of Crn7 in the Golgi export may be confined to the events downstream of the cargo-adaptor interaction. Such events include the generation of membrane curvature followed by membrane fission. Another such event may be interaction with (hypothetical) non-clathrin, non-COPI/II coat proteins.

Several proteins have been implicated in the membrane fission reaction in the trans-Golgi network (TGN). One of the most intriguing is protein kinase D (PKD), an enzyme localizing to the TGN by means of interaction with diacylglycerol and required for the formation of post-Golgi transport intermediates (29-32). The role of PKD can be precisely attributed to membrane fission as the overexpression of kinase-inactive PKD leads to the formation of long TGN-derived tubes containing cargo, but not resident enzymes or coat proteins (29,32). Our data allow us to speculate that Crn7 acts upstream of PKD in the secretory pathway as Crn7 KD does not result in the formation of stable cargo-containing tubules arrested in fission.

It remains to be established how exactly Crn7 is targeted to the Golgi, what other proteins it interacts with, and what the role of the cytosolic pool may be. Another important question arises from our earlier observation that Crn7 is tyrosine-phosphorylated on membranes (12), but not in the cytosol. Further studies will be required to find out whether phosphorylation is required for Crn7 targeting to Golgi membranes or for its function, and what kinase(s) participate in this event.
Acknowledgements—This work was supported by grants from the Imhoff Foundation and the Maria Pesch Foundation (to VR) and the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie (to AN). VR and NG are recipients of fellowships from Graduate Schools in Genetics and Functional Genomics, University of Cologne, and in Behavioural and Cognitive Neurosciences, University of Groningen. We thank Drs. J. Lippincott-Schwartz, M. Lowe, A. Shestakova and M. McNiven for generously sharing key reagents. D. Kalicharan and P. Wood are acknowledged for their help with microscopy. We thank Dr. Vivek Malhotra and members of Malhotra lab for their critical comments on the manuscript.

REFERENCES

1. Robinson, M. S. (2004) Trends Cell Biol 14(4), 167-174
2. de Hostos, E. L. (1999) Trends Cell Biol 9(9), 345-350
3. Rybakin, V., and Clemen, C. S. (2005) Bioessays 27(6), 625-632
4. Appleton, B. A., Wu, P., and Wiesmann, C. (2006) Structure 14(1), 87-96
5. Cai, L., Holoweckyj, N., Schaller, M. D., and Bear, J. E. (2005) J Biol Chem
6. Hasse, A., Rosentreter, A., Spoerl, Z., Stumpf, M., Noegel, A. A., and Clemen, C. S. (2005) Eur J Neurosci 21(5), 1155-1168
7. Nakamura, T., Takeuchi, K., Muraoka, S., Takezoe, H., Takahashi, N., and Mori, N. (1999) J Biol Chem 274(19), 13322-13327
