Early Embryonic Death of Mice Deficient in γ-Adaptin

(Received for publication, September 14, 1998, and in revised form, November 16, 1998)

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Intracellular protein transport and sorting by vesicles in the secretory and endocytic pathways requires the formation of a protein coat on the membrane. The heterotetrameric adaptor protein complex 1 (AP-1) promotes the formation of clathrin-coated vesicles at the trans-Golgi network. AP-1 interacts with various sorting signals in the cytoplasmic tails of cargo molecules, thus indicating a function in protein sorting. We generated mutants of the γ-adaptin subunit of AP-1 in mice to investigate its role in post-Golgi vesicle transport and sorting processes. γ-Adaptin-deficient embryos develop until day 3.5 post coitus and die during the preconditioning period, revealing that AP-1 is essential for viability. Heterozygous mice the amount of AP-1 complexes is reduced to half of controls. Free β1- or μ1 chains were not detectable, indicating that they are unstable unless they are part of AP-1 complexes. Heterozygous mice weigh less than their wild-type littermates and show impaired T cell development.

At several sites along the secretory and the endocytic routes the transport of membrane proteins and luminal cargo depends on the formation of carrier vesicles. The formation of these transport vesicles is facilitated by coat proteins. Budding from the Golgi and endoplasmic reticulum membranes involves the heptameric COP-I and COP-II complexes. Budding from the trans-Golgi network (TGN),† the plasma membrane, and also probably from membranes of the endosomal/lysosomal system involves heterotetrameric adaptor protein (AP) complexes of which three types are known at present and which show homology to the COP-I subunits (1–3).

AP-1 and AP-2 have been implicated in the formation of clathrin-coated vesicles at the TGN and the plasma membrane, respectively, but may participate also in vesicle formation at other sites (3–5). AP-3-mediated vesicle formation may take place at the TGN and/or endosomes (6–8).

Although most of our knowledge on AP function is derived from studies on their subcellular localization and interaction with membranes or cytoplasmic domains of membrane proteins, little is based on functional studies or genetic approaches. Deletion of genes of AP subunits in yeast did not reveal the function of AP-1 and AP-2 but was informative in the case of AP-3. Mutations of the four AP-1 and AP-2 subunits did not result in a mutant phenotype and only the combination of AP-1 mutations with a temperature-sensitive clathrin heavy-chain mutation enhanced the reduced growth rate observed in the clathrin heavy-chain mutant (9–11).‡ No biochemical data for cargo-AP-1 interactions are available in the yeast system. The AP-3 is needed for an alternative pathway from the TGN to the vacuole, which is utilized by the vacuolar alkaline phosphatase and the vacuolar t-SNARE Vam3p and does not depend on clathrin (12). In Drosophila melanogaster AP-3 is involved in the formation of pigment granules, as indicated by garten, an eye color mutant caused by mutations in one of the AP-3 subunits (8, 13). Further support for a role of AP-3 in pigment granule formation comes from the naturally occurring mouse mutants mocha and pearl, which belong to a larger group of mouse mutants with abnormal pigmentation, prolonged bleeding time, and altered lysosomal sorting. Mocha as well as pearl mice carry mutations in AP-3 subunits (14, 15).

In an in vitro system, synaptic vesicle formation from endosomes of PC12 cells is dependent on the neuronal isoform of AP-3 (7).

The AP-1 has been found in clathrin-coated buds on the TGN. Binding of AP-1 to the TGN depends on the GTPase ARF1 (16). The AP-1 consists of two 100-kDa adaptins, γ and β1, a 47-kDa adaptin, μ1, and a 19-kDa adaptin, σ1. (2) of the four AP-1 subunits, γ-adaptin as well as β1-adaptin interact with clathrin (3). The C-terminal 511 of 822 amino acids of γ-adaptin and the β1-adaptin subunit are dispensable for TGN binding (17). AP-1 recruits clathrin on to the membranes by binding to the clathrin heavy chain and to the cytoplasmic tails of membrane (cargo) proteins. The μ1 adaptin interacts with tyrosine-based sorting sequences in the cytoplasmic tails of cargo proteins (18). Leucine-based sorting signals in the cytoplasmic tails bind to μ1 or β1 adaptin (19, 20). AP-1 binding cargo molecules are the mannose-6-phosphate receptors, MPR-46 and MPR300, the lysosomal membrane protein, LAMP 1, the invariant chain of the major histocompatibility complex class II receptor, the CD3γ subunit of the TCRβ-CD3 receptor complex and the varicella zoster virus glycoprotein I (21–27).

