BmCREC Is an Endoplasmic Reticulum (ER) Resident Protein and Required for ER/Golgi Morphology

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The abbreviations used are: COPI, coat protein I; ER, endoplasmic reticulum; BmSCF, Bombyx mori supercoiling factor; EGFP, enhanced GFP; KDELR, KDEL receptor; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PSG, posterior silkgland.

Background: BmCREC was supposed to supercoil DNA in the nucleus.

Silkworm posterior silkgland is a model for studying intracellular trafficking. Here, using this model, we identify several potential cargo proteins of BmKinesin-1 and focus on one candidate, BmCREC. BmCREC (also known as Bombyx mori DNA supercoiling factor, BmSCF) was previously proposed to supercoil DNA in the nucleus. However, we show here that BmCREC is localized in the ER lumen. Its C-terminal tetrapeptide HDEF is recognized by the KDEL receptor, and subsequently it is retrogradely transported by coat protein I (COPI) vesicles to the ER. Lacking the HDEF tetrapeptide of BmCREC or knocking down COPI subunits results in decreased ER retention and simultaneously increased secretion of BmCREC. Furthermore, we find that BmCREC knockdown markedly disrupts the morphology of the ER and Golgi apparatus and leads to a defect of posterior silkgland tube expansion. Together, our results clarify the ER retrieval mechanism of BmCREC and reveal that BmCREC is indispensable for maintaining ER/Golgi morphology.

Results: BmCREC is retrieved to the ER by KDEL receptor- and COPI-mediated transport. Defects in ER retrieval of BmCREC lead to disrupted ER/Golgi morphology.

Conclusion: BmCREC, as an ER luminal protein, functions to maintain ER/Golgi morphology.

Significance: This study facilitates the understanding of the roles of ER luminal proteins in regulating ER/Golgi morphology.
ER Resident BmCREC Maintains ER/Golgi Morphology

EXPERIMENTAL PROCEDURES

Silkworm Strain and Cell Lines—Silkworm strain and BmN cells were used as reported previously (14).

Vector Construction, RNA Isolation, Quantitative Real-time PCR, and Antibodies—The CBD of BmKinesin-1 and full-length BmCREC were inserted into the vector pGEX-6P-1 (GE Healthcare). BmCREC-EGFP (C-terminal enhanced GFP-tagged) was inserted into the pFastBac-1-based pFastBac-hr5/IE1 vector as reported previously (4, 19). EGFP-BmCREC (EGFP inserted after the signal peptide), EGFP-BmCREC# (without HDEF), CFp-BmCREC and YFP-KDEL, with the fluorescent protein inserted after the signal peptide of BmCREC or KDEL, were inserted into the pFastBac-hr5/IE1 vector. See supplemental Table S1 for primer information.

For BmCREC rescue, the first 300 nucleotides of BmCREC coding sequence were mutated to ATGCTACGATTCTTTT-AAAATTGACTCCGATCAAGACGGATTCATTACATTG-AGGAGAGCAAACGACGGTTGGGTGAGATCGCTGAT-AAGATCAAGCGAAGACGTTTGACCAGTTGTCTCCGG-AAGATCGAAGCGACCGTTCGCTGCGTGTTGCTGCAAG-GAGAGCAGCAAGGCAGGTGTTGGTGAAGCCTGCTAT-AAATTGACTCCGATCAAGCGAAGGCAGTTACATTACATT-GTAAGAATGAAAGCCTGATCGGTACACCCAA, with the underlined nucleotides being mutated.

Isolation of total RNA and quantitative real-time PCR were performed as described previously (4). See supplemental Table S2 for primer information.

Anti-BmCREC and anti-EGFP rabbit polyclonal antibodies were produced and purified as described previously (14). Anti-silkworm α/β′-COP (4), anti-BmKinesin-1 (14), anti-calnexin (Abcam), anti-EGFP (MBL), anti-Drosophila GM130 (Abcam), anti-KDEL (Abcam), anti-lamin (20), and anti-tubulin (Sigma) antibodies were also used.

