A Single Point Mutation in \(\varepsilon\)-COP Results in Temperature-sensitive, Lethal Defects in Membrane Transport in a Chinese Hamster Ovary Cell Mutant*

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At the nonpermissive temperature of 39.5 °C, the Chinese hamster ovary cell conditionally lethal, temperature-sensitive (ts) mutant IdlF exhibits the following defects: rapid degradation of low density lipoprotein (LDL) receptors, disruption of ER-through-Golgi transport, and disintegration of the Golgi apparatus. All of these are corrected by transfection with an expression vector for wild-type \(\varepsilon\)-COP, a subunit of coatomers (Guo, Q., Vasile, E., and Krieger, M. (1994) J. Cell Biol. 125, 1213-1224). We now report the identification in IdlF cells of a point mutation in the \(\varepsilon\)-COP gene, Glu\(^{251}\) to Lys\(^{251}\), which prevents the corresponding cDNA from correcting the defects in transfected IdlF cells and the immunohistochemical analysis of the synthesis, structure, and stability of \(\varepsilon\)-COP. At the permissive temperature (34 °C), the steady state level of \(ts-\varepsilon\)-COP in IdlF cells was about half that of \(\varepsilon\)-COP in wild-type Chinese hamster ovary cells and the isoelectric point of \(ts-\varepsilon\)-COP was 0.14 pH units higher than that of the wild-type protein. The stability but not the biosynthesis of \(ts-\varepsilon\)-COP was temperature-sensitive (\(t_\text{50}>6\) h at 34 °C and \(1-2\) h at 39.5 °C), and this accounts for the virtual absence of detectable \(ts-\varepsilon\)-COP protein in IdlF cells after incubation at 39.5 °C for \(>6\) h. The steady state levels in IdlF cells of another coatomer subunit, \(\beta\)-COP, and the peripheral Golgi protein IdlCp were not temperature-sensitive. Thus, a mutation in \(\varepsilon\)-COP that causes instability at 39.5 °C is responsible for all of the temperature-sensitive defects in IdlF cells, and the stability of \(\beta\)-COP is not linked directly to that of \(\varepsilon\)-COP. IdlF cells should be useful for the future analysis of the structure and function of \(\varepsilon\)-COP, the assembly of COPs into coatomers, and the participation of coatomers in intracellular membrane transport.

The endocytic and secretory pathways of intracellular membrane traffic appear to depend on multisubunit protein complexes (e.g. coatomers, NSF/SNAPs/SNAREs) to catalyze and regulate the membrane fusions and fissions required for transport (Bennett and Scheller, 1993; Rothman, 1994; Pryer et al., 1992; Warren, 1993). At least some of the components of these complexes participate in reactions used throughout the secretory and endocytic pathways.

In the course of mammalian somatic cell genetic analysis of membrane traffic, we isolated and characterized a conditionally lethal, ts Chinese hamster ovary (CHO) cell mutant, called IdlF, which behaves normally at the permissive temperature (34 °C) but exhibits pleiotropic defects at the nonpermissive temperature (39.5 °C) (Hobbe et al., 1994; Guo et al., 1994). These include rapid degradation of LDL receptors, presumably due to misorting, disruption of ER-through-Golgi transport of integral membrane and secreted proteins, and disintegration of the Golgi apparatus in a manner that resembles the effects of the drug brefeldin A on wild-type CHO cells (Takeatsu and Tamura, 1985; Fujiiwara et al., 1988; Lippincott-Schwartz et al., 1989, 1990; Shiue et al., 1990; Orci et al., 1991). We cloned from a CHO cell cDNA expression library a cDNA that, when transfected into IdlF cells, corrected all of their ts pleiotropic defects in membrane transport (Guo et al., 1994). The predicted sequence of the protein encoded by this cDNA is virtually identical to that of bovine \(\varepsilon\)-COP, which was cloned contemporaneously (Hara-Kuge et al., 1994). \(\varepsilon\)-COP is one of seven coat proteins (\(\alpha, \beta, \beta', \gamma, \epsilon, \delta, \) and \(\gamma\)) that form a stable complex called the coatomer COP1. COP1 is distinct from a second coat complex, called COPII, which has also been implicated in membrane traffic (Barlowe et al., 1994; Bednarek et al., 1995). COP1 coatomers can be found in the cytoplasm and associated with the membranes of the Golgi apparatus, the ER or nonclathrin-coated (COP-coated) vesicles (Dudenh et al., 1991a; Waters et al., 1993; Serafini et al., 1991; Stanbeck et al., 1992, 1993; Pepperkok et al., 1993; Ostermann et al., 1993; Orci et al., 1994). They have been shown to be required for the formation of functional Golgi transport vesicles in vitro (Ostermann et al., 1993), and they may be involved in anterograde (ER to trans Golgi) (Bednarek et al., 1995) and retrograde (Golgi to ER) vesicular transport (Letourneur et al., 1994) and possibly in endosome function (Whitney et al., 1995).

