SEC23-SEC31 the Interface Plays Critical Role for Export of Procollagen from the Endoplasmic Reticulum

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Background: SEC23 plays a critical role in generating transport vesicles from the endoplasmic reticulum (ER).

Results: SEC23A M702V mutation at the SEC23-SEC31 interface causes a selective retention of procollagen in the ER. We correlate this defect with an enhanced SAR1 GTPase activity by M702V SEC23A through SEC13-SEC31.

Conclusion: The SEC23-SEC31 interface plays a critical role in capturing various cargo molecules.

Significance: SAR1 GTP hydrolysis is critical for cargo selection.

COPII proteins are essential for exporting most cargo molecules from the endoplasmic reticulum. The membrane-facing surface of the COPII proteins (especially SEC23-SEC24) interacts directly or indirectly with the cargo molecules destined for exit. As we characterized the SEC23A mutations at the SEC31 binding site identified from patients with cranio-lenticulo-sutural dysplasia, we discovered that the SEC23-SEC31 interface can also influence cargo selection. Remarkably, M702V SEC23A does not compromise COPII assembly, vesicle size, and packaging of cargo molecules into COPII vesicles that we have tested but induces accumulation of procollagen in the endoplasmic reticulum when expressed in normal fibroblasts. We observed that M702V SEC23A activates SAR1B GTPase more than wild-type SEC23A when SEC13-SEC31 is present, indicating that M702V SEC23A causes premature dissociation of COPII from the membrane. Our results indicate that a longer stay of COPII proteins on the membrane is required to cargo procollagen than other molecules and suggest that the SEC23-SEC31 interface plays a critical role in capturing various cargo molecules.

The majority of secretory proteins and cell surface membrane proteins are synthesized first in the endoplasmic reticulum (ER) and exit from ER exit sites, subregions of the ER, as passengers of transport vesicles. These transport vesicles coalesce into ER-Golgi-intermediate compartment (ERGIC) in mammalian cells (1). The ERGIC, then, mature into the cis-Golgi. The cargo molecules are subsequently modified during transit through the Golgi stacks and routed to final destinations (2, 3).

The transport vesicles specialized in ER export are generated by a set of cytoplasmic coat proteins (COPII proteins), which are comprised of SAR1, the SEC23-SEC24 complex, and the SEC13-SEC31 complex (4). These COPII proteins generate transport vesicles of an ~60–80-nm diameter that can accommodate most cargo molecules (4). The COPII proteins also are required for exporting an unusually large cargo, procollagen, from the ER (5–10), although it is unclear how these 60–80-nm vesicles can accommodate procollagen that is an ~300-nm-long rigid molecule (11, 12). Procollagen may be carried by a vehicle that depends on COPII only for fusion but not for budding (12). Alternatively, procollagen may be packaged into larger COPII vesicles/carriers.

Biogenesis of a COPII vesicle is initiated by activating SAR1, a small GTPase, through a guanine nucleotide exchange factor SEC12 at the ER exit sites. Membrane-bound SAR1-GTP then recruits the SEC23-SEC24 complex, which concentrates cargo molecules by recognizing sorting signal embedded in the cargo molecules. SEC24 has multiple cargo binding pockets in the membrane-facing side and is mainly responsible for cargo recognition (13, 14). The SAR1-SEC23-SEC24 complex recruits the SEC13-SEC31 complex (4). SAR1 and SEC13-SEC31 deform the membrane into tubular and spherical structures (15, 16). SEC23 has a GTPase-activating protein (GAP) activity for SAR1 GTPase (17). SEC13-SEC31 further stimulates this SEC23 GAP activity (18). Conversion of SAR1-GTP to SAR1-GDP leads to dissociation of coat proteins from the membrane because SAR1-GDP has a low affinity for SEC23-SEC24 and SEC13-SEC31 (18). Thus, for effective transport, the vesicles must be formed before the COPII proteins are displaced from the membrane. There are several paralogs of COPII proteins in vertebrate cells (19, 20). The redundancy of COPII proteins...
reflects the capacity of the COPII machinery to deliver a variety of cargo molecules out of the ER in a tissue-specific or developmentally regulated manner.

