Identification and Characterization of Novel Clathrin Adaptor-related Proteins*

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We have identified a human ~87-kDa protein, designated as γ2-adaptin, that is similar to γ-adaptin (called γ1-adaptin in this paper), a large chain of the AP-1 clathrin-associated adaptor complex, not only in the primary structure (60% amino acid identity) but also in the domain organization. Northern blot analysis has shown that its mRNA is expressed in a variety of tissues. Analysis using a yeast two-hybrid system has revealed that, similarly to γ1-adaptin, γ2-adaptin is capable of interacting not only with the σ1 chain (called as σ1A in this paper), the small chain of the AP-1 complex, but also with a novel σ1-like protein, designated as σ1B, which shows an 87% amino acid identity to σ1A; and that, unlike γ1-adaptin, it is unable to interact with β1-adaptin, another large chain of the AP-1 complex. Immunofluorescence microscopy analysis has revealed that γ2-adaptin is localized to paranuclear vesicular structures that are not superimposed on structures containing γ1-adaptin. Furthermore, unlike γ1-adaptin, γ2-adaptin is recruited onto membranes in the presence of a fungal antibiotic, brefeldin A. These data suggest that γ2-adaptin constitutes a novel adaptor-related complex that participates in a transport step different from that of AP-1.

In eukaryotic cells, transport of proteins between membrane-bound compartments along the exocytic and endocytic pathways involves coated carrier vesicles that bud from a donor compartment and fuse with a target acceptor compartment(s) (for review, see Refs. 1–5). The vesicle budding process is initiated by recruitment of coat proteins from the cytosol onto the donor membranes. To date, three classes of coated transport vesicles have been unequivocally characterized (1–5): COP II-coated vesicle coats assemble onto cisternae of the Golgi apparatus and onto the intermediate compartment between the ER and the Golgi; and clathrin-coated vesicles containing the AP-1 and AP-2 adaptor complexes bud from the trans-Golgi network (TGN) and the plasma membrane, respectively. Both the clathrin adaptor complexes are composed of two large subunits, a medium chain, and a small subunit: β1(β')-adaptin, γ-adaptin, μ1 (AP47), and σ1 (AP19) in AP-1; and β2 (β'-adaptin, α-adaptin, μ2 (AP50), and σ2 (AP17) in AP-2 (for review, see Refs 6 and 7). The clathrin-coated vesicles that bud from the plasma membrane play a direct role in receptor-mediated endocytosis, whereas those budding from the TGN are thought to be involved in sorting of lysosomal proteins into the lysosomal/endosomal system. Recently, the adaptor complexes have been shown to be capable of interacting through their medium chains (μ1 and μ2) with the Tyr-containing sorting signals within the cytoplasmic domain of various plasma membrane, lysosomal membrane, and TGN-resident membrane proteins (Refs 8–11, and reviewed in Ref. 12).

On the other hand, a morphological study has suggested the presence of another class of clathrin-coated vesicles on early endosomes that do not contain the AP-1 or AP-2 complex (13). Very recently, a novel adaptor-related complex, named AP-3, has been discovered and shown to be localized to the endosomal and TGN compartments and may function in protein transport from the Golgi to the lysosomal/endosomal system (14–20). The complex is a heterotetramer composed of δ, β3A, μ3A (also known as p47A), and σ3A or σ3B in humans.

In the present study, we identified a novel adaptor-related protein, designated as γ2-adaptin, that is highly similar to γ-adaptin (called as γ1-adaptin in this paper) not only in the primary structure but also in the domain organization. It is expressed in various tissues and is mainly localized to paranuclear vesicular structures, which are different from γ1-adaptin-containing structures. Analysis using the yeast two-hybrid system revealed that γ2-adaptin is capable of interacting with the σ1 chain and a novel σ1-like protein.

