Clint: A Novel Clathrin-binding ENTH-Domain Protein at the Golgi

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We have characterized a novel clathrin-binding 68-kDa epsin N-terminal homology domain (ENTH-domain) protein that we name clathrin interacting protein localized in the *trans*-Golgi region (Clint). It localizes predominantly to the Golgi region of epithelial cells as well as to more peripheral vesicular structures. Clint colocalizes with AP-1 and clathrin only in the perinuclear area. Recombinantly expressed Clint interacts directly with the γ -appendage domain of AP-1, with the clathrin N-terminal domain through the peptide motif ⁴²³LFDLM, with the γ -adaptin ear homology domain of Golgi-localizing, γ -adaptin ear homology domain 2, with the appendage domain of β 2-adaptin and to a lesser extent with the appendage domain of α -adaptin. Moreover, the Clint ENTH-domain associates with phosphoinositide-containing liposomes. A significant amount of Clint copurifies with rat liver clathrin-coated vesicles. In rat kidney it is preferentially expressed in the apical region of epithelial cells that line the collecting duct. Clathrin and Clint also colocalize in the apical region of enterocytes along the villi of the small intestine. Apart from the ENTH-domain Clint has no similarities with the epsins AP180/CALM or Hip1/1R. A notable feature of Clint is a carboxyl-terminal methionine-rich domain (Met⁴²⁷-Met⁶⁰⁵), which contains >17% methionine. Our results suggest that Clint might participate in the formation of clathrin-coated vesicles at the level of the *trans*-Golgi network and remains associated with the vesicles longer than clathrin and adaptors.

INTRODUCTION

Clathrin-coated vesicles participate in receptor-mediated endocytosis, in the transport of lysosomal enzymes from the *trans*-Golgi network (TGN) to the endosomes/lysosomes and in the sorting of receptors within the endosomal system (Stoorvogel *et al.*, 1996; Sorkina *et al.*, 1999; Kirchhausen,

2000; Raiborg *et al.*, 2001). The major structural components of the coat are the three-legged clathrin molecules and the adaptor proteins. Clathrin polymerizes into a polygonal lattice on the cytoplasmic side of the donor membrane, which upon the influence of additional factors is then forced to form a vesicle that includes the cargo. The interaction of clathrin with the membrane is not direct, but mediated by a complex network of adaptor proteins (AP) and accessory proteins. The combination of these proteins is thought to regulate where and when a coat is formed.

The conventional and most abundant adaptor proteins are AP-1 and AP-2. They are heterotetrameric complexes with the compositions $\gamma\beta_1\mu_1\sigma_1$ (AP-1) and $\alpha\beta_2\mu_2\sigma_2$ (AP-2). AP-1 functions at the TGN and probably also at endosomes (Folsch *et al.*, 2001), whereas AP-2 is found predominantly at the plasma membrane (Kirchhausen, 2000). The adaptors are capable of binding clathrin, membrane phospholipids, docking proteins, and cargo (Kirchhausen, 2000). A special domain at the carboxyl-terminal end of the α -subunit, known as the appendage domain, is capable of associating with numerous accessory proteins that play a pivotal role in the initiation of coat formation. A similar domain in the γ -subunit of AP-1 was shown to associate with γ -synergins (Page

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Abbreviations used: Arf, ADP-ribosylation factor-binding protein; BFA, brefeldin A; BSA, bovine serum albumin; CALM, clathrin assembly lymphoid myeloid leukemia protein; CLINT, clathrin interacting protein localized in the *trans*-Golgi region; EH-domain, Eps15-homology domain; ENTH-domain, epsin N-terminal homology domain; GAE, γ -appendage/ear; GGA, Golgi-localizing, γ -adaptin ear homology domain; GST, glutathione S-transferase; MDBK, Madin-Darby bovine kidney; PBS, phosphate-buffered saline; PI-(4,5)-P₂, phosphatidylinositol-4,5-bisphosphate; TD, clathrin amino-terminal domain; TGN, *trans*-Golgi network.

et al., 1999) and with auxilin 2, which participates in the uncoating reaction (Umeda *et al.*, 2000). More recently two related complexes, AP-3 and AP-4, have been identified. AP-3 has been implicated in membrane traffic originating from the TGN and early endosomes. AP-4 also seems to be located at the Golgi. However, it is not yet clear whether the coats of AP-4 vesicles contain clathrin (Robinson and Bonifacino, 2001).

The discovery of Golgi-localizing, γ -adaptin ear homology domain (GGA) proteins and their role in the sorting of lysosomal enzymes at the TGN has shown that besides the heterotetrameric adaptor-complexes there are also monomeric adaptors that are capable of fulfilling tasks ascribed to their tetrameric counterparts. GGAs are recruited to Golgi membranes by ADP-ribosylation factor 1 (Arf1). They bind both the mannose-6-phosphate receptors and clathrin and are necessary for the recognition and correct sorting of lysosomal enzymes at the TGN (Boman, 2001; Hirst *et al.*, 2001; Puertollano *et al.*, 2001; Zhdankina *et al.*, 2001; Zhu *et al.*, 2001).

Proteins with an epsin N-terminal homology domain (ENTH-domain) are among the growing number of accessory proteins that are involved in coat formation (Takei and Haucke, 2001). Founding and name-giving member of this family is epsin, of which three isoforms are known in humans (epsin 1–3). The amino-terminal ~150 amino acids comprise the ENTH-domain, which consists of eight super helically arranged α -helices (Hyman *et al.*, 2000), whereas the rest of the molecule lacks any ordered structure (Kalthoff *et al.*, 2001). However, this part of the molecule contains a number of short tandemly arranged binding motifs for clathrin, AP-2, Eps15, and intersectin as well as three ubiquitin-interacting motifs designed to interact with mono-ubiquitinated proteins (Hofmann and Falquet, 2001).

Overall, the molecular architecture of AP180 and its non-neuronal relative clathrin assembly lymphoid myeloid leukemia protein (CALM) is similar to that of the epsins, although with the exception of the ENTH-domains there is almost no sequence homology between them. However, it was recently shown that the AP180 segments containing the clathrin and AP-2 binding sites are similarly unstructured as the corresponding segments of epsin (Kalthoff *et al.*, 2001). The ENTH-domains of AP180 and CALM differ from those of the epsins in size (~260 residues that fold into 10 α -helices vs. 150 that form eight helices), but like them they are known to bind to phosphatidylinositol-4,5-bisphosphate [PI-(4,5)-P₂]. This rare membrane phospholipid is considered to be an important signal for clathrin coat formation at the plasma membrane (Takei and Haucke, 2001). Interestingly, the PI-(4,5)-P₂ binding site of epsin 1 differs from the ones in AP180 and CALM (Ford *et al.*, 2001; Itoh *et al.*, 2001). The epsin 1 ENTH-domain binds PI-(4,5)-P₂ in a pocket that is formed by basic residues from helices 3 and 4 upon which an arginine from a flexible amino-terminal tail folds back. In contrast, the ENTH-domains of AP180 and CALM bind the lipid through a basic cluster formed by helices 1 and 2 and the loop in between.