Cellular phenotypes elicited by alterations of the COPII proteins have been characterized extensively in yeast (21). For example, temperature-sensitive conditional mutants of <em>Saccharomyces cerevisiae</em> such as sec12–4, sec13–1, and sec23–1 secrete much reduced level of proteins at the non-permissive temperature (21). This ER export block induces vast proliferation of the ER network in the cytoplasm and enlargement of the ER cisternae. Similar cellular phenotypes have been observed in <em>Caenorhabditis elegans</em> sec23 mutants and in <em>Danio rerio sec23a</em>, or sec23b, or sec13 mutants. These mutants fail to secrete extracellular matrix proteins, including collagen and possibly other cargo molecules needed for normal craniofacial and skeletal morphogenesis, leading to abnormal skeleton and cuticle (an exoskeleton) morphogenesis (5, 6, 8, 22). In mice, reduction in the level of Sec23a causes an abnormal chondrogenesis and deletion of Sec24d causes severe skeletal and craniofacial dysmorphism (9, 10, 23). This implicates that the (exo)skeletal formation process (i.e. secretion of extracellular matrix including procollagen) is sensitive to these COPII defects.

Human SEC23A mutations cause a craniofacial disease, cranio-lenticulo-sutural dysplasia (CLSD) (6, 24). Two CLSD-causing SEC23A mutations (F382L and M702V) have been found at the SEC31 binding site (24, 25). SEC23A F382L mutant protein disrupts the interactions of SEC23A with SAR1B and SEC31A, resulting in defective COPII assembly and inefficient COPII vesicle formation, leading to extensive enlargement of the ER cisternae (6, 25). However, because SEC23A F382L causes a general ER export block, it is difficult to pinpoint the cargo molecule(s) whose ER export defect is responsible for CLSD phenotypes.

In this work, we characterize the recently discovered M702V SEC23A mutation. Surprisingly, we observe normal COPII assembly, but a selective ER export block of procollagen by M702V SEC23A, the SEC23A fragments were generated by digesting the pCF309 and pM702V SEC23A vectors with BamHI and HindIII and ligated into BglII and HindIII sites of pEGFP-C1 vector (Clontech, Mountain View, CA). SEC23A was in-frame with GFP.

Expression and Purification of Proteins—Rat liver cytosol was prepared as described previously (26). Human SAR1A and SAR1B proteins, wild-type and mutant SEC23A-His<sub>6</sub>SEC24D, and SEC13-His<sub>6</sub>SEC31A complexes were also purified as described previously (25–27).

Fluorescent Microscopy—Immunofluorescence labeling on fibroblasts was performed essentially as described previously (6, 24). For transient transfection, fibroblasts were plated in 24-well plate with washed glass coverslips 1 day before transfection, and transfected with indicated plasmid (0.5 μg for a coverslip) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 3 h of transfection, medium was replaced with fresh culture medium, and the cells were incubated for another 48 h. Transfected or untransfected fibroblasts were fixed with 4% paraformaldehyde in Dulbecco’s PBS for 30 min and permeabilized with 0.1% Triton X in Dulbecco’s PBS for 10 min at room temperature. The following primary antibodies were used in 0.3% BSA in Dulbecco’s PBS: polyclonal anti-ERGIC-53 (1/2,500), monoclonal anti-ER-GIC-53 (1/2,500), monoclonal anti-protein disulfide isomerase (1/2,500), and polyclonal anti-COL1A (LF-67, 1/2,500, a gift from Larry Fisher, National Institutes of Health) (29). We used Alexa Fluor 555 goat anti-rabbit IgG and Alexa Fluor 405 goat anti-mouse IgG (1/250, Molecular Probes, Invitrogen) as secondary antibodies. Images were processed with an Olympus BX61 spinning disc confocal microscope (Olympus, Center Valley, PA). Merges of images were processed with Olympus Slidebook software (version 5.0).

HTPC, a human fibrosarcoma cell line (HT1080) stably expressing α1(1) procollagen, was used for transfecting plasmids containing GFP-tagged WT SEC23A or M702V SEC23A. The HTPC cells were grown on 25 mm coverslips in DMEM + 10% FBS and were transiently transfected with 2 μg of DNA by using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Cells were grown for 18 h after transfection and fixed on coverslips with 3% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 15 min at room

**EXPERIMENTAL PROCEDURES**

Antibodies—Antibodies were used as described previously (26–28). The following antibodies were used for immunoblotting: rabbit anti-amyloid precursor protein C-terminal IgG (Sigma; 1/2,000), rabbit anti-ribophorin I antibody (1/5,000), rabbit anti-p58 serum (1/1,000), rabbit anti-SEC22 serum (1/500), mouse monoclonal anti-GFP antibody (clone 7.1 or 13.1, Roche Diagnostics 1/500), anti-rabbit IgG conjugated with HRP, 1/10,000. Rabbit anti-c-calreticulin antibody (Calbiochem; 1/500) and mouse monoclonal anti-fibronectin antibody (N-294, Calbiochem; 1/500) were also used for immunofluorescent labeling.