Pulldown Analysis and Immunoprecipitation—Pulldown assay with silkworm PSG homogenates was performed as reported previously (14). For immunoprecipitation in PSG, the homogenates were incubated with the affinity-purified antibodies in lysis buffer (20 mM HEPES, 320 mM sucrose, 120 mM NaCl, 2 mM EGTA, 1 mM MgCl₂, pH 6.8). The homogenates were then incubated with precleaved protein A-Sepharose beads, followed by extensive washing and sample preparation for SDS-PAGE separation. For immunoprecipitation in BmN cells, cells were washed in lysis buffer (20 mM HEPES, 120 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 6.8) and subjected to antibody incubation.

For identifying proteins from SDS-PAGE, the protein bands were separately excised and digested with sequence-grade modified trypsin (Promega). These samples were subjected to LCQ Deca XP Plus Analyzer liquid chromatography-tandem mass spectrometry (LC-MS/MS; Finngan). The endogenous BmCREC band was also excised for Edman N-terminal amino acid sequencing using the Procise 491 Protein Sequencer (Applied Biosystems).

Sample Preparation and Nuclear-Cytoplasmic Fractionation—For determining the secretion ratio of BmCREC, BmN cells were maintained at serum-free TC-100 medium (Sigma) for 24 h. The total cell lysates and the corresponding serum-free conditioned medium were collected 48 h after transfection and were compressed to the same volume (50 μl). One fifth of the total sample (10 μl) was loaded and subjected to Western blotting analysis. Western blotting and band intensity calculation were performed as described previously (21).

For nuclear-cytoplasmic fractionation, the BmN cells were lysed in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.05% Nonidet P-40, pH 7.9) by lysing with ~20 strokes in a glass Dounce homogenizer and were then subjected to centrifugation at 3,000 × g for 15 min to pellet the nuclei. The nuclear and cytoplasmic fractions were then subjected to Western blotting analysis.

Cell Transfection, RNAi, Immunostaining, and Fluorescence Resonance Energy Transfer (FRET) Assay—Cell transfection and immunostaining were performed as reported previously (14).

For RNAi, dsRNAs of BmCREC (1–300 nucleotides), β′-COP (661–961 nucleotides), BmKinesin-1 (2493–2793 nucleotides), KDEL#1 (32–291 nucleotides), KDEL#2 (300–561 nucleotides), and EGFP (1–300 nucleotides) were synthesized and extracted using a MEGAscript Kit (Ambion). See supplemental Table S3 for primer information. For knockdown in silkworm larvae, 1–2 μg of dsRNAs was injected into the hemolymph of the third-instar larvae, as described previously (4, 22). One week after injection, PSG were dissected and photographed, and the diameters of both glandular lumen and PSG tube were calculated using ImageJ (National Institutes of Health). For the pupation and cocoon analysis, dsRNAs were injected into silkworm larvae twice at the fourth and fifth instars. After pupation, the pupae were taken out, and the cocoons were collected and weighed.

Immunostaining and FRET assay were conducted as reported previously (23). For Pearson correlation analysis, cells immunostained with BmCREC and different markers were photographed with ZEISS LSM 710 confocal microscope, equipped with a 100×/N.A. 1.4 oil-immersion objective lens (Zeiss). The photographs were then analyzed using the colocalization finder plugin of ImageJ software as reported previously (24). In each case, >15 cells were measured.

Cryosection and Electron Microscopy—Cryosection and the following immunostaining, and electron microscopy were performed as reported previously (4). For Golgi morphology analysis in silkworm PSG, the numbers and areas of the Golgi structures were calculated using ImageJ. The diameter of the ER tubule was measured similarly.

Bioinformatics and Statistical Analysis—Silkworm cDNA sequences were obtained mainly from Silkworm Genome Database (25, 26) and SilkBase (27). The phylogenetic tree was constructed using MEGA 3.1 (28). The domain architecture, especially the signal peptide, was predicted by SMART (29). Statistical data were evaluated by unpaired two-tailed student’s t test.