The unstructured regions of AP180 and epsin 1 have been shown to promote the assembly of free clathrin triskelia into basket-like cages in vitro (Kalthoff *et al.*, 2001). Moreover, it was demonstrated that AP180 is able to induce the formation of clathrin lattices on artificial liposomes containing

PI-(4,5)-P₂, which seem to form buds when AP-2 is included in the system (Ford *et al.*, 2001). Thus, AP180 as well as the other ENTH-domain proteins are conjectured to drive the formation of clathrin coats at the plasma membrane either as accessory proteins (e.g., in the initiation of clathrin recruitment and coat assembly) or perhaps even as monomeric adaptor proteins themselves. The latter idea is supported by the finding that at least the epsins are capable of binding to mono-ubiquitinated transmembrane proteins and therefore could recognize cargo molecules, membranes, and clathrin itself, which might abolish the need for the classical heterotetrameric adaptor proteins (Ford *et al.*, 2001). Furthermore, the structure of the ENTH-domain is very similar to that of the Vps27p, Hrs, STAM (VHS)-domain (Hyman *et al.*, 2000; Mao *et al.*, 2001). The VHS-domain is found in other proteins such as the GGAs that have been suggested to function as monomeric adaptors. Another subgroup of ENTH-domain proteins encompasses the huntingtin-interacting proteins Hip1 and Hip1R (Engqvist-Goldstein *et al.*, 1999, 2001; Waelter *et al.*, 2001). Their central coiled-coil domain binds clathrin and in the case of Hip1R also AP-2 (Mishra *et al.*, 2001). An actin-binding talin-like domain near the C terminus of Hip1/Hip1R is able to form a link between clathrin coats and the actin cytoskeleton.

So far, the currently known ENTH-domain proteins are involved in clathrin coat formation at the plasma membrane. In this study, we have characterized a novel ENTH-domain-containing protein, which is predominantly located in the region of the *trans*-Golgi network. We demonstrate that it interacts with clathrin, Golgi-associated adaptor proteins, and GGA2. Our data suggest that it functions along the route between the TGN and the endosomal system.

MATERIALS AND METHODS

Sequence Analysis

Alignments were performed using the Clustal V method with a gap penalty of 15 and a gap length penalty of 10 by using MegAlign version 1.05 software (DNASTAR, Madison, WI). For protein matrix analysis we used MacVector 4.0 software with a pam250 matrix, a window size of 8, hash value of 2, and a min% score of 60. For modeling the three-dimensional structure of the sequence of Clint (1–160) algorithms from the SWISS-MODEL server were used (Guex and Peitsch, 1997). The obtained pdb-data sets were visualized using RasMol version 2.6 software. (Original author is Roger Sayle, Glaxo Wellcome Medicines Research Centre, Stevenage, Herts, United Kingdom; the software, freely distributed throughout the world, is available at: <http://www.umass.edu/microbio/rasmol/getras.htm>.)

Reagents

Enzymes and other reagents for molecular biology were purchased from MBI Fermentas (St. Leon-Roth, Germany). Phosphoinositides were obtained from Echelon (Salt Lake City, UT). Other laboratory chemicals, if not stated otherwise, were in the highest quality available and purchased from Sigma Chemie (Taufkirchen, Germany). The peptide NH₂-CASPDQNASTHTPQSS-COOH was used for custom immunization in rabbits by Eurogentech (Seraing, Belgium). To avoid cross-reactivity with unrelated proteins its sequence was checked for short nearly exact matches against all available databases by using the BLAST algorithm (Altschul *et al.*, 1997). No significant similarities to other proteins were found. To generate anti-Eps15 serum rabbits were custom immunized with the peptide NH₂-CQEDLELAIALSKSEISEA-COOH by Biosciences (Göttingen,

Germany). The sera were affinity purified on a SulfoLink column (Pierce Chemical, Rockford, IL), which contained 1 mg of peptide/ml of column material. The rabbit polyclonal serum against epsin 1 was described previously (Kalthoff *et al.*, 2001). Monoclonal antibodies (mAbs) to the clathrin heavy chain, dynamin 1, AP-3, and γ -adaptin were from Transduction Laboratories (Lexington, KY). mAb100/1 directed against the β -chains of AP-1 and AP-2 (Ahle *et al.*, 1988) were used. Monoclonal antibodies to clathrin (X22) and AP-2 (AP.6) were a gift from Frances Brodsky (University of California, San Francisco, CA). AP-1 was stained with mAb100/3 and AP-2 with mAb100/2 (Ahle *et al.*, 1988). Secondary horseradish peroxidase-coupled goat sera against mouse or rabbit IgG were from ICN Pharmaceuticals Biochemicals Division (Aurora, OH) and used for Western blots. The signals on blots were detected using enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany). Affinity-purified CY3-conjugated goat anti-rabbit and biotin-conjugated goat anti-mouse IgGs were used in immunocytochemical studies. Dichlorotriazinyl-fluorescein-labeled streptavidin was used to detect binding of the biotinylated anti-mouse IgG. All fluorochrome-conjugated affinity-purified goat anti-mouse and anti-rabbit IgG and biotin-conjugated goat anti-mouse IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). All commercially available antibodies were used according to the manufacturers' protocols. The noncommercial monoclonal antibodies were diluted to a concentration of $\sim 1 \mu\text{g/ml}$ for Western blots and immunofluorescence labeling, whereas the dilutions of affinity-purified noncommercial polyclonal sera were determined empirically.

Cloning and Expression of Recombinant Fusion Proteins

The cDNA clone ha02502 containing the gene KIAA0171 was obtained from Takahiro Nagase (Kazusa DNA Research Institute, Kisarazu, Japan) (Nagase *et al.*, 1996). The complete open reading frame as well as the codons 1–162, 163–337, 334–499, and 163–499 were amplified by polymerase chain reaction and ligated between the *Bam*HI and *Eco*RI-sites of pET32a (Novagen, Madison, WI) and pGEX-4T2 (Amersham Biosciences). The sequences of the amplified cDNAs were checked by automated sequencing. A point mutation was detected in fragment 163–499 at position 405 (A to V). Specific mutations were introduced using QuickChange site-directed mutagenesis kit from Stratagene (Amsterdam, The Netherlands). The construct encoding the fragment glutathione *S*-transferase (GST)-Clint-(245–625) was constructed by opening the vector containing the full-length sequence with *Bam*HI and *Bgl*III and religating it. Epsin 1 cDNA (provided by Pietro DeCamilli, Yale University, New Haven, CT) was first digested with *Pau*I and blunted with Klenow polymerase followed by digestion with *Sal*I. The fragment coding for amino acids 2–144 was inserted into *Sal*I and blunted *Not*I sites of pET 32c. Constructs for the expression of full-length GST-GGA2 and GST-GGA2-(473–613) were obtained from Stuart Kornfeld (Washington University, St. Louis, MO) (Zhu *et al.*, 2001). The construct for the expression of GST- α -appendage was provided by Richard Anderson [murine α_c -adaptin-(701–938)] (Wang *et al.*, 1995), the one for 6xHis- β 2-appendage is from Tomas Kirchhausen (Harvard Medical School, Boston, MA) [described as β 2-hinge/ear corresponding to rat brain β 2-adaptin-(592–951)] (Shih *et al.*, 1995), and the one for 6xHis- γ -appendage was described previously (Umeda *et al.*, 2000). For expression, all constructs were transformed into *Escherichia coli* strain BL21 (DE3)pLysS (Novagen, Madison, WI). The bacteria were grown to an optical density of approximately $A_{600 \text{ nm}} = 0.5$, upon which expression was induced by addition of 0.5 mM isopropyl β -D-thiogalactoside. After 3 h at room temperature the bacteria were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.9 mM KH_2PO_4 , 8.2 mM Na_2HPO_4), shock frozen, and stored at -80°C until further use. For protein purification the bacteria were thawed and lysed by addition of 1% Triton X-100 and sonication. The lysate was clarified by ultracentrifugation in a Type 70Ti rotor