The significance of the AP-1-mediated packaging into clathrin-coated vesicles at the TGN for the trafficking of these membrane proteins and their biological function is not clear. Most of the cargo proteins are found at the plasma membrane as well as in endosomal/lysosomal membranes. Disruption of their interaction with the AP-1 must not necessarily prevent the targeting to their destination, as has been demonstrated for LAMP 1 (28). Replacing the glycine residue in the GYxxI sorting signal by alanine, interferes with binding to AP-1, but not to AP-2, and allows transport to endosomes/lysosomes via the cell surface, albeit at the expense of an increased steady-state concentration at the plasma membrane (29).

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† The abbreviations used are: TGN, trans-Golgi network; AP, adaptor protein; AP-1, AP complex 1; CD, cluster of determination; MPR, mannose-6-phosphate receptor; neo*, neomycin resistance; kb, kilobase(s); bp, base pair(s); PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; FITC, fluorescein isothiocyanate; pc, post coitus.

* This work was supported by Grant SFB 523/A6 (to P. S.) from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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To obtain insight into the in vivo role of AP-1, we rendered the γ-adaptin gene of AP-1 nonfunctional in mice. AP-1-deficient mouse embryos die before nidation, and even heterozygous animals display a reduced growth rate during nursing. Although the life cycle of yeast does not depend on AP-1 function, it is essential for development of a higher eukaryotic organism such as mice.

**EXPERIMENTAL PROCEDURES**

**Isolation of Chromosomal γ-Adaptin Sequences and Gene Mapping**—A 1.3-kb Pst I fragment of the mouse γ-adaptin cDNA corresponding to the N-terminal half of the protein was used to screen a mouse phage cDNA library in EMBL3 (50). Seven library clones were isolated that contained two different chromosomal DNA fragments.

Adapt Mspl polymorphisms were detected between *Mus musculus* and *Mus spretus* with a 1-kb Pst I cDNA probe encoding the C-terminal half of the protein and with a chromosomal DNA probe (see Fig. 1A, probe 2). Hybridization of a 5.8-kb Mspl fragment of *M. musculus* and a 6.7-kb Mspl fragment of *M. spretus* was seen with the cDNA as a probe. The chromosomal DNA probe hybridized with a 5.6-kb fragment from *M. musculus* and with a 5-kb fragment from *M. spretus*. DNA from 94 animals, produced by crossing *M. spretus* with *M. musculus*, was probed with *M. spretus* or animals having a *M. musculus*/*M. spretus* genetic background, were hybridized with both probes after Mspl incubation. The hybridization pattern was analyzed using a computer program (Jackson Laboratory, Maine).

**Mutation of Embryonic Stem Cells, Generation of Mutated Animals**—The 5.5-kb Pst I fragment that contained the 123-bp exon was subcloned into the cloning plasmid pBluescript SKI+ (Stratagene). A BglII-restriction endonuclease site was introduced into the exon by oligonucleotide-directed mutagenesis (5′-GCCAATGCGTAGATCTGTGAATT-3′). A 1.3-kb *Pst* I fragment of *M. spretus* was seen with the cDNA as a probe. The chromosomal DNA probe hybridized with a 5.6-kb fragment from *M. musculus* and with a 5-kb fragment from *M. spretus*. DNA from 94 animals, produced by crossing *M. spretus* with animals having a *M. musculus/*M. spretus* genetic background, were hybridized with both probes after Mspl incubation. The hybridization pattern was analyzed using a computer program (Jackson Laboratory, Maine).

**Immunolabeling and Flow Cytometry Analysis**—Cells from a litter were analyzed in parallel when they were 5–7 weeks old. They were kept in the same or neighboring cages in the central animal facility of the university. Thymus and spleen were isolated, washed in HEPES/Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and broken up using a Tenbroek homogenizer (Teconomara AG, Huttangen). Cells were counted and washed twice in PBS. 106 cells were transferred to fluorescence-activated cell sorter tubes and labeled with antibodies for 1 h on ice: anti-CD4-FITC, anti-CD8-PE, anti-CD3-APC, anti-CD80-PE, and anti-CD86-APC (Sigma). Cells were washed twice with ice-cold PBS and analyzed using a FACScan and Lysis II software (Becton Dickinson).

