Traffic-independent function of the Sar1p/COPII machinery in proteasomal sorting of the cystic fibrosis transmembrane conductance regulator

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Newly synthesized proteins that do not fold correctly in the ER are targeted for ER-associated protein degradation (ERAD) through distinct sorting mechanisms; soluble ERAD substrates require ER-Golgi transport and retrieval for degradation, whereas transmembrane ERAD substrates are retained in the ER. Retained transmembrane proteins are often sequestered into specialized ER subdomains, but the relevance of such sequestration to proteasomal degradation has not been explored. We used the yeast Saccharomyces cerevisiae and a model ERAD substrate, the cystic fibrosis transmembrane conductance regulator (CFTR), to explore whether CFTR is sequestered before degradation, to identify the molecular machinery regulating sequestration, and to analyze the relationship between sequestration and degradation. We report that CFTR is sequestered into ER subdomains containing the chaperone Kar2p, and that sequestration and CFTR degradation are disrupted in sec12ts strain (mutant in guanine-nucleotide exchange factor for Sar1p), sec13ts strain (mutant in the Sec13p component of COPII), and sec23ts strain (mutant in the Sec23p component of COPII) grown at restrictive temperature. The function of the Sar1p/COPII machinery in CFTR sequestration and degradation is independent of its role in ER-Golgi traffic. We propose that Sar1p/COPII-mediated sorting of CFTR into ER subdomains is essential for its entry into the proteasomal degradation pathway. These findings reveal a new aspect of the degradative mechanism, and suggest functional crosstalk between the secretory and the degradative pathways.

Introduction

Quality control of newly synthesized membrane proteins occurs in the ER (Ellgaard and Helenius, 2001). Incorrectly folded proteins are targeted for ER-associated protein degradation (ERAD)* and are eliminated by the ubiquitin-proteasome pathway (Brodsky and McCracken, 1999). Because proteasomal degradation occurs in the cytoplasm, ERAD substrates must be retrotranslocated from the ER to the cytoplasm before digestion. The cellular machinery involved in the sorting and translocation of ERAD substrates is under active investigation. One of the key requirements is a fully competent early secretory pathway because mutations in proteins involved in ER-Golgi traffic cause disorganization of ER structure and inhibit ERAD (Taxis et al., 2002). The Sec61p/Sec63p translocon (Plemper et al., 1997) and auxiliary proteins Der1p (Knop et al., 1996), Der3p/Hrd1p (Bordallo and Wolf, 1999), and Hrd3 (Hampton et al., 1996) have been shown to be required for retrotranslocation of ERAD substrates. In addition, a subset of molecular chaperones such as calnexin (McCracken and Brodsky, 1996), BiP (Plemper et al., 1997; Nishikawa et al., 2001), Hsp70 (Zhang et al., 2001), and p97 (Rabinovich et al., 2002) participate in selecting ERAD substrates. Evidence indicates that two distinct mechanisms participate in directing different substrates for ERAD (Vashist et al., 2001). Soluble ERAD substrates such as carboxy peptidase Y (CPY), PrA, and KHN require functional transport between the ER and the Golgi (Caldwell et al., 2001; Vashist et al., 2001), and the degradation of these proteins is delayed when ER-Golgi traffic is inhibited in sec18–1 and sec12–4 strains. Additional components encoded by PER17 (Vashist et al., 2001), ERV14, and ERV29 (Caldwell et al., 2001) appear essential in degradation of soluble ERAD substrates, and appear to function by regulating ER-Golgi transport of specific soluble proteins. In contrast, the degradation of membrane ERAD substrates such as mutant forms of Ste6p, Yor1p, Sec61p, Vph1p, and cystic fibrosis transmembrane conductance regulator (CFTR) seem unaf-

*Abbreviations used in this paper: CFTR, cystic fibrosis transmembrane conductance regulator; CPY, carboxy peptidase Y; ERAD, ER-associated protein degradation.

Key words: ER sorting; proteasomal degradation; CFTR; ERAD; yeast

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fected by sec18–1, sec12–4, sec21–1, per17–1, erv14Δ, or erv29Δ mutations (Loayza et al., 1998; Katzmann et al., 1999; Caldwell et al., 2001; Kiser et al., 2001; Vashist et al., 2001). These findings suggest that transmembrane ERAD substrates do not enter post-ER compartments of the secretory pathway before degradation. In agreement, soluble ERAD substrates can be detected in post-ER transport vesicles, whereas transmembrane ERAD substrates are excluded (Vashist et al., 2001).