The correction of the ts defects of IdlF cells by transfection with an \(\varepsilon\)-COP cDNA suggested that these cells might carry a mutation in the \(\varepsilon\)-COP gene itself. However, the data did not rule out the possibility that \(\varepsilon\)-COP was functioning as an extragenic suppressor and that a mutation in some other gene in IdlF cells was responsible for their ts phenotypes. Here we show that there is a point mutation in the \(\varepsilon\)-COP gene in IdlF cells (ts \(\varepsilon\)-COP). The mutation converts Glu\(^{251}\) to Lys\(^{251}\), prevents the corresponding cDNA from correcting the defects in transfected IdlF cells, and results in the destabilization of the ts \(\varepsilon\)-COP protein at the nonpermissive temperature. Thus, IdlF cells provide direct genetic evidence that in animal cells \(\varepsilon\)-COP, and thus the COP1 coatomer complex (Kuge et al., 1993), is essential for establishing or maintaining Golgi structure and is

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1 The abbreviations used are: ts, temperature-sensitive; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; LDL, low density lipoprotein; kb, kilobase(s); PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
required either directly or indirectly for both ER-through-Golgi transport and normal endocytic recycling of LDL receptors.

**EXPERIMENTAL PROCEDURES**

**Materials—Reagents (and sources) were: Goat anti-rabbit IgG (Cappel/OrganonTeknika); Enhanced Chemiluminescence (ECL) detection kit (Amersham Corp.); methionine- and cysteine-free Ham's F-12 medium (Life Technologies, Inc.); [35S]methionine and [35S]-IAVPAT (DuPont NEN); cell culture media and supplements (Life Technologies, Inc. or JRH); DC Protein Assay Kit (Bio-Rad). Newborn calf lipoprotein-deficient serum (Gibco, Life Technologies, Inc.); Bacterial colonies from a cDNA library made from ldlF were screened (Libermann et al., 1987) from ts mutant IDIF cells was used to construct a size-selected, unidirectional cDNA expression library as described previously (Guo et al., 1994). Other reagents were obtained as described previously (Krieger et al., 1983) or were purchased from standard commercial suppliers.

**Cell Culture—** Wild-type CHO cells and the LDL receptor-defective and temperature-sensitive conditionally lethal mutant cells IDIF (clones 2; Guo et al., 1994)) were maintained in stock culture in medium A (Ham's F-12 containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) supplemented with 5% (v/v) fetal bovine serum (medium B) at 34°C in a 5% CO2/95% air humidified incubator.

**Cloning and Construction of the Mutant Expression Vectors pts-COP and pts-COPx—Poly(A) RNA prepared (Libermann et al., 1987) from ts mutant IDIF cells was used to construct a size-selected, unidirectional cDNA expression library as described previously (Guo et al., 1994). Other reagents were obtained as described previously (Krieger et al., 1983) or were purchased from standard commercial suppliers.

The resulting plasmids pts-COP and pts-COPx were purified using a QIAquick PCR purification kit from Qiagen and were ligated into the BamHI site of the expression vector pUC19 (Goff et al., 1984) and electroporated into Escherichia coli strain DM200 ( strain). The resulting plasmids were isolated after screening 53,640 colonies, and two colonies were picked containing cDNAs from the ldlF cells along with the plasmid (pLDL-1) and sequenced. The plasmids were electroporated into the E. coli strain DH10B and grown on Luria broth agar Petri dishes containing ampicillin (150 μg/ml) and tetracycline (8 μg/ml) (LB-amp/tet).