for 15 min in an Optima 80-LE centrifuge (Beckman Coulter, Fullerton, CA) at $117,000 \times g$. From the supernatant the GST- and 6xHis-tagged proteins were affinity purified on GSH-Sepharose or Ni^{2+} -nitrilotriacetic acid-agarose beads, respectively, according to the manufacturer's protocols. If needed, the proteins were further purified by gel filtration on a fast protein liquid chromatography system by using a Superdex 200 HR10/30 column (Amersham Biosciences). Recombinant epsin 1 and AP180 were expressed and purified as described previously (Kalthoff *et al.*, 2001). GST- or trx/6xHis-tags from expressed fusion proteins were removed with bovine thrombin (ICN Pharmaceuticals Biochemicals Division). The reaction was stopped by addition of 1 mM phenylmethylsulfonyl fluoride. The proteins were further purified by gel filtration as described above.

Pulldown Experiments with GST-Fusion Proteins

Pig-brain cytosol used in binding experiments was prepared as described elsewhere (Kalthoff *et al.*, 2001). Pulldown experiments with immobilized GST-fusion proteins and purified recombinant proteins were performed exactly as described recently (Scheele *et al.*, 2001).

Liposome Binding Assays

Synthetic liposomes were composed of phosphatidylcholin, phosphatidylethanolamin, and cholesterol in the ratio of 6:2.5:1. Where stated phosphoinositides were included in a concentration of 5%. The lyophilized lipids were dissolved in methanol:chloroform:water (2:1:0.8). The solvent was evaporated under a stream of dry nitrogen. The dried lipids were swollen overnight in 20 mM HEPES, 125 mM potassium acetate, 5 mM MgCl_2 , pH 7.1, and then vortexed and sonicated to near translucence. The liposomes were harvested by centrifugation for 10 min at $25,000 \times g$ and resuspended in assay buffer (25 mM HEPES, 125 mM NaCl, pH 7.4) at a concentration of 1 mg/ml. For binding experiments 25 μg of liposomes, 2×10^{-11} mol of fusion proteins, 0.5 mg/ml fat-free bovine serum albumin (BSA), and 1 mM dithiothreitol in 50 μl were used. Three independent experiments were performed. Error ranges were determined by calculating the SDs.

Tissue Distribution

To determine the amount of Clint expressed in different tissues, aliquots of mouse organs were homogenized in six volumes (6 μl of buffer/mg of tissue) of sample buffer (2.5% SDS, 2.5% β -mercaptoethanol, 12.5% glycerol, 25 mM Tris-HCl, 0.5 mM EDTA, 0.0625% bromphenol blue, pH 8.0; herein with additional 2% SDS) within 30 min after slaughter. Samples corresponding to 1.4 mg of tissue were subjected to SDS-PAGE and Western blotting. Blots were reacted with anti-Clint followed by horseradish peroxidase-conjugated secondary antibody. The blots were developed using enhanced chemiluminescence. Films were scanned with an Agfa Duoscan Instrument (Agfa, Mortsels, Belgium). Signals were quantitated densitometrically using NIH Image version 1.02 software and normalized to the total protein content of the tissue. This was determined by fractionating 0.2 mg of the respective tissue by SDS-PAGE followed by staining with Coomassie Blue. The total staining intensity of each lane was determined densitometrically.

Subcellular Distribution of Clint

One mouse kidney (320 mg) was homogenized in an equal volume (1 μl of buffer/mg of tissue) of 10 mM HEPES, 250 mM sucrose, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, by using a Potter S homogenizer (B. Braun, Melsungen, Germany) and spun for 15 min at $2700 \times g$ in an A-8-11 swinging-bucket rotor with a 5417R table top centrifuge (Eppendorf, Hamburg, Germany) to pellet nuclei and cellular debris. The supernatant was removed and centrifuged again for 1 h at $90,000 \times g$ in a TLA45 rotor with an Optima 100 TL

centrifuge (Beckman Coulter), whereas the pellet ("low-speed pellet") was resuspended in sample buffer (with a final SDS-concentration of 9.5%). The supernatant (cytosol) obtained by ultracentrifugation was removed. The pellet was extracted with 0.5 M Tris-HCl, 1 mM EDTA, pH 7.0, and the extract was subjected to ultracentrifugation as described above. The supernatant was removed ("Tris-extract"), and the pellet was dissolved in buffer G (25 mM HEPES, 125 mM potassium acetate, 5 mM magnesium acetate, pH 7.1) containing 1% Triton X-100, and spun again as described above. This supernatant is referred to as the "Triton X-100-extract." The corresponding pellet was resuspended in sample buffer (with a final SDS-concentration of 9.5%). To all supernatants, fourfold sample buffer was added. The volumes were adjusted and then applied to SDS-PAGE so that the amounts of supernatants and pellets were directly comparable. The distributions of Clint, clathrin, and β -adaptin were visualized by Western blot analysis.