8. Nal, B., Carroll, P., Mohr, E., Verthuy, C., Da Silva, M. I., Gayet, O., Guo, X. J., He, H. T., Alcover, A., and Ferrier, P. (2004) Int Immunol 16(2), 231-240
9. Spoerl, Z., Stumpf, M., Noegel, A. A., and Hasse, A. (2002) J Biol Chem 277(50), 48885-48887
10. Rappleye, C. A., Paredez, A. R., Smith, C. W., McDonald, K. L., and Aroian, R. V. (1999) Genes Dev 13(21), 2838-2851
11. Rothenberg, M. E., Rogers, S. L., Vale, R. D., Jan, L. Y., and Jan, Y. N. (2003) Neuron 39(5), 779-791
12. Rybakin, V., Stumpf, M., Schulze, A., Majoul, I. V., Noegel, A. A., and Hasse, A. (2004) FEBS Lett 573(1-3), 161-167
13. Majoul, I., Sohn, K., Wieland, F. T., Peperkok, R., Pizza, M., Hillemann, J., and Soling, H. D. (1998) J Cell Biol 143(3), 601-612
14. Tikkanen, R., Obermuller, S., Denzer, K., Pungitore, R., Geuze, H. J., von Figura, K., and Honing, S. (2000) Traffic 1(8), 631-640
15. Neubrand, V. E., Will, R. D., Mobius, W., Poustka, A., Wiemann, S., Schu, P., Dotti, C. G., Peperkok, R., and Simpson, J. C. (2005) Embo J 24(6), 1122-1133
16. Heldwein, E. E., Macia, E., Wang, J., Yin, H. L., Kirchhausen, T., and Harrison, S. C. (2004) Proc Natl Acad Sci USA 101(39), 14108-14113
17. Owen, D. J., and Evans, P. R. (1998) Science 282(5392), 1327-1332
18. Boll, W., Rapoport, I., Brunner, C., Modis, Y., Prehn, S., and Kirchhausen, T. (2002) Traffic 3(8), 590-600
19. Honing, S., Ricotta, D., Krauss, M., Spate, K., Spolaore, B., Motley, A., Robinson, M., Robinson, C., Haucke, V., and Owen, D. J. (2005) Mol Cell 18(5), 519-531
20. Hirschberg, K., Miller, C. M., Ellenberg, J., Presley, J. F., Siggia, E. D., Phair, R. D., and Lippincott-Schwartz, J. (1998) J Cell Biol 143(6), 1485-1503
21. Majoul, I. V., Bastiaens, P. I., and Soling, H. D. (1996) J Cell Biol 133(4), 777-789
22. Gallione, C. J., and Rose, J. K. (1985) J Virol 54(2), 374-382
23. Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J., and Lippincott-Schwartz, J. (1997) Nature 389(6646), 81-85
24. Polishchuk, E. V., Di Pentima, A., Luini, A., and Polishchuk, R. S. (2003) Mol Biol Cell 14(11), 4470-4485
25. Lauvrak, S. U., Torgersen, M. L., and Sandvig, K. (2004) J Cell Sci 117(Pt 11), 2321-2331
26. Shestakova, A., Zolov, S., and Lupashin, V. V. (2006) Traffic 7, 1-14
27. Bonifácino, J. S. (2004) Nat Rev Mol Cell Biol 5(1), 23-32
28. Grass, I., Thiel, S., Honing, S., and Haucke, V. (2004) J Biol Chem 279(52), 54872-54880
29. Liljedahl, M., Maeda, Y., Colanzi, A., Ayala, I., Van Lint, J., and Malhotra, V. (2001) Cell 104(3), 409-420
30. Maeda, Y., Beznoussenko, G. V., Van Lint, J., Mironov, A. A., and Malhotra, V. (2001) Embo J 20(21), 5982-5990
31. Baron, C. L., and Malhotra, V. (2002) Science 295(5553), 325-328
32. Yeaman, C., Ayala, M. I., Wright, J. R., Bard, F., Bossard, C., Ang, A., Maeda, Y., Seufferlein, T., Mellman, I., Nelson, W. J., and Malhotra, V. (2004) Nat Cell Biol 6(2), 106-112