RESULTS

BmCREC Is Associated with BmKinesin-1—To identify novel cargo molecules of BmKinesin-1, we performed pulldown analysis in silkworm PSG homogenates using prokaryotically
expressed GST-BmKinesin-1-CBD. We identified many potential BmKinesin-1-CBD-interacting proteins by LC-MS/MS assay (Fig. 1A). BmSCF was one of these candidates (Fig. 1A and supplemental Fig. S1A), and it was previously shown to be a DNA supercoiling factor in vitro (16). To investigate the in vivo characteristics of BmSCF, we produced a rabbit polyclonal antibody against the full-length BmSCF. The antibody specifically recognized a protein band of ~47.5 kDa in PSG homogenates as expected by Western blotting analysis (supplemental Fig. S1B). Both immunostaining and nuclear/cytoplasmic extraction results showed that BmSCF was not a nuclear protein (Fig. 1, B–D, and supplemental Fig. S1C), suggesting that BmSCF does not function to supercoil DNA in the nucleus as previously proposed (15, 16). As BmSCF does not belong to any subfamily of CREC proteins (supplemental Fig. S1D), we renamed BmSCF as BmCREC (GenBank accession number AEO79985).

Quantitative real-time PCR and Western blotting analysis showed that BmCREC was ubiquitously expressed in various silkworm tissues, including brain, Malpigian tubule, fat body, silk gland, and midgut, and was relatively higher in midgut in terms of both mRNA and protein levels (supplemental Fig. S1, E and F). The expression levels of BmCREC during the seven developmental days of the fifth-instar PSG were also determined. Although the mRNA levels of BmCREC were relatively higher from the 1st to the 3rd developmental days (supplemental Fig. S1E), the protein levels were stable, except for the last 2 days (supplemental Fig. S1G), possibly due to programmed cell death of PSG cells caused by pupal metamorphosis (30).

To confirm the association of BmCREC with BmKinesin-1, we performed pulldown analysis again with PSG homogenates, and the endogenous BmCREC was detected bound to GST-BmKinesin-1-CBD (Fig. 1E). Immunoprecipitation assay further confirmed the interaction of BmCREC with BmKinesin-1 in the PSG (Fig. 1F). Taken together, these data suggest that BmCREC is not a nuclear protein, but a novel associating protein of BmKinesin-1.

**BmCREC Is Localized in the ER Lumen**—To unravel the detailed localization of BmCREC, we communostained BmN cells for BmCREC and ER or Golgi marker. The results showed that BmCREC partially colocalized with the cis-Golgi marker GM130 (31) (Fig. 2A), whereas the majority of BmCREC was colocalized with the ER marker calnexin in BmN cells (Fig. 2B).

Next, using immunoelectron microscopy, we showed that the gold-labeled BmCREC proteins were preferentially localized on the membrane of both the ER and Golgi apparatus in PSG cells (Fig. 2, C and D), suggesting that BmCREC may attach to the membrane, possibly through interaction with some transmembrane proteins.

Based on the ER localization of BmCREC, its membrane attachment, and the bioinformatic prediction by SMART (29) that the N-terminal 16 amino acids of BmCREC were signal peptide, we assumed that BmCREC is an ER luminal protein. To explore this, we purified the endogenous BmCREC from PSG homogenates by immunoprecipitation with anti-BmCREC antibody (Fig. 2E) and determined its N-terminal amino acid sequence by standard Edman degradation. Matured BmCREC protein started from position 17 of its deduced amino acid sequence (Fig. 2F and supplemental Fig. S2A), implying that the N-terminal 16 amino acids of BmCREC were cleaved as a signal peptide. This result was consistent with the bioinformatic prediction. To further confirm this, BmN cells were permeabilized with Triton X-100 or digitonin before immunostaining. Triton X-100 permeabilizes both the plasma membrane and the intracellular membrane, whereas digitonin selectively permeabilizes the plasma membrane (32). The signal of BmCREC was detected only after Triton X-100 treatment (supplemental Fig. S2B). Collectively, these data suggest that BmCREC is localized in the ER lumen.