Immunohistochemical Localization of Clint

Young adult male Wistar rats were killed by cervical dislocation. Immediately after slaughter samples of small intestine and kidneys were removed and fixed in a solution of 4% PBS-buffered formaldehyde freshly prepared from paraformaldehyde. The specimen were minced into small blocks, transferred into fresh fixative solutions, and stored for 6 h at 4°C. After washing in PBS, the tissue blocks were dehydrated in ascending concentrations of ethanol and embedded in the paraffin-equivalent Histocomp (Vogel, Giessen, Germany). Paraffin sections (~4 μ m in thickness) were cut and collected on glass slides pretreated with 3-(triethoxysilyl)propylamine (Merck, Darmstadt, Germany). The sections were dewaxed in xylene and rehydrated in descending concentrations of ethanol. Subsequently, antigens were retrieved by heating the sections in 0.01 M sodium citrate buffer, pH 6.0, in a microwave oven (Sharp R-2S67; Sharp, Hamburg, Germany) at 700 W three times for 5 min and then allowing them to cool for 30 min (Cattoretti *et al.*, 1992). After washing in PBS and blocking nonspecific protein binding with 5% BSA in PBS for 30 min at room temperature, the sections were incubated overnight at 4°C with the primary antibody diluted in PBS that contained 1% BSA. Different primary antibodies were applied separately on adjacent sections as well as simultaneously on the same sections for colocalization studies. Secondary antibodies were also diluted in PBS with 1% BSA and incubated with the sections for 1 h at room temperature. To visualize the biotinylated secondary antibody the sections were additionally incubated for 30 min with dichlorotriazinyl-fluorescein-labeled streptavidin diluted in PBS. After washing in PBS the sections were mounted in Mowiol (Sigma Chemie). Control sections were treated in the same way as the experimental sections except that the primary antibodies were omitted. As additional controls preimmune-serum was used or the affinity-purified anti-Clint antibody was preincubated with the peptide NH₂-CASPDQNASTHTPQSS-COOH and then applied to the sections. In all instances only slight background staining was detectable. Madin-Darby bovine kidney (MDBK) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany), containing 10% fetal calf serum (Seromed, Berlin, Germany), and Caco-2 cells were cultured in the same medium plus 1 mM L-glutamine (Seromed). Immunofluorescence experiments were performed exactly as described elsewhere (Ahle *et al.*, 1988). The effect of brefeldin A (BFA; Sigma Chemie) on Clint was tested by incubating MDBK cells with 2 μ g/ml BFA for 2–5 min at 37°C. To reverse the effect of the drug cells were washed three times with fresh medium and incubated for 1 h before processing them for immunofluorescence. Labeled cells and tissues were viewed with an Eclipse E800 microscope (Nikon, Tokyo, Japan) with epifluorescence attachment and a confocal microscope (Leica, Heidelberg, Germany) with a krypton/argon Laser. Epifluorescence images were recorded with a charge-coupled device camera (Princeton Instruments, Trenton, NJ) by using IPLab software

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MLNMWVKVRELVDKATNVVMNYSEIESKVRREATNDDPWGPSQGLMGEIAKA 50
TFMYEQFPELMNMLWSRMLKDNKKNWRRVYKSLLLAYLIRNGSERVVTS 100
AREHIYDLRLSLNYHFVDEHGKDQGINIRQVKELVEFAQDDRLREERK 150
KAKKNKDKYVGVSSDSVGGFRYSERYDPEPKSKWDEEDKNKSAFPFSDK 200
LGELSDKIGSTIDDTISKFRKRDREDSPERCSDSDEEKARRGRSPKGEF 250
KDEEETVTTKHIHITQATETTTTRHKRTANPSKITDLGAAHYTGDKASP 300
DQNASTHTPQSSVKTSPVSSKSSGDLVDLFDGTSQSTGGSADLFGGFADF 350
GSAAASGSFSPSQVTATSGNGDFGDWSAFNQAPSGPVASSGEFFGSASQPA 400
VELVSGSQSALGPPPAASNSSDLDLMGSSQATMTSSQSMNFMSTNTV 450
GLGLPMSRSQNTDMVQKSVSKTLPTWSDPSVNIISLDNLLPGMQPSKPPQ 500
PSLNTMIQQNQMQPMNVMTQSFQAVNLSSPSNMLPVRPQTNALIGGPMF 550
MSMPNVMTGTMGMAPLGNTPMNQSMGMNMNIGMSAAGMGLTGTMGMG 600
PNIAMTSGTVQPKQDAFANFANFSK
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Figure 1. Amino acid sequence of Clint. Putative binding motifs are printed in bold. The methionine-rich domain is shaded.

(Scanalytics, Fairfax, VA) and confocal images with a photomultiplier. Final figures were arranged with Adobe Photoshop version 5.0 (Adobe Systems, San Jose, CA).

RESULTS

Characterization of a Novel ENTH-Domain Protein

Homology searches for yet uncharacterized hypothetical ENTH-domain proteins identified the product of the human gene KIAA0171 as a possible new member of the epsin/AP180 protein family (Ford *et al.*, 2001). The mRNA for the cDNA clone was originally isolated from the human immature myeloid cell line KG-1 and the expressed protein has a predicted molecular weight of 68,254 Da (Figure 1) (Nagase *et al.*, 1996). For reasons of clarity we will refer to the gene product of KIAA0171 as Clint. The sequence of Clint has a high amino-terminal homology with all epsins, especially with the human epsin 1 (similarity of 49.0% for the first 158 amino acids), but also with epsin 2 and epsin 3 (47.7% similarity in both cases) (Figure 2, A–C). In comparison, the homologies to the ENTH-domains of human AP180 and CALM are considerably lower. Only the first 100 amino acids of Clint, corresponding to the first five α -helices of its ENTH-domain, can be reasonably aligned with the ENTH-domains of AP180 (16.8% similarity) and CALM (17.9% similarity). The algorithm Clustal V failed to align the ENTH-domain of Clint with those of Hip1 and Hip1R (our unpublished data). This is not surprising because the sequence homology between Hip1 and Hip1R and the ENTH-domain of the epsins is already very low, although the fold might be similar. Using the automated protein-modeling server SWISS-MODEL (Guex and Peitsch, 1997) we obtained a hypothetical tertiary structure for the first 160 amino acids of Clint. The predicted structure revealed a fold, which is extremely similar to that of the ENTH-domain of epsin 1 (Figure 2D). It consists of eight α -helices and several loop structures in between them. As has been shown previously, epsin 1 contains a pocket of basic residues between helices 3 and 4, formed by the amino acids Arg⁶³, Arg⁷², and Lys⁷⁷, which is able to bind PI-(4,5)-P₂ (Itoh *et al.*, 2001). All crucial amino acids involved in binding the phospholipid are conserved in Clint (herein, Arg⁶⁷, Arg⁷⁷, and Lys⁸¹). Despite the insertion of Met⁶⁸ in the loop between α -helices 3 and 4 they are predicted to form a similar basic pocket in the hypothetical three-dimensional structure of Clint (shown in black in Figure 2D) with the backbone atoms almost exactly in the