**Bacterial DNAs from a cDNA library made from IDIF were screened by hybridization according to standard techniques (Sambrook et al., 1989). A 0.95-kb fragment of -COP cDNA was used as a probe. The probe DNA was labeled with [α-32P]-dATP using the megaprime DNA labeling system (Amersham Corp.). Hybridization was carried out at high stringency. In brief, after hybridization overnight at 61°C with hybridization buffer (500 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 7% SDS, 1% bovine serum albumin, and 100 μg/ml salmon sperm DNA), the filters were washed three times at room temperature with 300 mM phosphate buffer, two times at 60°C with wash solution A (150 mM phosphate buffer, 5% SDS, 0.5% bovine serum albumin, 1 mM EDTA), and two times at 60°C with wash solution B (150 mM phosphate buffer, 1% SDS, 1 mM EDTA). One positive clone was sequenced using 53,640 colonies, and two clones were isolated after a second screening. The plasmids (designated pts-COPx) containing cDNAs from the IDIF cells along with the plasmid (pe-COP, formerly called pLDL-1 (Guo et al., 1994)) containing the previously isolated cDNA for wild-type CHO e-COP were subjected to side-by-side DNA sequence analysis. Sequences were determined at least once on both strands using the dideoxy chain termination method with Sequenase (U.S. Biotechnical Corp.). Two differences between pe-COP and pts-COPx were noted, a point mutation at base 751 within a 0.3-kb PstI-BstEII fragment in the coding sequence and pts-COPx contained an extra 11 base pairs before the poly(A) tail. The predicted isoelectric points of the e-COP and ts-COP proteins were calculated using the program ISEOLECTRIC (Genetics Computer Group Sequence Analysis Software Package, version 7.3, Madison, Devoreux et al., 1984)) with an additional amino-terminal Cys corresponding to the predicted e-COP (NH2-CKENDFDRLALQYAPSA-COOH) corresponds to the predicted 16 amino acids in the carboxyl terminus of e-COP (amino acid residues 293-308 (Guo et al., 1994)) with an additional amino-terminal Cys added to permit cross-linking to carrier proteins. The peptide (a gift from Susan Hantman, Millipore Corporation) was coupled to activated keyhole limpet hemocyanin (Sigma) with the cross-linker m-maleimidobenzoyl-N-hydroxysuccinimide ester (Sigma) (Harlow and Lane, 1988). The antigen was injected into a New Zealand white rabbit, and a booster injection was administered after 6 weeks. The serum was collected after the IgG activity was purified using protein A-Sepharose (Pharmacia Biotech Inc.) (Harlow and Lane, 1988) and designated anti-e-COP.

**Preparation of Whole Cell Extracts—** Cells grown to subconfluence in medium B in 100-mm dishes were washed with PBS, released by incubation at 34°C for 15 min in PBS without Ca2+ and Mg2+, and collected by centrifugation at 500 g for 5 min at 4°C. The cells were then washed in PBS, released by lysis in buffer (50 mM Tris-HCl, pH 7.3, 1 mM MgCl2, 5% Triton X-100, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml Pefabloc, 1 μg/ml pepstatin A) and incubated on ice for 10 min. The lysates were subjected to centrifugation at 12,850 × g for 15 min at 4°C, and the resulting supernatants were used as whole cell extracts.

**Cytosols were prepared essentially as described by Spiro et al. (1995).** Cells grown as described above were released from the culture dishes by mild trypsinization, collected by centrifugation (5.0 min at 250 × g at 4°C), and washed three times with ice-cold PBS. Pellets were suspended in ice-cold breaking buffer (20 mM Hepes, pH 7.4, containing 0.10 mM KCl, 85 mM sucrose, 20 mM EDTA) and were homogenized in a stainless steel ball homogenizer until 80–90% of the cells were disrupted. Post-nuclear supernatants were prepared by centrifugation at 8,500 g for 5 min at 4°C and were subjected to centrifugation at 380,000 × g for 15 min at 4°C to yield cytosols. The protein concentrations of the whole cell extracts and cytosols were determined using the DC Protein Assay kit from Bio-Rad Laboratories. The whole cell extracts and cytosols were stored at −80°C prior to use.