Cell Culture—Control human dermal fibroblasts were obtained from the American Type Culture Collection (catalog no. 2091). Fibroblasts were maintained in DMEM medium with 10% fetal bovine serum in a water-jacketed incubator at 37 °C with 5% CO<sub>2</sub> enrichment and split 1:5 weekly or when confluent. NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% inactivated fetal calf serum (Thermo Fisher Scientific).

Plasmid Constructions—pCS2+rFZ1-GFP2 plasmid was a gift of R. T. Moon (University of Washington). pCS2+yF-Smo plasmid was a gift of M. P. Scott (Stanford University).

The pM702V SEC23A plasmid was generated by using primers (5′-AGGAAATTCTTCTACTCCAGATTCCAGT-GCCAAAGATACATTGA-3′ and 5′-TCAATGTTACGTA- GCACCTGGAATCTGAGTGAAAGAATTTCT-3′) from pcF309 (WT hSEC23A). The resultant plasmid, pM702V hSEC23A was identical to pcF309 (WT hSEC23A) except the M702V mutation. The sequence was confirmed by sequencing. pcF309 (WT hSEC23A) and pcF330 (F382L hSEC23A) were described previously (25).

To construct plasmids for GFP wild-type SEC23A and GFP-M702V SEC23A, the SEC23A fragments were generated by digesting the pcF309 and pM702V SEC23A vectors with BamHI and HindIII and ligated into BglII and HindIII sites of pEGFP-C1 vector (Clontech, Mountain View, CA). SEC23A was in-frame with GFP.
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temperature followed by blocking with 1% BSA for 30 min. Primary antibodies were used: rabbit polyclonal anti-COL1A (LF-67) (diluted 1:1000) and mouse monoclonal anti-PDI (diluted 1:1000). Secondary antibodies used were Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 647 goat anti-mouse IgG (diluted 1:200, Molecular Probes/Invitrogen). After incubating cells with the appropriate primary and secondary antibodies, coverslips were mounted on slides using Prolong Gold anti-fade mounting reagent from Invitrogen containing DAPI. Images were visualized with a Zeiss LSM710 confocal microscope (Thornwood, NY) using a 100× oil objective and captured with Zen10 software. Merges of images were performed with LSM Image Browser and Image J.

SAR1 shRNA Knockdown—HTPC, a human fibrosarcoma cell line (HT1080) stably expressing α1(1) procollagen, was used. The shRNA target sequences for individual human SAR1 paralogs were as follows: 5'-TCGAGAAAAAGATGACAGATG-CAATCAGTGATCTCTTGAATCACTGATTGCATCTGT-TCGGG-3' (SAR1A) and 5'-TCGAGAAAAAGAACAGATG-CTTGAGAACCCTCTGGAATGTGTCCTGATG-TCGGG-3' (SAR1B). Custom-made double strand DNA oligonucleotides with these sequences were cloned into pSuper.retro.puro shRNA expression vector using BglII and XhoI sites and transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. pSuper empty vector was used as a control. Cells were grown on 25-mm coverslips in six-well plates and were fixed 2 days after transfection of shRNA constructs. Collagen staining was performed using LF-67 (1:1000 dilution). The ER was stained using anti-PDI (1:1000 dilution) antibody. Cells on coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 15 min at room temperature followed by blocking with 1% BSA for 30 min. We used Alexa Fluor 568 donkey anti-rabbit IgG and Alexa Fluor 647 goat ant-mouse IgG (diluted 1:200) as secondary antibodies. After staining cells with appropriate primary and secondary antibodies, coverslips were fixed on slides using a mounting reagent containing DAPI. Images were visualized, and cells were quantified with a Zeiss LSM 710 confocal microscope and captured with Zen10 software. Merges of images were performed with LSM image Browser.