Figure 2. Sequence analysis of Clint. (A) Alignment of the ENTH-domains of epsins 1, 2, and 3; AP180; and CALM to the first 158 amino acids of Clint by using the Clustal V method (Higgins and Sharp, 1989). Boxed amino acid residues are identical to Clint. The highest homology is found in epsin 1 with 49% similarity. The epsins 2 and 3 have a similarity of 47.7%, and AP180 and CALM have similarities of 16.8 and 17.9%, respectively. (B) Protein matrix analysis of epsin 1 with Clint and with epsin 3. Note that the homology between epsin 1 and Clint is restricted to the ENTH-domain. (C) Phylogenetic tree of the ENTH-domains. It clearly shows that although the ENTH-domains of the epsins are the closest relatives of the one in Clint they are phylogenetically more distant from Clint than they are from each other. (D) Comparison of the predicted three-dimensional structure of the ENTH-domain of Clint (Guex and Peitsch, 1997) with the crystal structure of the ENTH-domain of epsin 1 (Hyman *et al.*, 2000). The overall fold of both structures is extremely similar. The side chains of the basic residues that are responsible for phosphatidylinositol-4,5-bisphosphate binding in epsin 1 and the conserved amino acids in Clint are shown in black. An additional basic residue (Arg⁶⁸) in Clint that protrudes into the basic pocket and might therefore influence the phospholipid binding properties of Clint is shown in gray.

same places as in the crystal structure of epsin 1. Nevertheless, the orientations of the side chains differ from those in epsin 1, probably due to differences in the space occupied by neighboring amino acids. Furthermore, Clint has an additional basic residue (Arg⁷⁸) that apparently protrudes into

the hypothetical phosphoinositide binding pocket (shown in gray in Figure 2D). Therefore, if Clint binds to phospholipids like epsin 1 does, the specificity of the binding pocket can be expected to differ somewhat from that of epsin 1. The basic cluster on α -helices 1 and 2, which is responsible for

Figure 3. Schematic view of the domain structure of Clint and the recombinantly expressed fragments used in this work. Putative binding motifs for clathrin as well as the ENTH- and methionine-rich domain are marked.

phosphoinositide binding in AP180 and CALM, is not present in Clint.

Outside the ENTH-domain, we were not able to identify any significant sequence homologies to other characterized proteins. In addition to that, all attempts to predict the secondary or tertiary structure in this region produced no reliable results. Therefore, the parts outside the ENTH-domain might either be poorly structured like the disordered segments of AP180 and epsin 1 (Kalthoff *et al.*, 2001) or they might have a novel unknown fold.

We also examined the sequence of Clint for the presence of protein-protein interaction motifs, which in other ENTH-domain-containing proteins mediate binding to clathrin, adaptors, and endocytic accessory proteins. We noted the sequences ³²⁶LVDLF and ⁴²³LFDLM, which are similar to the identified clathrin-binding motif LLDLL present in the γ -adaptin subunit of AP-1 (Doray and Kornfeld, 2001) (Figure 1). Similarities to other known motifs like the AP-2 binding DPF/W (found in AP180, CALM, epsins, and auxilins) and FXDXF (found in Hip1R and AP180; Mishra *et al.*, 2001), the EH-domain-binding motif NPF (found in epsins and CALM), or ubiquitin-interacting motifs (found in epsins) could not be identified. The DPW motif within the Clint ENTH-domain is conserved in epsin but was previously shown not to be involved in AP-2 binding (Chen *et al.*, 1998). A notable feature in the primary structure of Clint is segment Met⁴²⁷ to Met⁶⁰⁵ that contains 31 methionine residues, 17 of which are clustered in the short segment Met⁵⁴⁹-Met⁶⁰⁰ (Figure 1). We will refer to the segment Met⁴²⁷ to Met⁶⁰⁵ domain as the methionine-rich domain of Clint.

Protein-Protein Interaction Studies

To test whether any of the putative binding motifs are functional, full-length Clint and fragments of Clint were recombinantly expressed in bacteria as 6xHis- and as GST-tagged fusion proteins, respectively, and used for protein-protein interaction studies (Figure 3). Brain cytosol is a rich source for components of the endocytic machinery. Therefore, GST-Clint attached to GSH-Sepharose beads was incubated with pig brain cytosol and bound cytosolic proteins were analyzed by Western blotting, by using antibodies

Figure 4. Protein interactions of Clint. Pull-down experiments were carried out by incubating cytosol or purified recombinant proteins with GST-fusion proteins immobilized to glutathione-Sepharose. Beads with bound protein were pelleted by centrifugation (p), whereas unbound protein remained in the supernatant (s). Immobilized GST served as a control. The samples were analyzed by Western blotting by using antibodies against the proteins indicated left of the blots. (A) Incubation of pig brain cytosol with GST-Clint. Clathrin, AP-1, and also some of the AP-2 are pulled down, whereas dynamitin 1 and Eps15 remain in the supernatant. (B) Clint interacts directly with the terminal domain of clathrin (TD). (C) Pull-down of adaptin appendage domains with GST-Clint-(245–625). It shows that this part of Clint has a direct interaction with the γ - and β 2-appendage, whereas the association of α -appendage is barely above background. (D) Clint directly binds to GST-GGA2.

against several proteins known to be involved in clathrin-dependent membrane traffic. Indeed, we observed that clathrin, AP-1 and also some AP-2 were pulled out of the cytosol with immobilized GST-Clint, whereas dynamitin 1 and Eps15 remained quantitatively in the supernatant fraction (Figure 4A). To test whether the association of these proteins with Clint is direct, we also performed pull-down experiments with purified recombinant proteins. Because a number of interaction partners of clathrin bind to its globular amino-terminal domain (TD) we used a GST-TD fusion protein to test the interaction with 6xHis-tagged Clint in a pull-down experiment. We observed that Clint does indeed interact directly and specifically with the clathrin TD (Figure 4B).

To prove a direct interaction of the adaptors with Clint we concentrated on the appendage domains of the γ -subunit of AP-1 and the α - and β 2-subunits of AP-2, because it is well known that they are major platforms for protein-protein interactions (Takei and Haucke, 2001). Especially, the α -appendage domain has been shown to associate with a large number of diverse binding partners. We attached GST-Clint-(245–625) (Figure 3) to GSH-agarose beads and incubated them separately with untagged α -appendage, 6xHis- β 2-ap-

pendage, and 6xHis- γ -appendage. GST-Clint-(245–625) was used because it contained all the putative binding motifs and it was also obtained from the bacteria with higher yields than the full-length Clint. The β 2- and γ -appendage domains were readily pulled down by GST-Clint-(245–625), whereas the α -appendage associated only poorly with it (Figure 4C). When GST-Clint-(1–245) was used as the bait in pulldown experiments neither clathrin nor adaptors associated with it (our unpublished data).