**Electrophoresis, Isoelectric Focusing, and Immunoblot Analysis—** Samples for SDS-polyacrylamide gel electrophoresis were boiled for 5 min in 62.5 mM Tris-HCl, pH 6.8, containing 2.0% (w/v) SDS, 5.0% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.05% (w/v) bromophenol blue and analyzed following the method of Laemmli (1970). Isoelectric focusing was performed as described by O'Farrell (1975) with some minor modifications. Samples were solubilized at 1.0 mg protein/ml in 0.1 M Tris, pH 7.3, containing 0.2% (w/v) CHAPS, 0.5% (w/v) carrier ampholytes, and 20 μl of a 1:1 mixture of Bicine 4–6 and 6–8 (Bio-Rad). Samples were loaded onto a 4.0% acrylamide gel containing 8.0 μl urea, 2.0% (w/v) CHAPS, and 2.0% (w/v) carrier ampholytes, overlaid with 25 μl of a solution of 1.0 μl urea and 1.0% (w/v) carrier ampholytes, and then focused for 10 min at 500 V and 6 h at 750 V using a Bio-Rad Multiphor apparatus with 200 ml of a solution of 10 mM H3PO4 and 10 mM HCl as the anode and cathode solutions, respectively. After focusing, a gel was sliced into ten portions (approximately 0.1 ml each) that were crushed and incubated in 0.5 ml of water for 30 min with mixing prior to measuring their pH levels with a standard pH electrode.

For detection of radioactively labeled proteins, gels were treated with Amplify fluorography reagent (Amersham Corp.), dried, and exposed to Kodak XAR-5 x-ray film at −80°C. For immunoblot analysis, proteins
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in the gels were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell; pore size, 0.22 μm) as described by Towbin et al. (1979). The membranes were blocked overnight at 4 °C with 5% blocking reagent from the ECL detection system (Amer sham Corp.) in PBS containing 0.1% Tween 20 (PBS-T). Blocked membranes were probed with primary antibody (e-COP: 1 μg/ml of anti-e-COP; IdlCp: 3 μg/ml of anti-Cope (Podols et al., 1994); β-COP: 1:100 dilution of T. Kreis’s monoclonal antibody M3AS, a gift from R. Klausner and J. Donaldson) in PBS-T for 1 h at room temperature, then incubated with horseradish peroxidase-conjugated anti-rabbit (for anti-e-COP and anti-Cop) or anti-mouse (for M3AS antibody) in PBS-T for 1 h at room temperature, and developed using the ECL detection system following the manufacturer’s instructions.

RESULTS

We have previously shown that transfection with e-COP cDNA corrects all of the temperature-sensitive defects in membrane trafficking in the CHO cell conditional lethal mutant IdlF (Guo et al., 1994). This result suggested that the primary defect in IdlF cells was a mutation in the e-COP gene. However, the data did not rule out the possibility that e-COP was functioning as an extragenic suppressor and that some other defective gene in IdlF cells was responsible for their ts phenotypes. We directly addressed this question by cloning the e-COP gene from IdlF cells (see “Experimental Procedures”). From a single colony giving a strong hybridization signal, we isolated two clones. Both strands of the entire cDNA inserts of these two clones were sequenced and compared with the sequence of the wild-type e-COP. Fig. 1A shows the only portion of the coding region in which the sequences of the wild-type (wt) and mutant (ts) cDNAs differed. A single point mutation in the mutant sequence (C-to-T in the antisense strand shown in Fig. 1A) was observed. This substitutes Lys251 in the mutant for Glu251 in the wild-type protein (see Fig. 1A) and changes its net charge by two at physiologic pH. This mutation in IdlF cells was further confirmed by direct sequencing of polymerase chain reaction products amplified from genomic DNAs and from two independent cDNA libraries (data not shown). Unexpectedly, this mutant sequence was the only sequence of e-COP present in either the genomic DNA or cDNAs, suggesting that IdlF cells contain only one mutant allele at the e-COP locus.

To confirm the presence of this mutation in the protein product of the mutant e-COP gene in IdlF cells, we prepared a rabbit polyclonal antipeptide antibody (designated anti-e-COP) directed against the carboxyl terminus of hamster e-COP (see “Experimental Procedures”) and used immunoblotting of isoelectrically focused samples to compare the e-COP proteins in CHO and IdlF cells grown at the permissive temperature (Fig. 1B). The cells each expressed only one form of the protein: e-COP in CHO cells (apparent pl of 5.30 in this system); ts-e-COP in IdlF cells (apparent pl of 5.44). The observed difference in the apparent isoelectric points, 0.14 pH units, was virtually identical to that predicted from the protein sequences (0.15 units). The absolute pl values were somewhat higher than predicted (−0.45 units); this was presumably due to the nature of the isoelectric focusing system used. The presence of a single, abnormally basic form of e-COP in IdlF cells (ts-e-COP) is consistent with the observation of a single mutant allele of the e-COP gene determined by cDNA and genomic sequencing.