VSV-G ER-to-Golgi Transport Assay—Human dermal primary fibroblasts were transfected with tSO45 VSV-G-GFP constructs (a gift of Dr. William Brown at Cornell University, Ithaca, NY and Dr. Kai Simons at Max-Planck-Institute, Dresden, Germany) at 37 °C in a 5% CO2 incubator for 2 h and transferred to a 39.5 °C, 5% CO2 incubator. After 16 h, cells were placed in a 32 °C water bath and removed at indicated time points (8). The removed cells were washed with ice-cold PBS, solubilized with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1% Nonidet P-40, 1× protease inhibitor mixture), scraped, transferred to a microcentrifuge tube, and centrifuged at 20,000 × g in a refrigerated microcentrifuge for 20 min. The cleared lysate was quantified, mixed with SDS sample buffer, and analyzed by SDS-PAGE and immunoblotting.

In Vitro Vesicle Formation Assay—Semi-intact cells, rat liver cytosol, and recombinant COPII proteins were prepared as described previously (26, 27).

Liposome Flotation Assay—The liposome flotation assay was performed as described previously using 10% cholesterol-major–minor mix liposomes (25, 26, 28). For a mammalian COPII binding experiment, 15 μg of a liposome suspension was mixed with various combinations of human SAR1 (1.2 μg), wild-type or mutant human SEC23A-SEC24D (1.7 μg), and human SEC13-SEC31A (2.4 μg) in 75 μl of HKM buffer (20 mM HEPES (pH 7.2), 150 mM KOAc, 1 mM Mg(OAc)2) with an indicated nucleotide (0.1 mM).

Tryptophan Fluorescence GTPase Activity Assay—The tryptophan fluorescence GTPase activity assay was performed as described from Fromme and co-workers (25). Mixtures of indicated COPII proteins and major–minor mix synthetic liposomes containing 10% cholesterol were incubated in a stirred-cell cuvette at 37 °C. SAR1 was added at 1.5 μM (final concentration) to 0.3 μM liposomes in HKM buffer. After 1 min, GTP was added to 30 μM. Spontaneous exchange of GDP to GTP was complete after ~19 min. Wild-type or a mutant SEC23A-SEC24D complex was added to 32 nM. Where indicated, SEC13-SEC31A complex was added to 400 nM simultaneously with SEC23A-SEC24D.

Electron Microscopy—For conventional electron microscopy analysis, fibroblast cell cultures were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer and post-fixed cells with osmium tetroxide. En bloc staining with uranyl acetate was followed by dehydration in ethanol and embedding in Epon (30). Thin sections of fibroblasts were observed and photographed with a Philips EM 410 LS microscope.

RESULTS

Procollagen Is Accumulated in ER of Paternal Fibroblasts—We have previously shown that the CLSD patient with a heterozygous M702V SEC23A allele inherited the mutant allele from a clinically unaffected father (24). Interestingly, only the son is affected even though both the father and the son carry the same heterozygous mutant allele. When we examined the fibroblasts derived from the family of the patient, we observed a stronger ER distention and a more pronounced procollagen accumulation in the fibroblasts of the patient than the parental fibroblasts (24). Based on these results, we proposed that this disease is caused by compound heterozygous mutations: M702V SEC23A from the father and the other mutation probably in one of SEC genes of the early secretory pathway or in a cargo gene.

To measure the effect of M702V SEC23A on procollagen transport, we used paternal skin fibroblasts (Fig. 1). The majority of the paternal fibroblasts retained more procollagen in the ER than the control fibroblasts with significant statistical difference (Fig. 1, G and F). In fact, this is consistent with the previous finding that the paternal cells also display moderately distended ER cisternae (24). These results suggest that although the unknown mutation contributes to the stronger ER export block in the cells of the patient, M702V SEC23A alone can mildly block the ER export globally or specifically (i.e. procollagen).
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Recombinant M702V SEC23A Protein Has Normal Cargo Packaging Activity—According to the crystal structure of yeast Sec23p and a Sec31p fragment, the Met-702 residue in SEC23A is predicted to be in proximity to Phe-382 residue at the SEC31 binding surface (32). Using a recently solved crystal structure of human SEC23A, we confirmed this spatial configuration (Fig. 3A) (33). Based on this structural information, we speculated that M702V SEC23A disrupts SEC31 binding and vesicle formation. To evaluate the effect of M702V SEC23A on cargo packaging, we performed an in vitro COPII vesicle formation assay and monitored packaging of p58 into COPII vesicles (26). Surprisingly, M702V SEC23A was as active as wild-type SEC23A in cargo capture (Fig. 3B). Other cargo molecules such as amyloid precursor protein and SEC22 were also normally packaged into COPII vesicles in reactions containing M702V SEC23A (supplemental Fig. S1). However, as reported previously, F382L SEC23A displayed a pronounced cargo packaging defect (Fig. 3B). These results clearly indicate that M702V SEC23A is as active as wild-type SEC23A in cargo packaging activity. Because we have observed an ER export block of procollagen (Figs. 1 and 2) and a moderate ER distention in the paternal fibroblasts of the patient (24), these data suggest that M702V SEC23A blocks ER export of a subset of cargo molecules.