The exact pathway for degradation of transmembrane ERAD substrates from the ER is unclear, but there is evidence to suggest that at least some retained proteins are sorted into specialized ER subdomains before degradation (Kamhi-Nesher et al., 2001; Kiser et al., 2001; Zhang et al., 2001). It is unknown what molecular mechanisms regulate sequestration of ERAD substrates, and whether such sequestration is required for ER retrotranslocation and proteasomal degradation. To explore these questions, we used the yeast Saccharomyces cerevisiae and mutant strains defective in the Sar1p/COPII sorting machinery and a known ERAD substrate, CFTR, as our model system.

Sar1p/COPII-mediated selection of ER proteins for secretory traffic is one of the best understood mechanisms for selective recruitment of proteins in the ER (Antonny and Schekman, 2001). COPII sorting is initiated by Sar1p, a small GTPase (Veldhuisen et al., 1997) that is activated by Sec12p-mediated guanine-nucleotide exchange. Sar1p in its GTP-bound state associates with the ER membrane and recruits the Sec23p/Sec24p–COPII complex and subsequently the Sec13p/Sec31p–COPII complex to the membrane (for review see Barlowe, 2000). The assembly of the COPII coat on ER budding structures is coupled to the selection of transmembrane proteins into the nascent budding structures that will eventually bud from the ER as transport vesicles. COPII function has been examined to date exclusively in the context of secretory traffic.

CFTR is a chloride channel present on the apical surface of epithelial cells lining the respiratory, intestinal, and exocrine tissues (Kirk, 2000). In mammalian cells, only ~20% of newly synthesized wild-type CFTR folds correctly and is transported from the ER to the plasma membrane through the secretory pathway, whereas the remaining ~80% of CFTR is degraded from the ER through the ubiquitin-proteasome pathway (Jensen et al., 1995; Ward et al., 1995; Moyer et al., 1998; Riordan, 1999). In mammalian cells, CFTR is not sequestered into ER subdomains before degradation because inhibition of proteasomal activity by chemical inhibitors or overtaxing the proteolytic capacity by overexpressing CFTR leads to retrotranslocation of CFTR from the ER and its accumulation in cytosolic aggresomes (Johnston et al., 1998). In yeast, the majority (if not all) of newly synthesized CFTR is also degraded through the ubiquitin-proteasome pathway, but in contrast to mammalian cells, CFTR in yeast is not delivered to aggresomes, but appears sequestered in ER subdomains before degradation (Kiser et al., 2001; Zhang et al., 2001).

Here, we report that the Sar1p/COPII machinery functions in sorting CFTR into ER subdomains before proteasomal degradation, and that such sorting is required for CFTR entry into the degradative pathway. This function of Sar1p/COPII does not involve ER-Golgi traffic. Our results support a model in which the Sar1p/COPII machinery participates in sorting proteins to both the anterograde secretory pathway and the degradative pathway. These findings raise the possibility that all newly synthesized proteins are subject to a Sar1p/COPII sorting mechanism, irrespective of their ultimate secretory or degradative fate.

Results and discussion

GFP-tagged CFTR is a bona-fide ERAD substrate in yeast

To provide a substrate for our studies, we generated a construct containing EGFP fused in frame to the NH2-terminus of human CFTR in a yeast expression vector under a copper-inducible promoter. As shown in Fig. 1 A, EGFP-CFTR  

![Figure 1](Image)  