To determine if the substitution of Lys for Glu251 interfered with e-COP function, we constructed an expression vector, pts-e-COP, which contains this single point mutation, and examined its ability to correct the temperature-sensitive lethality of IdlF cells. Wild-type (pc-e-COP) or mutant (pts-e-COP) expression vectors were cotransfected with pSV2neo into IdlF cells. The survival of G418-resistant colonies after 14 days of incubation at either the permissive temperature (34 °C, a measure of transfection efficiency) or the nonpermissive temperature (39.5 °C, a measure of e-COP function) was assessed by staining fixed cells with crystal violet and visual inspection. Fig. 2 shows that although the transfection frequencies were similar for the two plasmids (34 °C, left panels), the mutant was far less efficient in correcting the temperature-sensitive lethality of IdlF cells than was the wild-type (39.5 °C, right panels). Whereas many wild-type cDNA transfected colonies survived at 39.5 °C (Fig. 2, upper right), only a few mutant-transfected colonies survived the initial selection at 39.5 °C (lower right), and only three out of eight that were tested could survive passage to mass culture (see below for further discussion of these unusual colonies). No colonies survived at 39.5 °C when pc-COP was substituted by the corresponding vector control containing no cDNA insert (data not shown). We conclude that the Glu251 to Lys251 mutation disrupts e-COP function at the nonpermissive temperature and that this is the mutation responsible for all of the temperature-sensitive defects in membrane traffic in IdlF cells.

The biochemical consequences of the Glu251 to Lys251 mutation in e-COP were characterized using the anti-e-COP antibody. In Fig. 3, CHO and IdlF cells were preincubated at the indicated temperatures for 12 h, then pulse-labeled at the same temperatures for 30 min with [35S]methionine, and lysed, and the lysates were subjected to immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography as described under “Experimental Procedures.” Although three major bands were observed in the immunoprecipitate from the CHO cells at 34 °C (lane 1), the immunoprecipitation of only one (−36 kDa, e-COP) was blocked by a large excess of the synthetic peptide against which anti-e-COP was raised (lane 2). In immunoblot analysis of proteins, only a single band of −36 kDa was recognized by anti-e-COP (data not shown). Because the mass of this protein is similar to that predicted from the primary sequence of e-COP, 34.5 kDa (Guo et al., 1994), and because its immunoprecipitation by anti-e-COP could be
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FIG. 2. Temperature-sensitive growth of ldF cells transfected with wild-type and mutant ε-COP cDNAs. On day 0, the ldF cells were plated at 34 ºC. On day 2, the cells were cotransfected with a mixture of either pε-COP or pts-ε-COP with pSV2neo (see “Experimental Procedures”). The cells were subsequently incubated in medium E containing 175 µg/ml G418 either at 34 ºC for 14 days to monitor transfection efficiency (left panels) or at 39.5 ºC for 14 days to test for reversion of their ts lethal phenotypes (right panels). Cells incubated at 39.5 ºC were plated at a density double that of those incubated at 34 ºC (see “Experimental Procedures”). Surviving colonies were visualized by staining with crystal violet.

FIG. 3. Immunoprecipitation of wild-type and ts-mutant ε-COPs from metabolically labeled wild-type CHO and ldF cells. On day 0, cells were plated in 6-well dishes at 150,000 cells/well in medium D at 34 ºC. On day 1, cells were either shifted to 39.5 ºC or were maintained at 34 ºC for an additional 12 h as indicated. On day 2, the cells were pulse labeled with [35S]methionine (400 µC/ml) in methionine-free medium F for 30 min, washed once with Ham’s F-12 medium, and lysed, and the lysates were immunoprecipitated with anti-ε-COP, and the precipitates were reduced and analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography. The band labeled x represents a protein of unknown identity. A, immunoprecipitation analysis. On day 0, CHO and ldF cells were plated in 3 ml of medium D in 6-well dishes (150,000 cells/well) at 34 ºC. On day 2, the cells were pulse labeled with [35S]methionine (350 µC/ml) for 30 min at 34 ºC and washed once with Ham’s F-12 medium. The cells were then lysed (time 0) or refed with medium D containing 1 mM unlabeled methionine prewarmed to 39.5 or 34 ºC and chased at 39.5 or 34 ºC for the indicated times. The cells were then lysed, the lysates were subjected to immunoprecipitation with anti-ε-COP, and the precipitates were reduced and analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography. The band labeled x represents a protein of unknown identity. B, immunoblot analysis. On day 0, ldF cells were plated in 5 ml of medium B in 60-mm dishes (570,000 cells/dish) at 34 ºC. On day 2, the cells were shifted to 39.5 ºC for the indicated times, then harvested, and lysed. The lysates were reduced and subjected to 8% SDS-polyacrylamide gel electrophoresis. Immunoblot analysis of a single filter divided to permit separate analysis of high (>43 kDa) and low (<43 kDa) molecular weight proteins was performed using either a mixture of antibodies to β-COP and ldF Cp (upper panel) or ε-COP (lower panel) and the ECL detection system as described under “Experimental Procedures.”