ER Export Block Is Cargo-specific in Fibroblasts of Patient—We tested the transport of other cargo molecules in the fibroblasts of the patient. To test this idea, we introduced fluorescently labeled receptor proteins such as Smoothened (GFP-Smo) and Frizzled 1 (YFP-Fz1) into control fibroblasts and the fibroblasts of the patient. These fusion proteins are known to receptors by monitoring colocalization of the fluorescent signals from these receptors with those of an ER marker, PDI (Fig. 4, A–D). Although we did not identify the compartment(s) in which these receptor molecules were localized, they were not retained in the ER of the fibroblasts of the patient. Apparently, unlike procollagen, cargo molecules such as Smo and Fz1 can exit the ER in the cells of the patient.

To test whether ER export of a secretory molecule is more sensitive than membrane proteins in the cells of the patient, we monitored the intracellular distribution of a secretory protein, fibronectin. Endogenous fibronectin was found in punctate...
structures that are distinct from the ER in the control fibroblasts (Fig. 4E). Similarly, fibronectin was not found in the ER in the fibroblasts of the patient and father (Fig. 4E). Our results show that the steady state distribution of most cargo molecules is not altered in the fibroblasts of the patient except that of procollagen.

We then considered a possibility that the M702V SEC23A in the fibroblasts of the patient affects the rate of the ER-to-Golgi transport. To examine the rate of ER export, we introduced tsO45 VSV-G-YFP into the control fibroblasts (supplemental Fig. S2A) (8, 36). Most VSV-G existed as an ER form after 39.5 °C incubation (supplemental Fig. S2A, time 0). Further incubation at 32 °C improved the conversion of VSV-G from the ER form to the Golgi form marginally. Perhaps, the rate of ER export is slow in the primary fibroblasts. In fact, we observed much lower packaging efficiency of p58 in the control human primary fibroblasts than in other cell lines such as HEK293, NIH3T3, CHO-K1, HeLa, embryonic stem cells, and embryonic fibroblasts (Fig. 3B and supplemental Fig. S2B) (25–28).

When we compared ER-to-Golgi transport of VSV-G in control and the fibroblasts of the patient, we observed statistically similar increase (18.6% in control and 14.3% in the fibroblasts of the patient) in the ratio of Golgi forms to the total VSV-G after the chase compared with time 0 (supplemental Fig. S2C). Our results suggest that ER export is not grossly blocked in the fibroblasts of the patient and suggest that only a subset of cargo molecules is affected. Obviously, procollagen is more sensitive to a moderate ER export defect than other cargo molecules.

M702V SEC23A Normally Interacts with Other COPII Proteins—To probe the mechanism used by M702V SEC23A to specifically block the collagen transport, we evaluated interaction of M702V SEC23A with other COPII proteins using a liposome floation assay. This assay allows us to monitor recruitment of SEC23-SEC24 and SEC13-SEC31 to the membrane bound SAR1 in a nucleotide-dependent manner (26, 37). In the presence of non-hydrolyzable GTP analog, GMP-PNP, SAR1 recruited SEC23-SEC24 and SEC13-SEC31A to the membrane (Fig. 5). Recruitment of SEC13-SEC31A by F382L SEC23A-SEC24D was less than that by wild-type SEC23A-SEC24D in reactions containing SAR1 or SAR1B. On the other hand, recruitment of SEC13-SEC31A by M702V SEC23A-SEC24D was as efficient as that by wild-type SEC23A-SEC24D. This result demonstrates that the apparent interactions of M702V SEC23A with SAR1 and SEC13-SEC31A are normal.