FIGURE LEGENDS
Fig. 1. Crn7 is required for the maintenance of Golgi architecture. A, Crn7 (red) co-localizes with the Golgi marker Erd2p (green) in fixed human HeLa cells. B, Downregulation of Crn7 protein by RNAi in HeLa cells. Upper panel, 48 hrs of RNAi treatment, middle and lower panels, 72 hrs. Upper and middle panels, the western blot was probed with Crn7 antibody to validate the knockdown. Lower panel, as siRNA specificity and loading controls, a mixture of coronin-3 and actin antibodies was used. See text for details. C, Morphology of the Golgi in HeLa cells at the electron microscopy level. Left, Typical appearance of the Golgi complex in a control HeLa cell. Note the membrane cisternae organized in a stack. Right, Disassembly of the Golgi ribbon in a HeLa cell treated with Crn7 siRNA. Note marked vesiculation of the Golgi and the presence of disorganized separate cisternae. D, Appearance of TGN and Golgi compartments in HeLa cells stained with anti-GM130 (red) and anti-TGN38 (green) antibodies. Left, High-magnification immunofluorescence image depicting a lateral part of a Golgi stack in a control cell. Right, Abnormal organization of TGN and Golgi in a siRNA-treated cell. Note the disorganization of the cisternae.

Fig. 2. Protein trafficking defects in Crn7 siRNA-treated cells. A, The export of VSVG-tsO45 from the Golgi in a control HeLa cell. Arrowheads point at GPC precursor-like tubules, arrows – at Golgi-derived vesicles. B, Block of the VSVG export from the Golgi in a Crn7 KD HeLa cell. Note the absence of GPC precursors and vesicles. Bar, 5 µm. C, The export of VSVG-tsO45 from the Golgi in a control Vero cell. Arrows point at Golgi-derived vesicles. D, Block of the VSVG export from the Golgi in a Crn7 KD Vero cell. See also Supplementary Movies 1 and 2. Bar, 5 µm. E, Dynamics of perimembrane VSVG-GFP fluorescence in control (blue) and Crn7 KD (red) HeLa cells during 30 min of the Golgi release. X-axis, time, min; Y-axis, cell surface VSVG fluorescence intensity, AU. Timescale as in A-D. F, G, Retrograde transport into the Golgi is preserved in Crn7 KD cells. HeLa cells were incubated in the presence of Alexa633-labelled B-subunits of cholera toxin, and could internalize and transport it to the Golgi in both untransfected and Crn7 KD (asterisk) cells. Crn7 staining (F), CTxB-Alexa633 signal (G). H, Trafficking defect in Crn7 siRNA cells is not due to a glycosylation deficiency. HeLa cells were treated with Crn7 siRNA for 10 days and assayed for LAMP2 glycosylation. No defect in LAMP2 glycosylation could be observed, in contrast to COG3 RNAi cells (positive control, see text for details). Western blot with Crn7 antibody (top) or LAMP2 antibody (bottom).

Fig. 3. Adaptor-binding properties of Crn7. A, Localization of putative tyrosine-based (YxxΦ) signals in the Crn7 protein. B, Surface plasmon resonance-based analysis of adaptor µ-chain binding to Crn7 tyrosine-containing and control peptides. Purified AP µ-chains were injected over the peptide-derivatized sensor surfaces. Only the peptide harbouring Y288 bound µ1 and µ2, while specific binding to µ3 was not detectable. The second peptide containing Y758 bound only the AP-2 µ-chain with very low affinity which may be insufficient to mediate binding in vivo. C, D, Examples of concentration-dependent binding of the AP-1 (C) and AP-2 (D) µ-chains to the Crn7 Y288-containing peptide. Purified AP µ-chains were injected at concentrations ranging from 15-500 nM over the peptide-derivatized sensor surfaces. The depicted sensorgrams were used to...
calculate the affinity rate constants for μ-chain binding to Crn7. E, Purity of μ-chain preparations as analyzed by polyacrylamide gel electrophoresis followed by Coomassie staining.

Fig. 4. A, Upper panel; Immunoprecipitation with adaptor-specific antibodies followed by detection of Crn7 by Western blotting supports the in vivo interaction of Crn7 and AP-1 (top), but not AP-2 (middle) or AP-3 (bottom). Lower panel; proof of AP-1 interaction with Crn7 by reverse immunoprecipitation. Crn7 was precipitated from DSS-treated HeLa cell lysate using Crn7 antibody, and γ-adaptin was detected in precipitate by Western blotting. B, Co-localization between γ-adaptin, a subunit of AP-1 adaptor complex (top left), and Crn7 (top right) in the Golgi area. Bottom left, merged image; co-localisation is reflected by yellow signal. Bottom right, visualization of co-localization points (CP) between AP-1 and Crn7 by the “Colocalization finder” module of the ImageJ program (white area). Note the absence of co-localization in any non-Golgi area. C, Both γ-adaptin and AP-1 cargo protein MPR46 are accumulated in scattered Golgi remnants in Crn7 KD cells. Upper panel, Demonstration of the independence of AP-1 targeting to Golgi membranes of Crn7. Left, control cells; Right, Crn7 KD cells. Note that the depletion of Crn7 leads to the enrichment of γ-adaptin on Golgi membranes. Lower panel, Distribution of MPR46 in control (left) and Crn7 KD (right) cells.

SUPPLEMENTARY INFORMATION
Movie 1. Visualization of VSVG export from the Golgi complex in Vero cells transfected with GFP-VSVG-tsO45 plasmid and control siRNA. The length of the video clip corresponds to 30 min of real time.

Movie 2. Visualization of VSVG export in Vero cells transfected with GFP-VSVG-tsO45 plasmid and Crn7 siRNA. The length of the video clip corresponds to 30 min of real time. Note the absence of any Golgi-derived export intermediates.
### B

| RNA duplexes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Mock |
|--------------|---|---|---|---|---|---|---|---|------|
| 48h Crn7     |   |   |   |   |   |   |   |   |      |
| 72h Crn7     |   |   |   |   |   |   |   |   |      |
| 72h Crn3 actin | | | | | | | | |      |

### C

[Images of electron microscopy](#)

### D

[Images of fluorescence microscopy](#)
Crn7 interacts with AP-1 and is required for the maintenance of Golgi morphology and protein export from the Golgi
Vasily Rybakin, Natalia V. Gounko, Kira Späte, Stefan Höning, Irina V. Majoul, Rainer Duden and Angelika A. Noegel

J. Biol. Chem. published online August 12, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M604680200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2006/08/15/M604680200.DC1