EXPERIMENTAL PROCEDURES
cDNA Cloning—Random-primed cDNA of human hepatoma HepG2 cells was amplified by polymerase chain reaction (PCR) with three sets of oligonucleotide primers synthesized on the basis of sequences of GenBankTM human expressed sequence tags (ESTs), accession numbers T49401, F07336, and R87719, the predicted amino acid sequences of which showed a significant homology but that was not identical to that of γ-adaptin. The amplified DNA fragments were separately subcloned into the pBluescript® II KS(+) vector (Stratagene), and their sequences were confirmed. A mixture of the three cDNA fragments were then used to screen ~4 × 109 phages of a HepG2 cDNA library (21). Six phage clones, which were hybridized with all the three probes, were isolated, and their cDNA insert was subcloned into the NotI site of pBluescript® II KS(+). A combination of three cDNA clones was presumed to cover the entire coding sequence, and their sequences were...
determined from both strands using a BcaBest sequencing kit (Takara Shuzo, Co.). The same HepG2 DNA library was also screened with a mouse γ-adaptin cDNA fragment as a probe, which was obtained by PCR amplification of mouse brain cDNA with primers synthesized on the basis of the published sequence (22), and 17 positive clones were obtained. A combination of these cDNA clones covered the entire coding sequence of human γ-1-adaptin.

Human γ1B DNAs were cloned from the HepG2 library by screening with a partial cDNA probe that was obtained by PCR amplification of random-primed cDNA of HepG2 cells with primers synthesized on the basis of an EST sequence (accession number, AA455805). An I.M.A.G.E. clone (clone 5468290) covering the entire coding sequence of human γ1A was obtained from UK Human Genome Mapping Resource Center.

**Northern Blot Analysis—**A human multiple tissue RNA blot (CLONTECH) was sequentially probed with a α32P-labeled cDNA fragment of human γ2- or γ1-adaptin, γ1A, or γ1B according to the instruction of the manufacturer. The probes used were: γ2-adaptin, a 550-bp StuI-Apal fragment (nucleotides 1902–2451); γ1-adaptin, a 2,204-bp NcoI-BglII fragment (nucleotides 580–2783); γ1A, a 494-bp KpnI-Apal fragment (nucleotides 83–576); and γ1B, a 591-bp fragment (nucleotides 28–618).

**Plasmid Construction—**For use in the yeast two-hybrid system, bait vectors of γ2- (pGBT9-γ2) and γ1-adaptins (pGBT9-γ1) were constructed by ligation of PCR-amplified cDNA fragments covering the entire coding sequence of human γ2- or γ1-adaptin. A (BFGA) vector containing the and either NotI or EcoRI sites of pGBT9-BEN, which is a derivative of pGBT9 (CLONTECH) constructed by replacing the original polynucleotide sequence between the EcoRI and BamHI sites with the sequence 5′-GAATTGGATCCGACTGGATCCGCGCGCCATGC-3′. Pre vectors of human β1- and β2-adaptins; mouse γ1A, β1A, and µ2 chains; human γ1B, β2, γ3A and γ3B chains; and rat µA and µB chains were constructed by ligation of PCR fragments of corresponding cDNAs into the BamHI and EcoRI sites of pGAD10 (CLONTECH).

A mammalian expression vector for γ2-adaptin (pcDNA3-γ2) was constructed by subcloning of a cDNA fragment covering the entire coding sequence of γ2-adaptin into the multiple cloning site of the pcDNA3 vector (Invitrogen). Construction of a variant of the pcDNA3 vector with an influenza virus hemagglutinin (HA) epitope sequence downstream of the cytomegalovirus promoter (pcDNA3-HA) was described previously (23). An expression vector for HA-tagged γ2-adaptin (pcDNA3-HA-γ2) was constructed by ligation of the human γ2-adaptin cDNA fragment described above into the BamHI and EcoRI sites of pGAD10 (CLONTECH).

**Antibodies—**Polyclonal γ2-adaptin antiserum was raised in two rabbits against synthetic 19-amino acid peptide conjugated with keyhole limpet hemocyanin, and one (no. 93) of them worked well. The antiserum was affinity purified as described (24). Polyclonal rabbit anti-lamp-1 and monolonal mouse anti-TGN38 were kindly provided by K. Akasaka (Pukuyama University, Japan; Ref. 25) and G. Banting (University of Bristol, UK; Ref. 26), respectively. Monolonal mouse anti-γ1-adaptin antibodies, clone nos. 88 and 100.3, were purchased from Ratheon (Ratheon, Bedford, MA), and monoclonal mouse 12CA5 and rat (3F10) antibodies against the HA epitope were from Boehringer Mannheim. All fluorescent and peroxidase-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories.