M702V SEC23A Aberrantly Activates SAR1B GTPase in Presence of SEC13-SEC31A—Because Met-702 of SEC23A is positioned away from the membrane surface, the Met-702 residue presumably will not interact with cargo molecules or cargo...
adaptors directly (32, 33), but it may influence the GAP activity of SEC23. Thus, we measured the activation of SAR1 GTPase by wild-type or mutant SEC23A in the absence or presence of SEC13-SEC31A (Fig. 6). In reactions with SAR1A, SEC13-SEC31A stimulated the GAP activity of wild-type and mutant SEC23A-SEC24D proteins comparably (Fig. 6A; wild-type was stimulated from \(2.4 \times 10^{-3}\) s\(^{-1}\) to \(4.7 \times 10^{-3}\) s\(^{-1}\); M702V was stimulated from \(2.4 \times 10^{-3}\) s\(^{-1}\) to \(4.9 \times 10^{-3}\) s\(^{-1}\); F382L was stimulated from \(2.5 \times 10^{-3}\) s\(^{-1}\) to \(4.1 \times 10^{-3}\) s\(^{-1}\)). Interestingly, however, M702V SEC23A stimulated the SAR1B GTPase activity further than wild-type SEC23A in the presence of SEC13-SEC31A (Fig. 6B; wild-type was stimulated from \(1.6 \times 10^{-3}\) s\(^{-1}\) to \(3.2 \times 10^{-3}\) s\(^{-1}\); M702V was stimulated from \(1.5 \times 10^{-3}\) s\(^{-1}\) to \(4.6 \times 10^{-3}\) s\(^{-1}\); F382L was stimulated from \(2.0 \times 10^{-3}\) s\(^{-1}\) to \(2.5 \times 10^{-3}\) s\(^{-1}\)). Clearly, SAR1B is more sensitive to mutations at the SEC23A-SEC31A interface than SAR1A. This is consistent with our results and the previous report that F382L SEC23A displayed more pronounced defects in combination with SAR1B than with SAR1A (Fig. 1) (25). Accelerated conversion of SAR1-GTP to SAR1-GDP can lead to faster release of the COPII proteins from the membrane, which will lower the effective concentration of the COPII proteins on the membranes (18). When the levels of SEC13 and SEC31 were reduced, ER export of procollagen but not VSVG protein was specifically hampered (8). Clearly, secretion of procollagen is more sensitive to the availability of COPII proteins than other cargo molecules. In this respect, faster GTP hydrolysis reduces the availability of COPII proteins on the membrane and contributes to exclusion of procollagen from transport carriers.

**Sizes of Vesicles at ER Exit Sites in M702V Fibroblasts Are Not Changed**—Alternatively, because formation of larger vesicles/tubules should require more time than smaller ones, faster release of COPII proteins from the membrane may cause premature vesicle formation, reduction of the vesicle size to smaller ones, and selective exclusion of procollagen. Procollagen is well known to form a 300-nm-long rigid triple helix (38, 39). Although COPII proteins usually generate 60–80-nm diameter vesicles, they may be able to generate large vesicles to accommodate procollagen. Cryo EM studies have suggested that the SEC13-SEC31 complex can polymerize into various cages (40, 41). Interestingly, SEC13-SEC31 can generate bigger cages when SEC23-SEC24 is included during the cage formation reaction (41). This result suggests that the interaction between SEC23 and SEC31 can influence the size of vesicles. Because Val-702 of SEC23A is implicated in SEC31 binding and also leads to an aberrant SAR1 GTPase activation, we consid-
ered the possibility that COPII vesicles became smaller and unable to accommodate procollagen. To test this idea, we measured the diameters of vesicles near the ER exit sites and near the Golgi stacks using electron microscopy. The diameters of these vesicles in fibroblasts carrying the M702V SEC23A allele (the father and the patient) were indistinguishable from those in control fibroblasts (Table 1). Furthermore, when we measured the diameters of buds at the ER exit sites, we did not detect any significant differences (Table 2). Thus, we conclude that faster release of COPII proteins from the membrane does not alter the size of the buds or the vesicles.

During the measurement of the vesicle size, we noticed that the fibroblasts of the patient showed abundant coated profiles (Fig. 7) (Table 3). Stability of COPII coats on the membrane is greatly influenced by the status of SAR1 (18, 42). Notably, this increase in the coated vesicular profiles was not observed in the paternal fibroblasts (a M702V SEC23A carrier), indicating that the increase of coated vesicles is not caused by M702V SEC23A (Table 3, Experiment 1). As we showed previously, the cells of the patient accumulate more procollagen and have more distended ER cisternae than the paternal cells (24). Thus, our data suggest that the second mutation exists perhaps in a component that influences the SAR1 GTPase cycle or uncoating.