The Golgi-localized protein GGA2 was recently shown to associate with clathrin, Arf1, and cargo (Hirst *et al.*, 2000; Puertollano *et al.*, 2001; Zhu *et al.*, 2001) and thus is believed to play a role as an adaptor-like protein in intracellular trafficking. Therefore, we considered this protein as a potential binding partner of Clint and included it in our binding studies. Indeed, we could show that GST-GGA2 efficiently pulls down 6xHis-Clint (Figure 4D). To further narrow down the binding sites to regions that contain the putative clathrin TD binding motifs ³²⁶LVDLF and ⁴²³LFDLM, we constructed 6xHis-tagged fragments of Clint, which all lacked the ENTH-domain, but encompassed the segments 163–499, 163–337, and 334–499, respectively (Figure 3). A pulldown experiment with GST-clathrin TD shows that Clint-(163–499) and Clint-(334–499), but not Clint-(163–337) bound efficiently to clathrin (Figure 5A). Therefore, major affinity sites seem to be located between the amino acids 334 and 499, thus apparently ruling out a high-affinity interaction of the clathrin TD with the ³²⁶LVDLF motif of Clint (see below). Performing a similar experiment with GST-GGA2 as the bait reveals that Clint-(163–499) has quite a strong affinity for GST-GGA2, whereas Clint-(163–337) and Clint-(334–499) either do not bind at all or only with negligible affinity (Figure 5A). This could either suggest that the binding site is disrupted by expressing the shorter fragments or that binding to GGA2 is mediated by several tandemly arranged low-affinity sites, which need to function cooperatively for a high-affinity interaction. A similar concept has been proposed for the binding of other proteins to the α -appendage domain of AP-2 or to the terminal domain of clathrin (Drake *et al.*, 2000; Kalthoff *et al.*, 2001; Scheele *et al.*, 2001).

GGA proteins are organized in distinct modules referred to as VHS-, GGA- and TOM1- (GAT), and γ -appendage/ear (GAE)-domain (Robinson and Bonifacino, 2001). Because of the homology between the GAE-domain and the γ -appendage we thought it likely that this part of the GGA2 molecule might bind Clint. To test this conjecture, GST-GGA2-(473–613), which corresponds to the GAE-domain, was attached to beads and incubated with the Clint fragments. Indeed, we observed strong binding of Clint-(163–499) to the GAE-domain, weak binding of Clint-(334–499), and no binding of Clint-(163–337) (Figure 5A).

To determine whether the short peptide motifs ³²⁶LVDLF and ⁴²³LFDLM are indeed involved in clathrin binding we mutated them to ³²⁶AVAAF and ⁴²³AFAAM, respectively. Pulldown experiments with GST-tagged clathrin TD showed that the association of Clint-(163–499) with clathrin was not significantly affected upon mutating the motif ³²⁶LVDLF. However, the mutations within the second motif (⁴²³LFDLM) drastically reduced binding of the Clint fragment to the clathrin TD (Figure 5B). When this motif was mutated in Clint-(334–499) binding was abolished (Figure 5B). Taken together, these results sug-

Figure 5. Localization of binding domains by using GST-pulldown experiments. (A) Principal binding site of Clint to clathrin TD is located between the amino acids 334–499 because only 6xHis-Clint-(334–499) and -(163–499), but not -(163–337), bind to GST-TD. GST-GGA2 only efficiently pulls down 6xHis-Clint-(163–499), whereas the shorter fragments alone hardly have an affinity for GGA2. As similar results are obtained by pulldown with GST-GGA2-(473–613), which encompasses the GAE-domain, the major affinity site for Clint in GGA2 is located within this part of the molecule. (B) Mutations in putative clathrin box motifs. The short peptide motifs ³²⁶LVDLF and ⁴²³LFDLM were altered to AVAAF and AFAAM, respectively. Only the mutations of ⁴²³LFDLM showed a reduction in clathrin TD binding.

gest that the motif ⁴²³LFDLM is mainly responsible for the interaction of Clint with the terminal domain of clathrin. However, our results do not rule out an ancillary function for the first motif.

Lipid Binding of Clint

Because the basic phosphoinositide binding pocket of epsin 1 is conserved in the ENTH-domain of Clint, we also investigated whether Clint would bind to phospholipids. For this purpose, we used liposomes that contained either no or 5% of phosphoinositides. Whereas thioredoxin-tagged epsin 1-(2–144) bound preferentially to liposomes that contained PI-(4,5)-P₂ (Itoh *et al.*, 2001), thioredoxin-tagged Clint-(1–162) associated with phosphoinositide-containing liposomes, but did not show any clear preferences for particular phosphoinositides (Figure 6).

Figure 6. Liposome binding of the thioredoxin-tagged ENTH-domains of epsin 1 and Clint. The recombinant ENTH-domains were incubated with liposomes containing 5% of different phosphoinositides. Whereas the epsin 1 ENTH-domain binds preferentially to PI-(4,5)-P₂ as described previously (Itoh *et al.*, 2001), the one of Clint does not seem to interact avidly with a specific phosphoinositide. The tag alone does not interact with any of the liposomes.

Tissue Distribution of Clint

A previously published systematic Northern blot analysis with a specific KIAA0171 probe suggested that the Clint message is ubiquitously transcribed at low-to-intermediate levels (Nagase *et al.*, 1996). We extended this analysis by determining the expression level of Clint by using Western blotting of lysates from different mouse organs. On SDS-PAGE Clint gave usually rise to a closely spaced doublet that migrates approximately like a polypeptide of ~80 kDa.

The band splitting might be due to posttranslational modifications, differential splicing, or proteolytic damage. The highest level of Clint was present in kidney, but it is also expressed in brain, spleen, lung, liver, and testes. Clint was not detected in heart and skeletal muscle (Figure 7A).

Clint in Subcellular Fractions

To determine which subcellular fraction contains Clint, mouse kidney tissue was homogenized in isotonic buffer and then subjected to differential centrifugation combined with various extraction procedures (Figure 7B). Clint was neither found in a low-speed pellet representing larger cellular debris and the nuclear fraction, nor in the 100,000 × *g* supernatant, which corresponds to the cytosolic fraction. When the 100,000 × *g* pellet was washed with 0.5 M Tris at pH 7.0, which is a commonly used procedure to remove peripheral membrane proteins such as clathrin coat components, Clint was solubilized quantitatively like most of the clathrin and AP complexes. Therefore, no Clint was found in the Triton X-100 extract or in the Triton-insoluble pellet of the Tris-extracted membranes. Thus, it can be concluded that Clint is a peripheral membrane protein. Because of these results and the fact that Clint interacts with clathrin, we asked whether Clint like conventional adaptors is a component of clathrin-coated vesicles (CCVs). We isolated CCVs from rat liver and found that ~10% of the total Clint indeed is present in a crude CCV fraction that was purified by differential centrifugation, which involved a Ficoll/sucrose step (Campbell *et al.*, 1983). To exclude the possibility of a fortuitous copurification of Clint-containing membranes with CCVs, the fraction was further purified by centrifugation.