**BmCREC Is Retrieved to the ER through COPI Transport** —To identify interacting proteins of BmCREC, we performed immunoprecipitation with anti-BmCREC antibody using PSG homogenates and identified α-COP by LC-MS/MS assay (Fig. 3A and supplemental Fig. S3A). Besides, β′-COP was also detected by LC-MS/MS assay in the pulldown precipitates of GST-BmKinesin-1-CBD (supplemental Fig. S3, B and C). As both α-COP and β′-COP are subunits of the COPI complex (33), we hypothesized that BmCREC is associated with the COPI complex. To this end, we performed GST-BmKinesin-1-CBD pulldown experiments again, and the results revealed interaction of COPI subunits with BmKinesin-1-CBD (Fig. 3B). Immunoprecipitation analysis further confirmed that both BmCREC and BmKinesin-1 were associated with COPI subunits (Fig. 3C). Consistently, immunostaining showed partial colocalization of
BmCREC with α-COP in BmN cells (Fig. 3D). Together, these data suggest that BmCREC is associated with the COPI complex. The major function of COPI vesicles is to retrieve escaped ER proteins from the Golgi apparatus back to the ER (2, 33). The association of BmCREC with COPI prompted us to investigate whether BmCREC is retrogradely transported by COPI vesicles. To address this, we first examined whether disruption of the COPI retrograde transport affects the subcellular localization of BmCREC. In normal BmN cells, only a little portion of BmCREC was secreted into the conditioned medium (Fig. 3E). However, knockdown of β'-COP resulted in a dramatic increase of secreted BmCREC protein level and a decrease of intracellular BmCREC protein level (Fig. 3F), with no significant alteration of its mRNA level (Fig. 3G). Moreover, immunofluorescence images showed that knockdown of β'-COP induced punctum accumulation of BmCREC in BmN cells (Fig. 3H and I). However, these enlarged cytoplasmic puncta of BmCREC were not colocalized with the cis-Golgi marker GM130 (supplemental Fig. S4), although COPI knockdown facilitated BmCREC entering the secretory pathway (Fig. 3F), which might lead to an increase of its localization in the Golgi apparatus. Thus, the identity of these BmCREC puncta needs to be further defined. To sum up, these data suggest that COPI deficiency leads to the failure of BmCREC to be transported back to the ER and the subsequent increase of its secretion.

BmCREC Is Recognized by the KDELR via Its C-terminal HDEF Motif—The endogenous BmCREC was localized in the ER lumen, but we found that a large proportion of BmCREC-EGFP (C-terminal EGFP-tagged) was secreted to the extracellular space (Fig. 4, A and B). The association of BmCREC with COPI prompted us to investigate whether BmCREC is retrogradely transported by COPI vesicles. To address this, we first examined whether disruption of the COPI retrograde transport affects the subcellular localization of BmCREC. In normal BmN cells, only a little portion of BmCREC was secreted into the conditioned medium (Fig. 3E). However, knockdown of β'-COP resulted in a dramatic increase of secreted BmCREC protein level and a decrease of intracellular BmCREC protein level (Fig. 3F), with no significant alteration of its mRNA level (Fig. 3G). Moreover, immunofluorescence images showed that knockdown of β'-COP induced punctum accumulation of BmCREC in BmN cells (Fig. 3H and I). However, these enlarged cytoplasmic puncta of BmCREC were not colocalized with the cis-Golgi marker GM130 (supplemental Fig. S4), although COPI knockdown facilitated BmCREC entering the secretory pathway (Fig. 3F), which might lead to an increase of its localization in the Golgi apparatus. Thus, the identity of these BmCREC puncta needs to be further defined. To sum up, these data suggest that COPI deficiency leads to the failure of BmCREC to be transported back to the ER and the subsequent increase of its secretion.

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was detected in the conditioned medium (Fig. 4C), indicating that HDEF motif is crucial for the ER retention of BmCREC.

The HDEF motif resembles the consensus ER retention signal KDEL, which is recognized by the KDELR and confers ER localization of target proteins (37). To study whether KDELR also recognizes the HDEF motif of BmCREC, we coimmunostained BmN cells for BmCREC and KDELR and showed their partial colocalization (Fig. 4D). We further used a FRET assay to confirm their interaction in BmN cells. The fluorescence intensity of CFP-BmCREC (donor) was increased after YFP-KDELR (acceptor) bleaching, and CFP-BmCREC and YFP-KDELR revealed approximately 15% FRET efficiency (supplemental Fig. S5), suggesting a direct interaction of BmCREC with KDELR. Moreover, knockdown of KDELR enhanced the secretion ratio of BmCREC (Fig. 4F). Also, less β'-COP was immunoprecipitated with BmCREC in KDELR knockdown cells than that in control cells (Fig. 4G). Taken together, these data indicate that BmCREC is recognized by the KDELR through its C-terminal HDEF motif and subsequently captured by the COPI vesicles.