**RESULTS**

**Isolation of Chromosomal γ-Adaptin Sequences and Genomic Mapping**—A genomic mouse phage DNA library was screened with a mouse γ-adaptin cDNA fragment. Two different chromosomal DNA fragments, each about 16-kb in length, were identified in seven purified phage clones. On each fragment only a single exon was found using cDNA as a probe. One clone contained a 123-bp exon, encoding amino acids 68–108 of 822 (see Fig. 1A). The other clone contained an exon of 75 bp (not shown) encoding amino acids 68–108.

*γ-Adaptin* gene *Adtg* could be localized on mouse chromosome 8 at 52 cM between the markers D8Bug1112e and D8Bir26 using two different Adtg Mspl polymorphisms, distinguishing *M. musculus* and *M. spretus* (see Materials and Methods, data accessible at mouse genomic data base, Jackson Laboratory, Maine). Neither pseudogenes nor homologues of *Adtg* were found in the mouse genome.

**Preparation of Protein Extracts, Western-, and Northern Blot Analysis**—Organs were homogenized in 0.1 M MES buffer, pH 6.5, 1 mM EGTA, 0.5 mM MgCl2, 0.62% NaCN, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 5 mM iodoacetamide. Tissue fragments and unbroken cells were removed by centrifugation at 1000 × g for 10 min. The supernatant was separated into a membranous and cytosolic fraction by centrifugation at 100,000 × g for 45 min. Gel filtration was done on a Superdex-200 column (Smart-System, Amersham Pharmacia Biotech) at a flow rate of 40 μl/min and a sample volume of 50 μl with 5 μg/ml protein. Fraction volume was 30 μl. Column was calibrated with blue dextran, thryoglobin, ferritin, catalase, aldolase, bovine serum albumin, ovalbumin, and cytochrome C. Proteins (50 μg of crude cell extract and the entire gel filtration fractions) were resolved on a Laemmli gel and transferred onto nitrocellulose membranes (Sartorius). Adaptins were detected with anti-γ- and anti-β-adaptin mouse monoclonal antibodies (both 1:5,000 dilution) (Transduction Laboratories) and an anti-mouse μ1 polyclonal rabbit antisera (1:500 dilution) raised against amino acids 291–310. Anti-LAMP1 (1D1B) was a rat monoclonal antibody (1:1,000 dilution) (Developmental Studies Hybridoma Bank, University of Iowa). Protein-antibody complexes were made visible by chemiluminescence (SuperSignal®, Pierce) and x-ray film exposure. X-rays were scanned and quantified (Wincam software). Membranes were stripped by incubation in 0.2 M NaOH over 5 min and blocked again with 5% milk powder and 0.1% Tween 20 in PBS.

Total fibroblast RNA was isolated using a kit (Qiagen) according to the manufacturer’s protocol. 10 μg of RNA were separated on a 1% agarose gel, transferred onto a nylon membrane (Amersham), and UV cross-linked. CDNs were labeled with [α-32P]dCTP using a labeling kit (Amersham). Membranes were stripped by boiling in 0.1% SDS for rehybridization of the membrane. Signals were quantified by phosphorimage analyses (Fujitsu).

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**Preparation of γ-Adaptin Expression**—The exon encoding amino acids 68–108 was choosen to construct the targeting vector (see Fig. 1A). The exon 45 bp after the start of the exon corresponding to bp 245 of the cDNA by oligonucleotide-directed mutagenesis, and the neomycin-resistant (*neoR*) gene was cloned into the BglII site as a 1.2-kb BamHI fragment. To select against nonhomologous recombination events the thymidine kinase gene was in-

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3 Generous gift of A. Berna, Amsterdam.
The lower part shows the targeting construct with the inserted neo<sup>R</sup> gene and the BglII site used for neo<sup>R</sup> insertion. Arrows indicate orientation of the neo<sup>R</sup> gene open reading frame (top) and of the exon open reading frame (bottom). The position of the exon in the γ-adaptin cDNA is indicated by a black bar. B, Southern blots of HindIII- and EcoRV-digested chromosomal DNA hybridized with probe 2 are shown, recognizing the wild-type 6-kb EcoRV and above the mutated 7.2-kb HindIII fragment, and the mutated 3.6-kb fragment.