Fig. 3 shows that the electrophoretic mobility in SDS-polyacrylamide gels of the mutant form of ε-COP in ldF cells, ts-ε-COP (lanes 5 and 6), is indistinguishable from that of wild-type ε-COP (lanes 1, 3, and 4). Strikingly, the rate of synthesis of either ε-COP in CHO cells or ts-ε-COP in ldF cells was not temperature-sensitive (compare intensities of the bands in lanes 3 and 4 with those in lanes 5 and 6). Thus, substantially reduced synthesis of ε-COP at the nonpermissive temperature cannot account for the defects in ldF cells. Interestingly, at both 34 and 39.5 ºC, ts-ε-COP in ldF cells appeared to be synthesized at approximately one-half the rate of ε-COP in wild-type CHO cells. In addition, quantitative immunoblot analysis (data not shown) was used to show that for cells grown at 34 ºC, the ratio of the steady state level of ε-COP in CHO cells to that of ts-ε-COP in ldF cells was 2:1. These results are consistent with the possibility that wild-type CHO cells express ε-COP from two loci, whereas in ldF cells there is only a single, mutant locus for ts-ε-COP.

Because ts-ε-COP was synthesized normally in ldF cells, we examined the possibility that the defects in ldF cells were due to instability of ts-ε-COP at the nonpermissive temperature. We compared the rates of degradation of newly synthesized ε-COP in CHO cells at 34 and 39.5 ºC with those of newly synthesized ts-ε-COP in ldF cells. Fig. 4A shows the results of an experiment in which cells were pulse-labeled for 30 min...
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with [35S]methionine at 34 °C and then chased in medium containing unlabeled methionine for the indicated times at either 34 or 39.5 °C, prior to immunoprecipitation with anti-e-COP, electrophoresis, and autoradiography. In wild-type CHO cells at 34 °C (Fig. 4A, top panel, left), e-COP was relatively stable and readily detected throughout the 6-h chase at comparable levels at both 34 and 39.5 °C. Similar results were observed for e-COP at 39.5 °C (top panel, right) and for the background band x at both 34 and 39.5 °C. In IdlF cells at 34 °C (Fig. 4A, bottom panel, left), ts-e-COP was almost as stable as e-COP in CHO cells. However, in IdlF cells at 39.5 °C (bottom panel, right), ts-e-COP was much less stable than e-COP in CHO cells. There was a significant decrease in the intensity of the band after 1 h of chase at 39.5 °C and very little signal after 3 h of chase. In contrast, there was no significant temperature dependence of the stability of background band x.

The results of the immunoprecipitation experiments were confirmed by analysis of the steady state levels of ts-e-COP using immunoblotting. Fig. 4B shows that after shifting IdlF cells to 39.5 °C, the cellular pool of ts-e-COP could be seen to drop after 1 h and was virtually completely depleted after 6 h (Fig. 4B, bottom panel). The rate of degradation of ts-e-COP determined using immunoblotting (Fig. 4B, bottom panel) was somewhat lower than that of newly synthesized ts-e-COP observed using immunoprecipitation (Fig. 4A, bottom panel, right). To determine if the temperature-dependent instability of ts-e-COP in IdlF cells was protein-specific, we examined the steady state levels both of another coatomer subunit, β-COP, and of an independent, brefeldin A-sensitive peripheral Golgi protein, IdlCp (Podos et al., 1994). Neither β-COP nor IdlCp exhibited significant temperature-dependent instability (Fig. 4B, top panel). Taken together, these results indicate that 1) ts-e-COP is abnormally unstable at the nonpermissive temperature in IdlF cells, 2) its temperature-dependent rapid degradation does not represent a general destabilization of Golgi-associated proteins, and 3) the instability of ts-e-COP at the nonpermissive temperature is apparently responsible for the pleiotropic ts defects in IdlF cells.