Figure 1. EGFP-CFTR represents yeast ERAD substrate. (A) Yeast transformed with pCU426CUP1 (EGFP-CFTR, – lane) or pCU426CUP1/EGFP-CFTR (EGFP-CFTR, + lanes) were induced, labeled with [35S]methionine for 20 min, and lysed. Lysates were immunoprecipitated with anti-CFTR, anti-F1β, or anti-GFP pAbs. A band of 175 kD, appropriate for a chimera of EGFP and CFTR, can be immunoprecipitated with anti-CFTR and anti-F1β antibodies from cells expressing EGFP-CFTR. (B) Wild-type, pre1–1, and ubc6Δ yeast were transformed with pCU426CUP1/EGFP-CFTR, induced, and pulse-labeled with [35S]methionine for 20 min. An equal amount of culture was taken at each chase time, lysed, and the lysates were immunoprecipitated with anti-CFTR antibody. Relative intensities of EGFP-CFTR bands were quantitated. EGFP-CFTR is stabilized in pre1–1 and ubc6Δ strains.
is detected after immunoprecipitation with anti-CFTR antibody and anti-GFP antibody after copper induction of wild-type yeast carrying the EGFP-CFTR plasmid (Fig. 1 A, lanes 1 and 4), but not from yeast carrying an empty vector (Fig. 1 A, lane 3). EGFP-CFTR is not seen in a sample precipitated with anti-F1β antibody. The mobility of EGFP-CFTR (~175 kD) is appropriate for a chimera of EGFP (~27 kD) and full-length human CFTR (~145 kD).

To ensure that EGFP-CFTR behaves as an ERAD substrate, we examined its degradation rate in mutant strains lacking either a functional proteasome subunit (pre1–1) or an ubiquitin-conjugation enzyme (ubc6Δ). Both proteins have been previously shown to be required for degradation of CFTR tagged at the COOH terminus with GFP (Kiser et al., 2001) or HA (Zhang et al., 2001) in yeast. As shown in Fig. 1 B, EGFP-CFTR in wild-type yeast is digested rapidly, with a half-life of ~10–15 min (Fig. 1 B), slightly more rapidly than previously published (~20 min, Kiser et al., 2001; ~30 min, Zhang et al., 2001). All CFTR appears to be degraded in yeast (Kiser et al., 2001; Zhang et al., 2001), as opposed to mammalian cells where ~20% is transported to the plasma membrane. It is possible that yeast does not have sufficient machinery to efficiently fold overexpressed CFTR, or that even correctly folded CFTR is "read" by yeast as defective. In contrast to rapid degradation in wild-type yeast, EGFP-CFTR remains stable in pre1–1 mutant yeast even after 90 min of chase, and the half-life of EGFP-CFTR in the ubc6Δ strain is extended to ~40 min. Because our EGFP-CFTR construct is degraded through the ubiquitin-proteasome pathway in yeast, we use it as an ERAD substrate to test Sar1p/COPII involvement in proteasomal targeting.

**EGFP-CFTR is sorted to subcompartments of ER before degradation**

Previous studies have shown that CFTR tagged at the COOH terminus with GFP (Kiser et al., 2001) or HA (Zhang et al., 2001) is degraded as an integral membrane protein and colocalizes with the ER chaperone Kar2p in punctate ER structures before degradation. To analyze our EGFP-CFTR construct, we first tested its association with membranes. As shown in Fig. 2 A, EGFP-CFTR is detected exclusively in the membrane pellet fraction after medium speed centrifugation, indicating that it is degraded as a membrane-associated form. We performed immunofluorescence with known organelar markers to localize EGFP-CFTR. As shown in Fig. 2 B, EGFP-CFTR is not detected in small punctate structures containing the late Golgi marker Kex2p (Preuss et al., 1992). This result agrees with findings that CFTR expressed in yeast undergoes ER-type N-glycosylation, but does not
EGFP-CFTR is not detected on the plasma membrane, and does not colocalize with the fluorescent dye FM 4–64 (Fig. 2 B) shown to be transported to endosomal (unpublished data) or vacuolar compartments of yeast as a function of incubation time and temperature (Vida and Emr, 1995; the minimal colocalization in the vacuole is due to bleed-through from the strong EGFP-CFTR signal and is not visible in cells without EGFP-CFTR). The lack of vacuolar localization is consistent with lack of degradation of CFTR via the Pep4p/Prb1p vacuolar proteases (Kiser et al., 2001; Gelman et al., 2002).