γ-Adaptin-deficient Embryos Die before Nidation—To determine the stage where embryonic development ceases, pregnancies were terminated starting at day 13.5 pc, the last day of organogenesis in mouse development, to 8.5 pc. None of 20 embryos was homozygous for the targeted Adtg allele (see Table I). Twelve of 58 blastocysts isolated at day 3.5 pc were homozygous and 31 were heterozygous for the targeted Adtg allele, revealing an almost mendelian distribution of the γ-adaptin mutation (see Table I and Fig. 2). The blastocysts displayed a normal morphology with a zona pellucida, trophoderm, inner cell mass, and a blastocoele. Shortly before nidation at day 4.5 pc to 5 pc, blastocysts hatch from their zona pellucida and are characterized by an elongated shape. Blastocysts with these characteristics were found also among the day 3.5 pc blastocysts because of the asynchrony of the early stages in development. None of the 29 embryos with this morphology turned out to be of the −/− genotype, suggesting that γ-adaptin deficiency is not compatible with development to day 4.5 pc.

To establish −/− cell lines from the inner cell mass, day 3.5 pc blastocysts were transferred onto feeder cell cultures and cultivated for 2 days in the presence of leukemia inhibitory factor, which stimulates growth and preserves the pluripotent state of cells of the inner cell mass. Sixteen of 23 blastocysts attached to the feeder cells and formed colonies. They were transferred into feeder cell-free plastic Petri dishes and allowed to attach and expand. Twelve of these could be successfully genotyped. The inner cell mass of wild-type and −/+ embryos developed on the feeder cells into colonies, attached to the plastic surface, and formed monolayers. Three of the blastocysts were γ-adaptin-deficient. The trophoblast cells and cells of the pluripotent inner cell mass enlarged significantly, indicating differentiation or cell death (not shown). The −/− cells failed to spread onto the plastic surface, divide, and establish colonies.

γ-Adaptin and AP-1 in γ-Adaptin −/+ Cells and Tissues—Analysis of expression levels of the γ-adaptin protein in cultured day 12.5 pc embryonic fibroblasts and in liver, brain, and thymus of adult animals by Western blotting revealed a decrease of 50% in −/+ cells compared with the +/+ cells (see Fig. 3). γ-Adaptin mRNA levels were reduced to 30% in cultured embryonic fibroblasts, liver, kidney, and brain. In all tissues, a 2.8-kb γ-adaptin transcript was seen. Fig. 3B shows mRNA
levels of adaptins in embryonic fibroblasts. \( \mu \)1 and \( \sigma \)1 adaptin mRNAs were not altered in fibroblasts.

Cytosolic proteins of cultured \( \gamma / \beta \)1/2 embryonic fibroblasts were separated by gel filtration to analyze whether the excess of \( \beta \)1, \( \mu \)1, and \( \sigma \)1 adaptins of the AP-1 complex form smaller or larger complexes or accumulate as free subunits. Column fractions were analyzed by Western blotting (see Fig. 4). The wild-type AP-1 complex was detected by anti-\( \gamma \) mouse monoclonal antibody, a mouse monoclonal antibody recognizing \( \beta \)1 and \( \beta \)2 or an antiserum specific for \( \mu \)1. The AP-1 complex was found in fractions 7–10 (\( M_r \) of 400,000). No adaptor complexes of a smaller or larger size or free \( \beta \) and \( \mu \)1 adaptins were found in the other fractions of the gradient. This suggests that the excess of \( \beta \)1 and \( \mu \)1 are not stable.

Pulse-chase experiments were performed with the fibroblasts to analyze the MPR-dependent sorting of cathepsin D to lysosomes. No differences were observed between the \( \gamma / \beta \)1/2 and \( \gamma / \beta \)2/2 cell lines (not shown).