**Cell Fractionation and Western Blot Analysis—**pcDNA3-γ2 or pcDNA3-HA-γ2 was transfected into human embryonic kidney 293 cells using a Cellfection transfection kit (Amersham Pharmacia Biotech) and cultured for 48 h. The cells were washed twice with ice-cold phosphate-buffered saline and lysed in ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 2.5 mM EDTA, 0.25 mM NaCl) containing a Complete™ protease inhibitor mixture (Boehringer Mannheim) by repeated vortexing. The homogenate was centrifuged by centrifugation at 10,000 × g for 10 min at 4 °C.

For preparation of the cytosol and membrane fractions, 293 cells or HL-60 cells washed twice with phosphate-buffered saline were homogenized in ice-cold cytosol buffer (10 mM HEPES-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1.0 mM diithiothreitol, 1 mg/ml glucose) containing the Complete™ mixture by 5 × 30 strokes with a Dounce homogenizer. The homogenate was centrifuged at 500 × g for 10 min at 4 °C to remove nuclei and cell debris. The supernatant, nuclear supernatant, was centrifuged at 10,000 × g for 40 min at 4 °C to obtain cytosol and membrane fractions. The pellet was washed once with cytosol buffer and lysed in the cell lysis buffer.

The membrane fraction of HL-60 cells was further fractionated by a modified method of Simpson et al. (15). Briefly, the pellet of the membrane fraction was resuspended in ice-cold cytosol buffer. The suspension containing ~1.0 mg of protein was loaded onto 12 ml of 0–40% Nycodenz (Nycodema Pharma AS) linear gradients in cytosol buffer. The gradients were centrifuged in a Beckman SW41Ti rotor at 37,000 rpm for 4 h at 4 °C, and 0.6-m1 fractions were collected from the bottom. The fractions were diluted with four volumes of cytosol buffer and centrifuged at 120,000 × g for 60 min, and the pellets were subjected to SDS-PAGE and Western blotting.

The cell lysates and the cell fractions were boiled in an SDS-PAGE sample buffer, electrohoresed on a 7.5% SDS-polyacrylamide gel, electrophobulated onto an Immobilon-P membrane (Millipore). The blot was incubated sequentially with either anti-γ2-adaptin, anti-HA (3F10) or anti-γ1-adaptin (100.3) antibodies, and peroxidase-labeled secondary antibodies (Jackson ImmunoResearch Laboratories) and developed using a Renaissance Chemiluminescence Reagent Plus (NEN Life Science Products) according to the instruction of the manufacturer.

**Recruitment Assay—**Recruitment experiments were performed by the method of Robinson and Kreis (27). Briefly, normal rat kidney (NRR) cells grown on 9-cm culture dishes were washed twice with cytosol buffer and then frozen by floating the dishes in liquid nitrogen. After thawing, the permeabilized cells from two dishes were scraped, divided into six aliquots, and washed twice with cytosol buffer. The pellet was resuspended in 100 μl of cytosol buffer or cytosol (~250 μg of protein) containing 1 mM EGTA and an ATP regenerating system (1 mM ATP, 5 mM creatine phosphate, 80 μg/ml creatine kinase) and incubated at 37 °C for 20 min. When indicated, 1 mM GTP, 100 μg/ml GTP-β-S, or 100 μg/ml GTP-γ-S was included. After a 20 min incubation, the reaction mixture was centrifuged at 500 × g for 10 min, and the pellet was boiled with SDS-PAGE sample buffer and subjected to SDS-PAGE and Western blot analysis using polyclonal anti-γ2-adaptin antibodies or monoclonal anti-γ1-adaptin antibody (no. 100.3) as described above.

**Indirect Immunofluorescence Analysis—**Rat hepatocyte Clone 9 cells, NRR cells, or monkey kidney Vero cells grown in wells of 8-well Lab-Tek-II chamber slides (Nunc) were transfected with pcDNAs-γ2 or pcDNAs-HA-γ2 using a TransIT-TL1 polyanime transfection reagent (PanVera Corp.). The transfected cells were cultured for 12 h and processed for indirect immunofluorescence analysis as described previously (23, 28). Briefly, the cells fixed and permeabilized with methanol at −20 °C for 5 min were incubated with anti-γ2-adaptin or anti-HA (3F10) primary antibodies in combination with monoclonal antibodies against γ1-adaptin (no. 88), TGN38, or lamp-1 at 4 °C overnight.