Figure 7. Tissue distribution and subcellular localization of Clint. (A) Tissue distribution of Clint. Comparable amounts of lysed mouse organs were loaded onto SDS-PAGE and analyzed by Western blotting against Clint. The protein is most abundant in kidney but also present in brain, spleen, lung, liver, and testes. The intensities of the bands were analyzed densitometrically, normalized to the protein content of the samples, and depicted in the diagram. (B) Subcellular distribution of Clint. A mouse kidney was homogenized and fractionated by differential centrifugation combined with several different chemical extractions of the high-speed pellet. Clint is neither found in a low-speed pellet nor in the cytosolic fraction. It can be quantitatively extracted with 0.5 M Tris, pH 7.0, from the 100,000 × *g* pellet and, therefore, none is found in a Triton X-100 extract of the Tris-treated membranes or in the corresponding Triton-insoluble pellet. (C) Clint copurifies with clathrin-coated vesicles. Shown are the fractions of the Ficoll/D₂O-gradient as the final step of coated vesicle preparation from rat liver. Clint is found in the same fractions as clathrin.

gation on a Ficoll/D₂O gradient (Pearse, 1983). Western blotting of the fractions showed that all of the Clint still copurified with clathrin, which suggests that a significant subpopulation of Clint is tightly associated with CCV (Figure 7C).

Immunolocalization of Clint

Because kidney tissue seemed to be highly enriched in Clint (Figure 7A), we stained kidney-derived epithelial cell lines with antibodies to Clint, clathrin, AP-1, and AP-2. In MDBK cells, Clint concentrates perinuclearly and overlaps with clathrin and AP-1 staining in this region (Figures 8, A–F). On close inspection, many structures in the perinuclear area were observed to contain Clint and clathrin or Clint and AP-1, respectively. This region probably corresponds to the TGN. Outside the perinuclear area, the anti-Clint antibody gives rise to punctuate staining of the cytoplasm, suggesting that Clint is also a component of more peripheral vesicular structures. This staining pattern does not match that of AP-1, AP-2, AP-3, or clathrin (Figures 8, A–O), which suggests that Clint is a component of a distinct class of transport vesicles. We also detected Clint in the human enterocytic line Caco-2. The staining pattern was very similar to that in MDBK-cells, including the partial colocalization of Clint with AP-1. Double labeling with AP-3 shows that although both proteins are enriched in the perinuclear area only little overlap in staining with Clint could be detected (Figures 8, M–O). For lack of suitable reagents we were unable to perform double-labeling immunofluorescence with anti-Clint and anti-GGA2 antibodies. Recruitment of AP-1, AP-3, and GGAs to the TGN is an Arf1-dependent process, which is efficiently inhibited by the fungal metabolite BFA (Stamnes and Rothman, 1993; Traub *et al.*, 1993; Ooi *et al.*, 1998; Puertollano *et al.*, 2001). To determine whether the association of Clint is also dependent on Arf1, we treated MDBK cells with BFA for 5 min and then stained them for AP-1 and Clint. Both, AP-1 and Clint became rapidly redistributed upon exposure to the drug (Figures 8, P–Q). Within 60 min of incubating the cells at 37°C in the absence of the drug, the Clint- and AP-1 staining returned to that of untreated cells (our unpublished data). This result strongly suggests that the recruitment of Clint to the TGN is directly or indirectly an Arf1-driven process.

Next, we stained paraffin sections from rat kidney with antibodies to Clint, clathrin, AP-1, and AP-2, respectively. The basic unit of the mammalian kidney is the nephron, which consists of the glomerulus, the proximal tubule, the loop of Henle, and the distal tubule. The latter is connected with the collecting duct. A major function of the proximal tubule is the active resorption of nutrients and sodium chloride. Furthermore, it is important in regulating pH. The distal tubule and the collecting duct are predominantly involved in pH regulation and in controlling the water and electrolyte balance. Clathrin and Clint are present in all tubular cells, but clathrin staining is the strongest in cells of the proximal tubule, where it is presumably involved in the uptake of nutrients. In contrast, strongest expression of Clint was detected in cells lining the collecting ducts. Within these cells the labeling is most prominent in the apical region where it colocalizes with the staining of clathrin (Figures 9, A–C). In contrast to the bright staining in the collecting ducts, the epithelium of the proximal tubules reveals only

faint Clint staining. It is predominantly present in a line-like pattern underneath the brush border (Figure 9A). Double-labeling experiments with anti-Clint and anti-clathrin antibodies indicate a similar distribution for clathrin and overlays of the images reveal a close spatial relationship of clathrin and Clint in the proximal tubule epithelium, but only little overlapping staining (Figures 9, A–C).

In cortical collecting duct cells, AP-1 was detected within the narrow region between the apical domain of the plasma membrane and the nucleus (Figure 9H). Colocalization studies showed an intense overlapping staining in these cells (Figure 9I). In contrast, AP-2 labeling was strong below the brush border of proximal tubule cells. An overlay of confocal images showed no evidence of colocalization with Clint (Figure 9, M–O).

Because Clint was abundant in Caco-2 cells we extended our histological analysis to epithelial cells that line the villi of the rat small intestine. With the exception of apoptotic cells at the very tip of the villi Clint occurs evenly distributed (Figure 9D). Within the enterocytes it is predominantly present in a supranuclear region where the Golgi is located. Labeling of clathrin (Figure 9E) reveals a similar distribution with additional faintly stained dots close to the plasma membrane. The merged image (Figure 8F) demonstrates strong colocalization of the two proteins in the region of the Golgi. A similar Golgi staining pattern was found after application of the antibody against AP-1. Merging of confocal images obtained after double labeling showed a strong colocalization of Clint and AP-1 in small intestinal epithelial cells (Figure 9, J–L).

DISCUSSION

We have characterized a novel ENTH-domain protein that binds in pulldown experiments well to clathrin, to the conventional adaptor AP-1, and the adaptor-related protein GGA2. We also detected a weak association with the AP-2 adaptor that seems to be mainly mediated by the β -subunit, because the recombinant α -appendage domain binds only poorly to Clint. Immunofluorescence studies corroborated a close functional relationship of Clint with AP-1 in the region of the TGN and therefore we refer to the new protein as Clint. Like AP-1, the association of Clint with the TGN seems to directly or indirectly involve Arf1, because the fungal metabolite BFA causes the rapid redistribution of Clint. A very recent database entry (GenBank accession AF43813) lists the sequence of Clint as epsin 4. However, we do not think that Clint is an epsin, because the primary structure of its ENTH-domain sets it apart from the ENTH-domains of the archetypical epsins 1–3. Moreover, outside the ENTH-domain the sequence of Clint bears no homology to the corresponding regions of the epsins (Figure 2B). So far, the ENTH-domain proteins epsin 1/2, Hip1, Hip1R, and AP180/CALM have been characterized as essential components of the endocytosis machinery. The function of epsin 3, which is expressed only by keratinocytes in wounded skin or in culture when migrating on a collagen matrix has not yet been determined (Spradling *et al.*, 2001), but it is probably related to the regulation of the interaction between cells and the extracellular matrix. The preferred association of Clint with AP-1 and GGA2 *in vitro*, suggests that Clint is not part of the endocytic machinery, but might function between