EGFP-CFTR is detected in relatively large punctate structures that contain Kar2p (Fig. 2 B). The punctate Kar2p distribution in EGFP-CFTR—expressing yeast is distinct from its normal cage-like perinuclear localization in control yeast (Paddon et al., 1996). The effects of EGFP-CFTR expression on intracellular morphology were analyzed by electron microscopy of yeast transformed with either empty or EGFP-CFTR–containing plasmids. As shown in Fig. 2 C, normal nuclear membrane and sub–plasma membrane ER structures are evident in the control cell (arrows), in agreement with published images (Zhang et al., 2001). In contrast, EGFP-CFTR cell shows clusters of membranous structures in proximity to the perinuclear ER (arrowheads). Higher magnification shows them to be accumulations of tubular and vesicular elements, morphologically analogous to ER extensions observed in yeast expressing CFTR (Zhang et al., 2001). The relative size of such clusters (average diameter ∼0.5–1 μm) corresponds to the approximate size of the fluorescent Kar2p/EGFP-CFTR puncta. Significantly, membrane amplification induced by CFTR (Zhang et al., 2001) or EGFP-CFTR (this work) is distinct from multi-layered ER “karmellae” induced by expression of HMG-CoA reductase (Koning et al., 1996). Together, the data suggest that EGFP-CFTR localizes to subdomains of the ER network that represent penultimate stations before degradation. The results also imply that sequestration into the ER domains is more efficient than subsequent removal by degradation.

**Functional Sar1p/COPII machinery is required for sorting EGFP-CFTR to ER subdomains**

To determine if the Sar1p/COPII machinery participates in sorting EGFP-CFTR, we examined its localization when COPII function is disrupted. First, we tested the localization of EGFP-CFTR in wild-type, ubc6Δ, and sec18–1 yeast strains when grown at 24°C (permissive temperature for strains defective in COPII components) and when grown at 39°C (restrictive temperature for strains defective in COPII components). As shown in Fig. 2 D, EGFP-CFTR in the wild-type strain remains as punctate spots at both temperatures. Similarly, the pattern of EGFP-CFTR in the ubc6Δ strain defective for proteasomal degradation is analogous to that in the wild-type strain. This strongly suggests that EGFP-CFTR is sorted into the punctate structure and maintained there before it is targeted for ubiquitination and degradation. Our data, together with the finding that CFTR remains sequestered in Kar2p-containing subdomains in yeast strain (pre1–1pre2–2) defective for proteasomal degradation (Zhang et al., 2001), suggest that the catalytic activity of ubiquitin-conjugating enzyme Ubc6p is not sufficient to remove EGFP-CFTR from ER subdomains, and that ubiquitination and proteasomal degradation are tightly coupled. The same punctate EGFP-CFTR pattern is observed in sec18–1 strain grown at either temperature, indicating that block in vesicular fusion during ER-Golgi protein transport does not significantly affect EGFP-CFTR sequestration.

Analysis of EGFP-CFTR localization in the sec23–1ts strain (Kuehn et al., 1998) showed significant temperature-dependent changes (Fig. 2 D). At the permissive temperature, EGFP-CFTR is sequestered into ER subdomains, but at the restrictive temperature, EGFP-CFTR is diffusely distributed throughout the ER in a pattern characteristic of ER proteins (compare enlargement with Kar2p staining in inset; Fig. 2 C). Similar results were obtained when yeast strains mutant in other components of the Sar1p/COPII machinery (described below) were analyzed (unpublished data).

**Functional Sar1p/COPII machinery is required for EGFP-CFTR degradation**

To uncover whether the Sar1p/COPII machinery is also involved in CFTR degradation, we compared the degradation rate of EGFP-CFTR at permissive (24°C) and restrictive (39°C) temperature in yeast mutant in Sar1p/COPII components. In wild-type strain, EGFP-CFTR is degraded rapidly at both temperatures, with a half-life of 10–15 min (Fig. 3 A). The temperature-sensitive strain, sec12–4ts, is defective in catalyzing GDP/GTP exchange on Sar1p at the restrictive temperature due to a P73L mutation (Barlowe and Schekman, 1993). EGFP-CFTR is degraded rapidly in the sec12–4ts strain at permissive temperature; with a half-life (∼10 min) analogous to that in wild-type strain (Fig. 3 B). In contrast, the degradation rate is delayed significantly when the yeast is shifted to the restrictive temperature, with the half-life extending to 40–45 min, a threefold increase compared with the permissive temperature. The temperature-sensitive strains sec23–1ts and sec13–1ts (Salama et al., 1997) are defective at the restrictive temperature due to an S382L mutation (Yoshihisa et al., 1993), and a mutation that has not yet been identified, respectively. In both cases, COPII function is compromised. EGFP-CFTR is degraded rapidly in both sec23–1ts and sec13–1ts strains at the permissive temperature, with half-lives of <15 min (Fig. 3 C and D). In contrast, the degradation rates are significantly delayed in both strains at the restrictive temperatures, with a half-life of ∼50 min. The significant inhibition of EGFP-CFTR degradation suggests that functional COPII machinery is required for proteasomal degradation of EGFP-CFTR.