The cells were then stained with fluorescein isothiocyanate-conjugated and Cy3-conjugated secondary antibodies. The stained cells were observed with a laser-scanning confocal microscope (TCS NT, Leica LaserTech).

**RESULTS**

**Identification of a cDNA for a γ-Adaptin-like Protein**—We exploited the availability of large data bases of ESTs to identify adaptor-related proteins. As a search of the GenBank™ EST database using the TCONS alignment revealed that several human ESTs encoded sequences homologous but not identical to γ-adaptin. We obtained partial cDNA fragments by PCR amplification of human hematopoietic HepG2 cell cDNA using sets of primers specific to these ESTs and used them as probes to screen a HepG2 cDNA library. Sequence analysis of the positive cDNA clones revealed that a combination of three...
cDNAs had an open reading frame of 785 amino acids. The predicted human protein shows 60% identity to mouse γ-adaptin. A rare possibility that the human protein was a counterpart of mouse γ-adaptin is excluded by the fact that, from the same human cDNA library, we cloned a cDNA that encodes a protein with 99% identity to mouse γ-adaptin. Therefore, we hereafter refer to the novel γ-adaptin-like protein as γ2-adaptin and γ-adaptin as γ1-adaptin.

**Fig. 1.** Comparison of the sequence of γ2-adaptin with those of other adaptins. A, alignment of human γ1- and γ2-adaptin amino acid sequences deduced from the cloned cDNAs. Conserved residues are boxed, and gaps introduced into the alignment are indicated by hyphens. B, schematic representation of the structures of human γ2- (hγ2) and γ1- (hγ1) and mouse A- (mA) and C- (mC) adaptins. The percentage amino acid identity in each domain of each adaptin with γ2-adaptin is shown.

**Fig. 2.** Northern blot analysis of γ1- (A) and γ2- (B) adaptin mRNAs. Human multiple tissue RNA blot was analyzed with a γ2- or γ1-adaptin cDNA fragment as described under “Experimental Procedures.” The positions of the RNA size markers are indicated.

cDNAs had an open reading frame of 785 amino acids. The predicted human protein shows 60% identity to mouse γ-adaptin. A rare possibility that the human protein was a counterpart of mouse γ-adaptin is excluded by the fact that, from the same human cDNA library, we cloned a cDNA that encodes a protein with 99% identity to mouse γ-adaptin. Therefore, we hereafter refer to the novel γ-adaptin-like protein as γ2-adaptin and γ-adaptin as γ1-adaptin.

Fig. 1A shows the deduced amino acid sequences of human γ1- and γ2-adaptins. The overall amino acid identity between the two γ-adaptins was 60%. The primary structure of adaptins is generally dissected into three domains (7): the N-terminal head (core/trunk) domain, the C-terminal ear (appendage) domain, and the Pro-rich hinge region connecting the two domains (Fig. 1B). Between γ2- and γ1-adaptins, most conserved is the head domain with a 69% amino acid identity (Fig. 1B). In this domain, γ2-adaptin is also significantly similar to other adaptin family members (Fig. 1B). In the ear domain, γ2-adaptin is highly similar to γ1-adaptin (49% identity) but not significantly to other adaptins (<15% identity). In the primary structure and in length, the hinge region is not significantly conserved.

Northern Blot Analysis—Northern blot analysis was performed to examine whether γ2-adaptin shows tissue-specific expression because some adaptor components are expressed ubiquitously, whereas others are expressed in specific tissues. As shown in Fig. 2B, two mRNA species of 2.8 and 3.8 kilobases were detected for γ2-adaptin in all but one (skeletal muscle).
tissues examined. In the brain, another species of ~8.5 kilobases was also detected. When the same blot was reprobed for $\gamma_1$-adaptin, a 7.5-kilobase mRNA was detected in all tissues (Fig. 2A). The widespread expression of $\gamma_1$- and $\gamma_2$-adaptins suggests that both play a fundamental role in most tissues.