Figure 8. Immunolocalization of Clint in cultured mammalian cell lines. (A–C) Clint and clathrin colocalize in the perinuclear area of MDBK cells. C shows the merged image. In more peripheral parts of the cells a distinct punctate staining of Clint can be seen, which does not overlap with that of clathrin. (D–I) Perinuclear staining for Clint also colocalizes with AP-1 both in Caco-2 (D–F) and MDBK cells (G–I). Again, no colocalization in the peripheral vesicles can be seen. (J–L) Clint shows no overlapping staining with AP-2. (M–O) Although Clint and AP-3 are both found in the perinuclear area they do not exactly colocalize. (P–Q) Treatment of MDBK cells with BFA redistributes Clint from the Golgi area to the cytosol as it does AP-1. Bars, 10 μ m.

the TGN and the endosomal system. Its localization at or near the TGN as seen by immunofluorescence microscopy supports this conjecture, as does the apparent lack of colocalization with the AP-2 adaptor. However, Clint is also a component of numerous more peripheral vesicular struc-

tures that cannot be stained with antibodies to AP-1 or AP-3. The observation that Clint associates with clathrin, AP-1, and GGA2 suggests that it might be involved in early steps of transport vesicle formation, but that in contrast to clathrin and the adaptors it remains associated with the vesicle for

Figure 9. Immunolocalization of Clint in rat tissue sections. (A–C) Collecting duct (cd) cells of rat kidney sections show positive labeling at their apical pole for Clint and clathrin. Overlay of both stainings (C) reveals a colocalization of Clint and clathrin in this region. In the proximal tubule (pt) both proteins show a linear distribution pattern below and in parallel to the brush border but only little overlap in staining. (D–F) Clint and clathrin show a regular supranuclear distribution in cells lining a small intestinal villus. It is notable that the cells at the villus tip lack staining for Clint. Overlay of both images (F) confirms that Clint and clathrin colocalize in the supranuclear region of small intestinal epithelial cells. (G–I) Confocal images. In cortical collecting ducts Clint and AP-1 show identical distribution. They are located between the nucleus and the apical plasma membrane. This colocalization is confirmed by the overlay of the images (I). (J–L) Confocal images. The same is evident in small intestinal epithelial cells. Clint and AP-1 are both localized in the supranuclear region of the cells and the overlay of the images (L) shows a strong colocalization of the proteins in this tissue. gc, goblet cell. (M–O) Clint and AP-2 showed divergent distributions in the rat kidney. AP-2 labeling (N) is strong below the brush border of proximal tubule cells. The merged image shows only occasional overlapping staining. Bars, 10 μ m.

Figure 10. Summary of binding data obtained for Clint and its fragments. The results of GST-pulldown experiments with purified components are shown. The sequences of the mutated putative clathrin box motifs are listed next to the arrows on the left.

much longer. In analogy to the role of AP180/CALM and epsin on the plasma membrane Clint might help to recruit other coat components to the TGN membrane. Alternatively, AP-1 or GGA2 might recruit Clint, which then helps to orchestrate clathrin binding. Electron microscopy on immunogold-labeled sections will allow a more definitive identification of the structures that bind Clint.

After γ -synergin and auxilin 2 (cyclin G-associated kinase), Clint is the third interaction partner of the γ -adaptin appendage domain, which like the α -adaptin appendage domain, seems to be a major platform for protein-protein interactions with accessory proteins. Because of the homology of the γ -appendage to the ear domain of GGA2 it is not surprising that we also detected binding of Clint to the ear domain of GGA2.

The role of the ENTH-domain in membrane binding or membrane selection is currently difficult to assess. The ENTH-domain of Clint displays only a low affinity and a broad specificity for phosphoinositides. Therefore, more important for its Golgi-association is probably a direct or indirect interaction with Arf1 or other factors.

Paraffin sections of rat kidney revealed that Clint is highly expressed in the epithelial cells lining the collecting duct. Within these cells antibody staining is the strongest in the apical region. The staining pattern of Clint in the collecting duct is reminiscent to that of aquaporin 2 and the V-ATPase (our unpublished data). Aquaporin 2 is localized in apical vesicles and in the apical plasma membrane (Hayashi *et al.*, 1994; Sasaki *et al.*, 1994). In type A intercalated cells of the collecting duct the V-ATPase also localizes to apical vesicles and to the apical plasma membrane (Brown and Breton, 2000). The question whether the presence of Clint in apical vesicles of the collecting duct reflects a functional relationship to aquaporin 2 and/or the V-ATPase needs to be addressed by double labeling studies by using light- and electron microscopy. In enterocytes of the small intestine Clint colocalizes perfectly with the bulk of the clathrin and AP-1

in the region of the TGN. At the tip of the villi where cells undergo apoptosis, the intensity of the Clint fluorescence tended to be relatively reduced compared with that of clathrin. This suggests that Clint might be either degraded or its expression down-regulated when the cells reach the end of their natural life span. However, Clint has no cleavage sites for caspases 1–10.

Despite our limited knowledge about the structure of Clint we noted significant differences to the endocytic ENTH-domain proteins AP180 and epsin 1 that are disordered except for their ENTH-domains (Kalthoff *et al.*, 2001). Preliminary results suggest that Clint is probably folded in the regions outside its ENTH domain. First, unlike the C-terminal segments of AP180 and epsin 1 Clint-(245–625) precipitates upon boiling and shock cooling. Second, its behavior during gel filtration is inconsistent with that of an unfolded protein (our unpublished data). A remarkable feature of Clint that is not shared by epsin 1 and AP180 is the unusually high number of 39 methionine residues, 17 of which cluster between Met⁵⁴⁹ and Met⁶⁰⁰. A BLAST search with the use of the sequence of this segment identified an epsin-related protein in Arabidopsis (GenBank accession T48997) with an ENTH-domain and a methionine-rich domain near its carboxyl-terminal end. Despite its much larger size we consider it a Clint ortholog.

Although Clint lacks archetypical clathrin box motifs it binds well to the clathrin terminal domain. Strong clathrin binding could be demonstrated for the Clint fragment 334–499. Based on our mutation studies the pentapeptide 423LFDLM is mainly responsible for clathrin TD binding. The LFDLM sequence is related to the LLDLL motif that was recently shown to mediate binding of a GST- γ -ear/hinge fusion protein to the clathrin terminal domain (Doray and Kornfeld, 2001). The Clint segment 163–337, which also contains a pentapeptide with similarity to the LLDLL motif (326LVDF), failed to bind the clathrin TD on its own. Furthermore, the elimination of this motif from Clint-(163–499)

did not decrease the affinity of the fragment for clathrin in a pulldown assay.

A principal binding site of the GAE-domain of GGA2 is also located in the Clint segment 334–499. We do not yet know whether the γ -appendage domain binds to the same site on Clint, but we think that this is possible, because of the homology between the GAE-domain and the γ -appendage domain. An overview of the binding results is given in Figure 10. Our current work focuses on the Clint regions that contain determinants for γ -appendage binding, which also might be shared by γ -synergism and auxilin 2.

Note added in proof. While this article was in print, the identification of a protein identical to Clint was reported. It was given the name enthoprotin. (Wasiak, S., *et al.* (2002). Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics. *J. Cell Biol.* 158, 855–862.)

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