Phenotype of Heterozygous Animals—We observed differences in size between 4-week-old \( +/+ \) and \( +/- \) littermates. A cohort of 9 \( +/+ \) male mice and 6 \( +/+ \) males of the outbred (C57/Bl6) colony were weighed from day 6.5 postnatal to 100 postnatal, and a second group of 27 \( +/- \) and 11 \( +/+ \) mice were weighed between 3 and 10 months of age. Data of the periods between day 6–21 and day 100–320 were analyzed by least square linear regression (Fig. 5A). The weight difference developed within the first 3 weeks after birth. Between day 6–21 postnatal the body weight of \( +/+ \) male mice increased by 0.23 g/d compared with 0.40 g/d in \( +/+ \) males. At day 21 postnatal, litters were separated from their mothers, and weaning caused a growth arrest for 2 to 4 days. Thereafter \( +/- \) mice grew as fast as \( +/+ \) mice, thus maintaining their 2.5 g lighter body weight for at least up to 10 months of age. This growth difference was independent of the genetic background, although animals of the inbred colony grew slower then outbred animals, and females grew slower then males. Histological analysis of liver, thymus, spleen, and kidney did not reveal morphological abnormalities in heterozygous animals.

Fig. 2. Genotyping of day 3.5 pc blastocysts. Chromosomal DNA was isolated and neo\(^{\text{R}}\) (top) and exon (bottom) sequences were amplified in two separate polymerase chain reactions. Shown is a representative experiment. Lane 1, size standard 1-kb DNA ladder; lane 2, no sample loaded; lanes 3–12, amplification products; lane 13, no sample loaded; lane 14, negative control (no chromosomal DNA); lane 15, positive control (DNA from a heterozygous animal). Resulting genotypes are given below each lane.

Fig. 3. A, Western blot analysis of \( \gamma \)-adaptin protein levels in crude cell extracts of liver and thymus prepared from 2 \( +/+ \) and 2 \( +/- \) adult mice. Numbers identify the individuals. LAMP 1 protein was used as internal control. B, Northern blot analysis of adaptin mRNA levels in embryonic fibroblasts. 10 \( \mu \)g of total RNA were loaded per lane. Numbers give the relative signal intensities between the cell lines in percent. Membranes were stripped for successive hybridization experiments with \( ^{32} \)P-labeled cDNAs. Glyceraldehyde-3-phosphate dehydrogenase (\( \text{GAPDH} \)) was used as internal control. Signals were visualized and quantified by Phospholmager analysis.

Fig. 4. AP-1 adaptins in cultured \( +/+ \) embryonic fibroblasts. Elution profile at 280 nm and molecular mass calibration for the gel filtration (see Experimental Procedures). Fractions containing adaptins are indicated by a bar. Western blot analysis of these fractions is shown. Others did not contain antigenic material. a, \( \gamma \)-adaptin; b, \( \beta \)-adaptin; c, \( \mu \)1-adaptin. Membranes were stripped and reprobed with the respective anti-adaptin antibody.

Fig. 5. Lethality of \( \gamma \)-Adaptin Deficiency in Mice
transport and its biological significance are poorly understood (3). We rendered the γ-adaptin gene of AP-1 nonfunctional in mice. γ-Adaptin −/− embryos were found at day 3.5 pc, but they died within the following 24 h before nidation. At day 2.5 pc of mouse development, translation of maternal mRNA stops and zygotic mRNA appears (31). Thus protein levels of γ-adaptin will begin to decline in −/− blastocysts at day 2.5 pc. Apparently development of −/− blastocysts ceases, when γ-adaptin declines below a threshold because of the lack of embryonically encoded γ-adaptin.

This phenotype is probably not caused by absence of just the γ-adaptin subunit, but by the absence of the entire AP-1 complex. In +/− cells the amount of γ-adaptin is reduced to 50%. We tested whether β1, µ1, and σ1 are in excess in these cells. mRNA levels of these subunits are not altered, but we failed to detect β1 or µ1 as free subunits or as partial complexes missing the γ-adaptin. We conclude from this that γ-adaptin governs AP-1 complex formation, probably by acting as a scaffold, and that other adaptins are unstable in the absence of γ.