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![FIGURE 5. Differential binding of mutant SEC23A-SEC24D proteins. A and C, purified recombinant COPII proteins were incubated with liposomes at 37 °C for 20 min. Reaction mixtures were subject to flotation on a sucrose gradient as described previously (26, 37). Proteins in the floated fractions were normalized to the lipid recovery, resolved by SDS-PAGE, stained by SYPRO Red (Invitrogen) and visualized using a Typhoon 9400 image analyzer. B and D, fluorescent intensities of floated SEC23A and SEC31A bands were measured to calculate the ratio of SEC31A recruited to membrane-bound SEC23A (Student’s t test; p, * < 0.05 (n = 3); p, ** < 0.025 (n = 3).](image)

**DISCUSSION**

We have characterized the cellular and molecular consequences of SEC23A mutations identified from CLSD patients. M702V SEC23A clearly affects the exit of procollagen from the...
ER but not that of other cargo molecules we tested. This specific trafficking defect was correlated with the accelerated rate of SAR1-GTP to SAR1-GDP conversion. Notably, M702V SEC23A did not influence apparent binding of SEC31. Thus, our results suggest that a change in the SAR1 GTPase activity itself influences cargo selection. A direct proof of this notion requires a measurement of procollagen packing into the COPII vesicles with recombinant COPII proteins, which has not been established so far. Nevertheless, our data clearly indicate that M702V abrogates the ER export of procollagen.

F382L SEC23A disrupts binding of SEC31A and grossly inhibits COPII vesicle formation. On the other hand, M702V SEC23A does not affect apparent SEC31A binding but increases the rate of SAR1B-GTP to SAR1B-GDP conversion. Interestingly, M702V SEC23A blocks transport of procollagen, whereas it does not affect the transport of small cargo molecules such as p58/ERGIC53/LMAN1, SEC22, and amyloid precursor protein, indicating that the action of M702V SEC23A differs from that of F382L SEC23A. Our results suggest that this mild mutation at the SEC23-SEC31 interface can specifically affect cargo selection and suggests that the SEC23-SEC31 interface, which is positioned away from the membrane surface, is exquisitely optimized to accommodate various cargo molecules.

The finding that M702V SEC23A influences SAR1 GTPase activation and procollagen export from the ER shows that normal SAR1 GTPase activity is critical for procollagen export. In addition, a recent study has shown that a defect in Sar1 assembly can also block ER export of procollagen (7). Furthermore, depletion of SEC24D or SEC13-SEC31 blocks secretion of the extracellular matrix or procollagen (8–10) and TANGO1, which plays a critical role in ER export of procollagen interacts with SEC24C (43, 44). Thus, the data established by us and others (7–10) clearly show that all COPII proteins (SAR1, SEC23-SEC24, and SEC13-SEC31) are required for procollagen export from the ER.

Clathrin alone can self-assemble into a cage structure in the absence of lipid bilayer with a diameter ranging from 70 to 120 nm and inclusion of adaptor proteins reduces the size of the cages to 80 nm in diameter (45). In case of COPII, the COPII outer coat (SEC13-SEC31) can form cages with diameters of 50–80 nm and cages with diameters of 60–100 nm in the presence of SEC23-SEC24 (41, 46). In fact, cages with ~100 nm diameter are the most abundant, suggesting the coat proteins appear to prefer a particular dimension that is consistent with the standard dimension of vesicles (~60 nm) (41). Thus, coat proteins appear to have an inherent propensity to polymerize into a structure that is consistent with the standard size of a vesicle. However, large clathrin vesicles (~200-nm diameter) do exist (47). In addition, COPII proteins can decorate the surface of relatively large liposomes in the presence of nonhydrolyzable GTP analog (100 nm or larger in diameter) (16, 18, 37). Clearly coat proteins can induce or conform to various sizes/shapes of membrane structures.

It has been suggested that SAR1 GTP hydrolysis is critical for cargo proofreading (48). A cargo molecule (Emp47-Δ281–333)
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that fails to form a complex with Emp46p stimulates the GAP activity of Sec23p and is loaded inefficiently into COPII vesicles. According to this kinetic proofreading model, GTP hydrolysis ensures loading of proper cargo molecules. Then, faster SAR1 GTP hydrolysis may preferentially hamper loading of cargo molecules with a lesser affinity for the COPII machinery. Perhaps, type I procollagen has a low affinity for COPII machinery/cargo adaptor and requires sufficient amount of SEC13-SEC31 for ER export. In fact, knocking down the levels of SEC13-SEC31 preferentially disrupts the export of type I procollagen (8). Thus, it is possible that M702V SEC23A causes a more stringent cargo selection.