Western Blot Analysis—To characterize the $\gamma_2$-adaptin protein, an antiserum (no. 93) was raised in a rabbit against a synthetic peptide corresponding to the C-terminal 19 amino acids of $\gamma_2$-adaptin and affinity purified. Western blot analysis with the antibodies (Fig. 3A) revealed a protein of ~87 kDa, which is approximating the calculated molecular weight of $\gamma_2$-adaptin ($M_r = 87,116$), in a lysate of human embryonic kidney 293 cells (lane 1). In a lysate of 293 cells transfected with the expression vector for HA-tagged ($HA-\gamma_2$) or untagged ($\gamma_2$) $\gamma_2$-adaptin were subjected to Western blotting. The blot was probed with anti-$\gamma_2$-adaptin, no. 93 (lanes 1–3), anti-HA, 3F10 (lanes 4–6), or anti-$\gamma_1$-adaptin, no. 100.3 (lanes 7–9). B, postnuclear supernatant (PNS), cytosol (Cyt.), and membrane (Mem.) fractions (containing 30 μg of protein) from 293 cells analyzed for the presence of $\gamma_2$-adaptin with anti-$\gamma_2$-adaptin. The positions of the protein size markers are indicated.

To examine if $\gamma_2$-adaptin was present in the cytosol or associated with membranes, cytosolic and membrane fractions were prepared from 293 cells or HL-60 cells and subjected to Western blot analysis. As shown in Fig. 3B, a ~87-kDa protein was detected in both the fractions of 293 cells. Similar results were obtained with the HL-60 cell fractions (data not shown). The data suggest that, like other adaptor proteins, $\gamma_2$-adaptin might cycle between the cytosolic and membrane pools.

Indirect Immunofluorescence and Cell Fractionation Analyses—To examine the subcellular localization of $\gamma_2$-adaptin, we performed indirect immunofluorescence analysis. Because the anti-$\gamma_2$-adaptin antibodies we prepared were unable to detect the endogenous $\gamma_2$-adaptin protein by immunofluorescence (data not shown), cells were transfected with the expression vector for HA-tagged $\gamma_2$-adaptin tagged with (pcDNA3-HA-$\gamma_2$) or without (pcDNA3-$\gamma_2$) the HA epitope sequence and examined with anti-HA or anti-$\gamma_2$-adaptin antibodies, respectively (Fig. 4). In transfected rat Clone 9 hepatocytes, $\gamma_2$-adaptin, whether HA-tagged or not, was localized to paranuclear structures, which...
were often observed as large vesicles (panels A, D, and G). A similar staining pattern of γ2-adaptin was observed in transfected NRK and Vero cells (data not shown). Double labeling experiments revealed that γ2-adaptin was not colocalized with γ1-adaptin (panels A-C) or TGN38 (panels D-F), a TGN membrane protein. These observations indicate that γ2-adaptin, unlike γ1-adaptin, is not associated with TGN membranes. Furthermore, the γ2-adaptin staining was not superimposed on that of β-COP, a COP I component (data not shown), or α-mannosidase II, a Golgi marker protein (data not shown), indicating that the γ2-adaptin-containing structures differ from Golgi compartments. Because the large vesicular staining often observed for γ2-adaptin was reminiscent of the pattern for markers for the lysosomal/endosomal system, double labeling with antibodies against lamp-1, a marker for lysosomes and late endosomes, was also performed. However, the staining of γ2-adaptin differed from the vesicular staining of lamp-1 (panels G-I).

Although the immunofluorescence analysis using transfected cells indicates that γ2-adaptin is associated with subcellular structures distinct from those of γ1-adaptin, there remained a possibility that the overexpression of γ2-adaptin might cause aberrant aggregation of the protein. To circumvent this problem, we further fractionated the membrane fraction of HL-60 cells by Nycodenz gradient centrifugation and examined whether endogenous γ2- and γ1-adaptins were associated with distinct membrane compartments by Western blotting. As shown in Fig. 5, γ2-adaptin was present in lighter membrane fractions than γ1-adaptin. Taken together with the immunofluorescence observations, the data show that γ2- and γ1-adaptins are associated with distinct subcellular compartments.

We then examined effects of BFA on the localization of γ2-adaptin because this drug is known to cause dissociation of AP-1 and COP I from their target membranes through inhibiting membrane binding of a small GTP-binding protein, ADP-ribosylation factor (ARF), the membrane binding of which triggers recruitment of coat proteins onto membranes (for review, see Refs. 2, 30, 31). As shown in Fig. 6, treatment of γ2-adaptin-transfected Clone 9 cells with BFA resulted in dissociation of γ1-adaptin from TGN membranes (compare panels B and D). By contrast, the same treatment did not affect the staining pattern of γ2-adaptin (compare panels A and C). Thus, membrane association of γ2-adaptin appears to be regulated in a manner different from that of AP-1 and COP I.