We tried to establish −/− cell lines by cultivating cells of the inner cell mass from day 3.5 pc blastocysts. The inner cell mass of +/+ and +/− blastocysts grew in vitro, but cells from −/− blastocysts did not proliferate. The severity of the γ-adaptin −/− phenotype in mice is surprising given the absence of any phenotype of the yeast AP-1 mutants. It is suggestive to ascribe the lethality to the missorting of some functionally important proteins caused by the absence of AP-1. Receptors that normally interact with AP-1 are expected to become transferred in the absence of AP-1 to the plasma membrane rather than to endosomes. This occurs for example when, in these transmembrane proteins, domains interacting with AP-1 are deleted (33, 34). Lethality could thus be due to the abnormal presence of these transmembrane proteins at the cell surface and/or from their deficiency at the normal site of their action. The MPRs are responsible for the recruitment of the majority of AP-1 complexes to the TGN (35), and they serve as receptors for soluble lysosomal enzymes and insulin-like growth factor II. Mice lacking both MPRs are viable albeit at a reduced rate (36, 37), and embryonic development is normal in I-cell disease (38), in which M6P-containing ligands are missorted because of the absence of the M6P recognition marker. AP-1 could be important for prohormone processing and secretion of proteins critical for embryonic development. AP-1 is found on maturing secretory granula where it binds to the prohormone-processing protease furin (5). The presently known growth factors controlling embryonic development during the pre nidation period are provided to the embryo by the mother. The failure of proper pre implantation development of −/− embryos is therefore unlikely to result from defects in growth factor processing and secretion (39, 40). It could, however, also result from a failure of growth factor trafficking. Blastocyst development depends on maternal growth factors, which stimulate blastocyst growth and maintain the pluripotent state of the inner cell mass. These factors are transcytosed through the trophctoderm (41). At the day 3.5 to 4.5 of mouse development, blastocysts hatch out of the zona pellucida. Cells of the mural trophectoderm produce a trypsin-like protease, which generates an opening in the zona pellucida through which the blastocyst hatches, leaving an empty zona pellucida behind (42). γ-Adaptin-deficient blastocysts are not able to proceed through these developmental steps. That AP-1 is dispensable for the unicellular yeast in fact argues for an essential role of AP-1 during the development of a multicellular organism.

It was surprising to see a phenotype in γ-adaptin +/− heterozygotes, in which AP-1 is reduced to half the normal level. The growth deficiency in heterozygotes as well as the decrease in the number of CD4+ single-positive T cells was reduced by 20%, whereas the number of CD8+ single-positive T cells was hardly affected (see Fig. 5B). Cell surface expression of CD4 and CD8 on CD3+ cells was not altered. No abnormalities were found for splenocytes or the tissue architecture of thymus and spleen.

**DISCUSSION**

The AP-1 complex of clathrin-coated vesicles at the TGN has a function in protein sorting, but its role in post-Golgi vesicular
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ef CD4+/CD8− T-cells in the thymus points to a rate-limiting function of AP-1 in some tissues, which cannot be explained by the known AP-1 transport functions. However, the transport of lysosomal enzymes in γ-adaptin +/− embryonic fibroblasts was normal. This indicates that AP-1 is not rate limiting for trafficking of MRPs, despite the fact that MRPs constitute the major cargo proteins recruiting AP-1 to the TGN (35). Related adapter complexes exist, which may compensate the loss of AP-1. The sequences of a human and mouse γ-adaptin homologue, named γ2-adaptin, have recently been published,4 which are 60% homologous to γ-adaptin. Obviously this protein cannot compensate for the loss of γ-adaptin and must therefore fulfill a different function.

This study has shown that the AP-1 complex has a unique and essential role in mice at the TGN or on endosomes, which cannot be fulfilled by known or unknown adaptor complexes. That γ-adaptin has a particular role is demonstrated by a mouse knock-out of the μ1-adaptin subunit. These mice develop until midorganogenesis. In cell lines established from the embryos, a trimeric Δμ1AP-1 complex is formed, indicating that this complex is able to fulfill some AP-1 function in Δμ1 cells.5

Application of antisense technology to cell lines from γ-adaptin +/− heterozygotes may be helpful to achieve a graded reduction in the γ-adaptin levels so that the critical threshold of AP-1 for viability could be determined, and the consequences of AP-1 deficiency could be analyzed.

Acknowledgments—We thank A. Berns (Amsterdam) for the generous gift of a DNA library, M. Robinson (Cambridge) for the γ-adaptin cDNA, A. Wobus (Gatersleben) for technical advice for in vitro blastocyst cultivation, P. L. Chiodera (Brescia) for histological analyses, and R. Dressel (Göttingen) for technical assistance. Special thanks go to M. Pauly-Evers for the introduction into handling the blastocysts and M. Horst for critical reading of the manuscript. Image processing was done using Adobe Photoshop 3.0 and Deneba Canvas 5.0.

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