It must be stressed that EGFP-CFTR, like other transmembrane ERAD substrates, does not enter the ER-Golgi recycling degradative pathway. Like the previously characterized CFTR (Kiser et al., 2001), EGFP-CFTR is degraded with normal kinetics at permissive and restrictive temperatures in a sec18–1 strain (Fig. 3 E). The strain is defective in Sec18p function, as shown by a block in maturation of the vacuolar CPY at the restrictive temperature (Fig. 3 E). Together, the results indicate that ER-Golgi transport and recycling are not required for EGFP-CFTR degradation. Our findings suggest a novel role for the Sar1p/COPII sorting machinery. In addition to selecting correctly folded sub-
strates into the forward secretory pathway (Schekman and Orci, 1996; Bannykh et al., 2000) and selecting misfolded soluble ERAD substrates for the ER-Golgi recycling degradative pathway (Vashist et al., 2001), Sar1p/COPII also sorts misfolded substrates into subdomains of the ER from which they are subsequently degraded. How does the same machinery sort proteins into three distinct pathways? A common sorting mechanism for both correctly folded and misfolded membrane proteins presents a spatial problem because these classes must occupy distinct regions of the ER. It is likely that additional targeting mechanisms coordinate with Sar1p/COPII to direct the proteins to ER exit or retention. It appears that a primary distinction is made at the level of packaging into COPII vesicles; although correctly folded soluble and transmembrane proteins for secretory traffic and misfolded soluble ERAD substrates for ER-Golgi recycling degradative traffic are sorted into COPII vesicles, misfolded transmembrane ERAD substrates are excluded (Vashist et al., 2001). There is evidence that transmembrane ERAD substrates are prevented from entry into COPII vesicles and are retained in the ER through the action of luminal ER proteins. For example, mutants of the transmembrane ATPase Pma1p appear to be retained and degraded in the ER through the action of a protein disulfide isomerase–related protein, Eps1p (Wang and Chang, 1999). Similarly, the retention of a transmembrane H2a precursor in the ER is determined by a pentapeptide sequence present on the luminal side of the ER (Shenkman et al., 1997). In addition, ERAD substrates can be retained by binding to calnexin. It has been shown that calnexin binds to wild-type and folding compromised ΔF508CFTR in the ER, but the association with ΔF508CFTR is prolonged and might facilitate ER retention (Pind et al., 1994). ERAD substrates can be also retained by association with the translocon (Loayza et al., 1998; Kamhi-Nesher et al., 2001). Cytoplasmic chaperones may also participate in ER retention (Brodsky et al., 1999). The finding that strains inactivated in the cytosolic Hsp70 Ssa1p do not sort CFTR into ER subdomains supports the view that ER sequestration requires the coordinate action of Sar1p/COPII and accessory factors.

That Sar1p/COPII is likely to participate in the secretory and the degradative pathways is supported by data obtained from whole genome microarray analysis where many genes involved in secretory traffic were found to be UPR targets (Travers et al., 2000). Initially, the up-regulation was viewed as means to increase traffic in the ER-Golgi recycling degradative pathway for soluble ERAD substrates (Caldwell et al., 2001). However, our work suggests that the up-regulation is also a means to increase components required for ER sequestration of transmembrane ERAD substrates. Specifically relevant to our analysis is the up-regulation of SEC12, SEC13, and SEC24. Thus, it may be that the UPR-mediated up-regulation of these genes may not be a cellular strategy to remove protein from the ER by exporting them, but additionally might promote sorting of retained proteins toward degradation.