To corroborate the above data, we utilized an in vitro system originally designed to study the recruitment of γ1-adaptin onto membranes (27). Permeabilized NRK cells were incubated with HL-60 cytosol under various conditions. Fig. 7 shows typical Western blots. Without addition of cytosol, a small amount of γ2-adaptin was detected in NRK cell membranes, whereas γ1-adaptin was not (lane 1) because the anti-γ2-adaptin antibodies cross-react with rat γ2-adaptin and the monoclonal anti-γ-adaptin antibody (100.3) cannot recognize rodent γ1-adaptin (27). When the cell membranes were incubated with the donor cytosol in the presence of GTP, both γ1- and γ2-adaptins were recruited onto the membranes (lane 2). When incubated in the presence of GTPγS, which activates GTP-binding proteins including ARF, an increase in the γ1-adaptin recruitment was observed (lane 3). Under the same incubation conditions, however, recruitment of γ2-adaptin was not enhanced. Pretreatment with BFA before addition of GTPγS largely inhibited the γ1-adaptin recruitment (lane 5), and pretreatment with GTPγS negated the effect of BFA (lane 4). By contrast, BFA did not inhibit the recruitment of γ2-adaptin (lane 5). Thus, the data on effects of BFA on membrane recruitment of γ1- and γ2-adaptins (Fig. 7) agree well with the immunofluorescence data (Fig. 6).

Identification of a Protein Interacting with γ2-Adaptin Using the Yeast Two-hybrid System—In an attempt to identify protein components that constitute a putative adaptor-related complex containing γ2-adaptin, we made use of the yeast two-hybrid system. Reporter yeast cells that were first transformed with a plasmid encoding a fusion between the GAL4 DNA-binding domain and γ2-adaptin were transformed with a mouse liver or brain cDNA library encoding proteins as C-terminal fusions with the GAL4 activation domain. Screening of \(-2 \times 10^6\) and \(-4 \times 10^6\) transformants from the liver and
brain libraries yielded 4 and 20 positive clones, respectively, showing a His$^1$ and β-galactosidase$^1$ phenotype (for example, see Fig. 8). Restriction endonuclease mapping and sequence analyses of rescued plasmids revealed that 17 plasmids contained cDNA inserts encoding the mouse σ1 chain (AP19, Ref. 32) and were derived from at least seven independent cDNA clones.

**Interactions with Adaptor Components**—We further examined whether γ2-adaptin was capable of interacting with small chains of other adaptor complexes using the two-hybrid system. As shown in Fig. 8, yeast cells cotransformed with γ2-adaptin and the σ1 chain grew efficiently on a plate lacking His and showed a high level of β-galactosidase activity, indicating a strong interaction between γ2-adaptin and the σ1 chain; for the reason described below, σ1 is hereafter called σ1A. By contrast, γ2-adaptin did not show an interaction with the σ2, σ3A, or σ3B chain. Similar results were obtained using γ1-adaptin as a bait (Fig. 8). We then examined if γ2-adaptin was able to interact with β1- and β2-adaptins because Page and Robinson have shown using the two-hybrid system that γ1-adaptin is able to interact with β1-adaptin, although with a relatively low affinity (33). As shown in Fig. 8, γ2-adaptin failed to show a significant interaction with β1- and β2-adaptins, whereas γ1-adaptin showed a significant interaction with β1-adaptin as described by Page and Robinson (31). We also examine whether γ2-adaptin was capable of interacting with medium chains (μ1, μ2, μ3A, and μ3B) but failed to show the interaction (not shown), as expected from the report of Page and Robinson (33) showing that neither γ1- or α1-adaptin can interact with medium chains.

**Identification of a σ1-like Protein**—When we performed the above experiments, we noticed by searching the EST database the presence of several human ESTs that were deduced to...
encode parts of a protein, the sequence of which was highly similar but not identical to the reported mouse σ1 (σ1A) sequence (32). We therefore cloned and sequenced a cDNA for the σ1-like protein. As shown in Fig. 9, the predicted σ1-like protein, named σ1B, consists of 157 amino acid residues and shows an 87% overall amino acid identity to mouse σ1A. The human σ1B is not a counterpart of mouse σ1A, since we also cloned and sequenced a human σ1A cDNA, the deduced amino acid sequence of which was identical to that of mouse σ1A. Northern blot analysis revealed that both σ1A and σ1B are expressed ubiquitously (Fig. 10). A two-hybrid analysis revealed that σ1B was capable of interacting with both γ1- and γ2-adaptins (Fig. 8).