SEC23-SEC24 and SEC13-SEC31 can stably remain on the surface of COPII vesicles in the presence of GTP (42). However, hydrolysis ensures loading of proper cargo molecules. Then, faster SAR1 GTP hydrolysis may preferentially hamper loading of cargo molecules with a lesser affinity for the COPII machinery. Perhaps, type I procollagen has a low affinity for COPII machinery/cargo adaptor and requires sufficient amount of SEC13-SEC31 for ER export. In fact, knocking down the levels of SEC13-SEC31 preferentially disrupts the export of type I procollagen (8). Thus, it is possible that M702V SEC23A causes a more stringent cargo selection.

**TABLE 2**

| Diameter (nm) of coated buds at the ER exit sites |
|-----------------------------------------------|
| Mean ± S.D.                                   |
| Control 52 ± 8 (n = 21)                      |
| Patient 53 ± 4 (n = 34)                      |
| Father 51 ± 7 (n = 23)                       |

$n$ represents the number of buds. The measures were performed on printed pictures at a calibrated magnification of 125,809-fold.

**FIGURE 7. Increased coated vesicular profiles in the fibroblasts of the patient.** A, electron micrograph of control fibroblast. The amount of coated vesicular profiles (arrows) associated with the Golgi stacks (G) appears less important compared with the mutant. B, electron micrograph of a Golgi area from a M702V fibroblast showing stacks of cisternae (G) with many vesicular profiles (arrows) surrounding the stacks. The inset shows a high magnification view of coated vesicular profiles (vesicle is indicated by arrow, and bud is indicated by an asterisk).
this stable association of SEC23-SEC24 and SEC13-SEC31 with membrane should occur after complete cage formation. In other words, once COPII proteins complete the cage formation on a vesicle surface, this structure may remain relatively stable even after liberation of SAR1 from the vesicle. On the other hand, monomeric or oligomeric SAR1, SEC23-SEC24, and SEC13-SEC31 complexes stay only transiently on the membrane in the presence of GTP (18).

As mentioned above, a mutation in the second gene should be responsible for the increase in the volume density (Vv) of the coated vesicular profiles of the fibroblasts of the patient. There are two obvious explanations. First, uncoating is ineffective because of a defect in the SAR1 GTPase cycle or uncoating step. We already sequenced SEC23A, SEC23B, SEC31A, and SEC13 of the patient and found no mutations. Thus, it is possible that a mutation exists in SAR1 paralogs or in SEC31B. Second, vesicle fusion is less than normal because of an alteration in the fusion machinery, resulting in an increase of the Vv of overall vesicles (coated and uncoated vesicles). Perhaps fusion machinery may also serve as candidate genes for CLSD or other craniofacial diseases.

It is not clear why F382L SEC23A or M702V SEC23A is more defective with SAR1B than SAR1A. In addition, we do not know how SEC31B interacts with these mutant proteins. Further research is required to fully understand the interactions among these COPII paralogs in the context of cargo selectivity and diseases. Nevertheless, we have clearly shown that the SEC23-SEC31 interface plays a crucial role not only in COPII vesicle assembly, but also in procollagen selection.

Our previous and current data establish that CLSD can be caused by homozygous SEC23A mutations or by heterozygous SEC23A mutation in combination with another mutation in the early secretory machinery. According to the NCBI human single nucleotide polymorphism data base, there are mutations in SEC23A (rs35459408), SEC24B (rs17040495), SEC24C (rs34729114), SEC24D (rs34551429, rs34707306), and SEC31B (rs35004459) that result in production of truncated proteins. Perhaps, these alleles constitute as a genetic background for many skeletal-related diseases.

The importance of studying rare genetic syndromes is well illustrated by the fact that analysis of the two mutations identified in patients with CLSD has provided critical insights into understanding COPII vesicle biogenesis and cargo selection. Because CLSD should be caused by ER export failure of a subset of proteins critical for development of the face, head, and bone, identification of such molecules that are specifically retained in the ER of the patients’ cells will help understand the pathogenesis of CLSD and human craniofacial development.

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