DISCUSSION

A single point mutation in the e-COP gene in IdlF cells results in the substitution of Lys251 in the mutant (ts-e-COP) for Glu251 in the wild-type protein. This mutation can account for the pleiotropic ts defects in IdlF cells, including defects in membrane trafficking (secretion and LDL receptor instability), disruption of the structure of the Golgi apparatus, and instability of the ts-e-COP protein itself. The mutation in ts-e-COP did not alter the stabilities of two other cytoplasmic, peripheral Golgi proteins, β-COP (Duden et al., 1991a, 1991b) and IdlCp (Podos et al., 1994). Thus, the current work supports the conclusion that IdlF cells provide the first genetic evidence that e-COP and thus COPI coatomers in animal cells are essential for establishing or maintaining Golgi structure and are required either directly or indirectly for both ER-through-Golgi transport and normal endocytic recycling of LDL receptors (Guo et al. 1994). Furthermore, these findings suggest that IdlF cells should be useful for studies of the relationship of e-COP’s structure to its function. Analysis of the effects of site-specific mutagenesis on the ability of e-COP to correct the multifaceted membrane transport defects in IdlF cells in vivo and in vitro should help define the molecular mechanisms underlying e-COP’s function.

In this regard, additional studies will be required to define precisely how the Lys251 to Glu251 mutation affects the function and stability of ts-e-COP. There was a very low but significant and reproducible level of survival at 39.5 °C of IdlF cells transfected with pts-e-COP. (Fig. 2, bottom right panel). Using immunoblot analysis, we found that two out of three of the surviving transfected colonies examined overexpressed ts-e-COP at 39.5 °C when compared with untransfected controls (data not shown). The increased expression of ts-e-COP in these transfecants raises the possibility that the mutant protein may be at least partially functional and that overexpression provides a sufficient steady state level of e-COP activity at 39.5 °C for the membrane traffic required for survival and growth. We have previously observed in a different mutant CHO cell line (IdlC) the suppression of a mutant phenotype (LDL receptor deficiency) due to overexpression of an unstable protein (abnormally glycosylated LDL receptor) (Reddy and Krieger, 1989).

The molecular mechanism responsible for the thermal instability of ts-e-COP is unknown. The mutation might directly result in intrinsic temperature instability of the ts-e-COP protein, regardless of its interactions with other cellular components. Alternatively, the mutation might interfere with the incorporation of ts-e-COP into coatomers (or some other complex) at the nonpermissive temperature. The uncomplexed but perhaps otherwise normal protein might then be subject to abnormally rapid degradation. To address this issue, we attempted an immunological analysis of cells that were expected to simultaneously express e-COP and ts-e-COP at the permissive temperature by examining IdlF cells transfected with the wild-type pβ-COP cDNA (IdlC[DLDL] cells; Guo et al., 1994). The normal phenotypes of IdlF[DLDL] cells at the nonpermissive temperature are due to the expression of wild-type e-COP (Guo et al., 1994). Using isoelectric focusing and either immunoprecipitation or immunoblotting, we could readily detect e-COP but were unable to detect significant levels of ts-e-COP in the cells grown at the permissive temperature (data not shown). The mechanism (transcriptional, translational, or post-translational) for the suppression of ts-e-COP expression in the presence of e-COP expression has not yet been determined. It is possible that the incorporation of e-COP protein into coatomers was strongly favored over that of ts-e-COP and that the unincorporated ts-e-COP was unstable. Additional experiments will be required to resolve this issue.

Because e-COP is an integral component of COPI coatomers (Hara-Kuge et al., 1994), IdlF cells may be helpful in studying the assembly and functions of coatomers. Here we found that the stability of at least one coatomer component, β-COP, was not significantly altered in the absence of normal levels of e-COP. This finding is similar to the observation by Duden et al. (1994) that in Saccharomyces cerevisiae deletion of β-COP (Sec26p) does “not lead to the loss of other coatomer subunits.” Additional studies will be required to determine 1) if the stabilities of other COPs are affected at the nonpermissive temperature in IdlF cells, 2) if the intracellular distribution of the other coatomer subunits is altered in the absence of e-COP, 3) if stable coatomer-like complexes can assemble in the absence of e-COP, and 4) if such e-COP-deficient complexes can exhibit any coatomer activity (e.g. ARF-dependent membrane attachment).

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