**DISCUSSION**

In this study, we identified a ubiquitously expressed, novel human adapter large chain-like protein, designated as γ2-adaptin. This protein is most similar to γ-adaptin, which is called γ1-adaptin in this paper, not only in the primary sequence but also in the domain organization. The similarity suggests that γ2-adaptin is also a component of an adaptor or adaptor-related complex. By using the yeast two-hybrid system, we showed that γ2-adaptin as well as γ1-adaptin is capable of binding to the σ1 (σ1A) chain, which is the small chain of the AP-1 adaptor complex (Fig. 8). Furthermore, both γ1- and γ2-adaptins are able to interact with a novel small chain-like protein, named σ1B, which shows 87% amino acid identity to the σ1A chain. The interaction seems to be specific, because neither γ1- nor γ2-adaptin was able to interact with other examined small chains, and because Page and Robinson also showed by the two-hybrid analysis that γ1-adaptin is able to bind to σ1 but not to σ2, whereas the opposite is the case for α-adaptin (33). It is also interesting to note that γ2-adaptin, unlike γ1-adaptin, did not show a significant interaction with β1-adaptin in the two-hybrid analysis. Taken together, these data suggest that, although γ1- and γ2-adaptins share the same small chain, σ1A or σ1B, they use distinct β-chains and possibly distinct medium chains to form the adaptor complexes. However, we cannot exclude the possibility that the two-hybrid analysis could not reveal additional interactions because of conformational differences between native adaptor proteins and the fusion proteins or because of environmental differences between the cytoplasm of mammalian cells and the yeast nucleus. Although we failed to identify the β-like chain and the medium chain that form a complex with γ2-adaptin, there are several ESTs in the database that can code for parts of proteins homologous but not identical to the known β-chains and medium chains. Cloning of cDNAs for such candidates are currently underway in our laboratory.

Immunofluorescence analysis revealed that γ2-adaptin was not colocalized with γ1-adaptin; the former was localized to structures around the nucleus often observed as relatively large vesicles, whereas the latter was to typical Golgi-like paranuclear structures (Fig. 4). Furthermore, Nycodenz gradient centrifugation was able to separate a membrane compartment containing γ2-adaptin from that containing γ1-adaptin (Fig. 5). These observations suggest that the putative γ2-adaptin-containing complex is responsible for formation of coated vesicles different from the AP-1 clathrin-coated vesicles. In addition, the vesicular structures associated with γ2-adaptin appear to be different from lysosomal/endosomal compartments. Another observation that discriminates between γ1- and γ2-adaptins is that membrane recruitment of γ2-adaptin was not affected by BFA (Figs. 6 and 7). BFA inhibits guanine-nucleotide exchange on a small GTP-binding protein, ARF, and thereby inhibits its binding to Golgi membranes, which is a prerequisite process for membrane binding of the AP-1 and COP I complexes (2, 30, 31). Furthermore, recruitment to membranes of AP-3 has recently been shown to be sensitive to this drug (16). The data therefore indicate that recruitment from the cytosol to membranes of the putative γ2-adaptin-containing complex is regulated in a manner different from that of the AP-1 and AP-3 adaptors, possibly in an ARF-independent manner.

Delivery of proteins to the lysosomal/endosomal system and to the cell surface involves sorting into distinct pathways from the TGN. On the other hand, the endocytic pathway may involve many functionally distinct compartments, although it is difficult to determine a precise relationship between the compartments because of their dynamic nature. Furthermore, there may be interactions between the biosynthetic and endocytic pathways (for review, see Ref. 34). Taking into account the subcellular localization of γ2-adaptin, we speculate that the putative γ2-adaptin-containing complex may function at some trafficking step in the complex pathways between the TGN and the cell surface. It is, however, clear that further studies, for example using mutagenesis of the adaptor components in an attempt to affect the complex, will be required for uncovering the function of the complex.

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