Materials and methods

Antibodies and plasmids

Anti-CFTR NBD1 pAb was described previously (Bebok et al., 1998) and was a gift from Dr. David Bedwell (University of Alabama at Birmingham, Birmingham, AL). Anti-Kar2p pAb was a gift from Dr. Jeffrey Brodsky (University of Pittsburgh, Pittsburgh, PA). Anti–Kex2p mAb was a gift from Dr. David Bedwell (University of Alabama at Birmingham, Birmingham, AL). Anti–COOH-terminal CFTR mAb was purchased from Molecular Probes, Inc. Goat anti-rat and anti–mouse antibodies conjugated with fluorescein isothiocyanate or rhodamine were purchased from Jackson Immunoresearch Laboratories. To generate an inducible construct expressing GFP–tagged CFTR in yeast, pEGFP-CFTR plasmid (Moyer et al., 1998) was first digested with SacII, and then treated with Klenow fragment followed by digestion with Nhel. The pRSETB/EGFP plasmid (provided by Dr. David Bedwell) was first digested with HindIII and then treated with Klenow fragment followed by digestion with Nhel. The EGFP-containing fragment from pRSSETB/EGFP and CFTR-containing fragment from pEGFP-CFTR were gel-purified, ligated, and transformed into Eschericia coli. The resulting plasmid was then digested with Nhel and EcoRV, and treated with Klenow fragment. The EGFP-CFTR-containing fragment was gel-purified and cloned into the
Small site of pCL426CU1 (Labbe and Thiele, 1999). The final construct was sequenced to ensure the correct open reading frame.

**Yeast strains**

CTY182 (MATa, ura3–52, his3–200, lys2–800), CTY252 (MATa, ura3–52, sec12–4), CTY253 (MATa, ura3–52, sec13–4), and CTY260 (MATa, ura3–52, sec13–4, leu2–3, 112, sec23–3) were a gift from Dr. Vytas Bankaitis. YH290 (MATa, ura3–52, sec13–11, 112, his3–11, 15, can, GAL1), YH291 (MATa, prel–1, ure3–2, 112, his3–11, 15, can, GAL1), and YWO0346 (MATa, ura3–1, leu2–3, 112, his3–11, 15, ade2–1 ocre, trp1–1, can1–100, abc6::LEU2) were a gift from Dr. Dieter Wolfl (Universität Stuttgart, Stuttgart, Germany). SEY6210 (MATa ura3–52 leu2–3, 112 his3Δ200 trp1Δ901 lys2–801 suc2–3) and SEYS186 (MATa sec18–1 ura3–52 leu2–3 gal1+) were a gift from Dr. David Bedwell.

Yeast media were prepared as described previously (Rose et al., 1990). Unless specified, in all experiments, cultures were grown for a minimum of 5–6 generations to an A600 of no more than 1.0.

**Fractionation, immunofluorescence, and electron microscopy**

Yeast expressing EGFP-CFTR was fractionated as described previously (Padden et al., 1996). In brief, cells grown to exponential phase were converted to spheroplasts using yeast lytic enzyme (ICN Biomedicals) in the presence of 20 mM potassium phosphate buffer, pH 7.0, and containing 1.2 M sorbitol. The spheroplasts were washed and disrupted using a Dounce homogenizer in 25 mM Hepes-NaOH buffer, pH 7.4, plus protease inhibitors (Sigma-Aldrich). Cell lysates were centrifuged at 4°C at 500 g for 10 min, and the resulting supernatant was spun at 25,000 g for 15 min. The resulting supernatant was centrifuged at 130,000 g for 1 h. Pellet from each spin was washed with Hepes buffer and saved as P1, P2, and P3, respectively. Protein concentration was measured using a Detergent Compatible Protein Assay kit (Bio-Rad Laboratories).

For immunofluorescent studies, yeast were grown in synthetic complete medium lacking uracil to an OD600 of no more than 1.0. Yeast were fixed spectrally. Protein concentration was measured using a Detergent Compatible Protein Assay kit (Bio-Rad Laboratories). IpLab Spectrum software (Signal Analytics Corp.) was used to normalize data.

Immunoprecipitates were separated on 6% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics, Inc.). EGFP-CFTR signal was quantified using ImageQuant Software (Molecular Dynamics, Inc.), and relative amounts were calculated and plotted using Excel software (Microsoft). For CPY analysis, pulse-chase was done as above, and lysates were immunoprecipitated with anti-CPY antibodies. Immunoprecipitates were resolved on 10% SDS-PAGE and analyzed as above.

**SDS-PAGE and immunoblotting**

Samples were resolved on 6% SDS-PAGE as described previously (Bebok et al., 1998). Some gels were electrotransferred to nitrocellulose membrane and immunoblotted with anti–COOH-terminal CPY mAb as described previously (Bebok et al., 1998).

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