**BmCREC Is Required for ER/Golgi Morphology and PSG Tube Expansion**—To investigate the physiological function of BmCREC, we knocked down BmCREC in BmN cells by dsRNA transfection (Fig. 5A). In BmN cells, there are two types of Golgi apparatus distribution patterns. The Golgi apparatus in most cells was dispersed throughout the cytoplasm, whereas in approximately 20% of cells it accumulated around the nucleus (Fig. 5, B and C). Intriguingly, only BmCREC, but not

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**FIGURE 3. BmCREC is retrieved to the ER via COPI transport.** A, SDS-PAGE analysis of the immunoprecipitates (IP) from PSG homogenates with anti-BmCREC antibody. Gel was stained with Coomassie Blue. α-COP was identified by LC-MS/MS. B, Western blotting analysis of GST or GST-Kinesin-1-CBD pulldown precipitates from PSG homogenates. C, immunoprecipitation (IP) analysis of PSG homogenates with anti-BmKinesin-1 or anti-BmCREC antibody. The immunoprecipitates were analyzed by Western blotting with the indicated antibodies. D, coimmunostaining of BmCREC (green) and α-COP (red) in BmN cells. Rectangle areas are magnified. Pearson correlation coefficient R(r) is shown as mean ± S.D., n = 10. Arrows indicate colocalized dots. Scale bar, 10 μm. E, Western blotting analysis of BmCREC in BmN cells. TCL, total cell lysate; CM, conditioned medium. F and G, Western blotting (F) and quantitative real-time PCR (G) analysis of BmN cells treated with control or β'-COP dsRNA. Coomassie blue staining (Co.St.) and tubulin were used as a loading control in F. Data in G are shown as mean ± S.D. (error bars); n.s. means not significant, n = 3. H, control- or β'-COP-dsRNA-treated BmN cells immunostained with anti-BmCREC antibody. Scale bar, 10 μm. I, quantification of the percentage of cells with accumulated BmCREC distribution pattern in H. Data are shown as mean ± S.D.. ***, p < 0.001, n = 20 or 23 fields, ≥20 cells examined per field.
BmCREC# (without HDEF), recovered this phenotype (Fig. 5D and supplemental Fig. S6A). These results imply that HDEF-mediated ER retrieval of BmCREC is responsible for the morphology of the Golgi apparatus.

To investigate further the physiological function of BmCREC in vivo, we knocked down BmCREC by injecting its dsRNA into silkworm larvae (Fig. 5E and supplemental Fig. S6B and C) and examined the subcellular structures in PSG cells by electron microscopy. After BmCREC dsRNA treatment, the ER network appeared indistinct and disordered (Fig. 5F), and the diameter of the ER lumen decreased markedly (Fig. 5G). Moreover, the bubble-like Golgi structures in the BmCREC-knockdown PSG cells became fewer and smaller compared with those in the control PSG cells (Fig. 5F). We measured the numbers and areas of the Golgi structures (>0.1 μm²) and further confirmed that knockdown of BmCREC reduced both the population and the size of Golgi structures in PSG cells (Fig. 5H). Collectively, these data suggest that BmCREC is indispensable for maintaining the morphology of the ER and Golgi apparatus.

A previous report showed that a collapsed endomembrane system causes a defect in PSG tube expansion (4), prompting us to examine whether the PSG with deficient BmCREC is abnormal in tube expansion. Knockdown of BmCREC resulted in significantly narrower PSG glandular lumens (Fig. 5I and supplemental Fig. S6D). Further, we measured the diameter of both the glandular lumen (arrow a in Fig. 5I) and the PSG tube (arrow b in Fig. 5I), and determined the ratio of a to b (a/b) and found that it decreased dramatically after BmCREC knockdown (Fig. 5I), further confirming that BmCREC is required for...
PSG tube expansion. Knockdown of BmCREC also led to reduced cocoon weight after pupation (supplemental Fig. S6E), possibly due to the decreased secretion of fibroin (38). Therefore, we conclude that BmCREC is crucial for maintaining the morphology of the ER and Golgi apparatus, and its deficiency triggers a defect in PSG tube expansion (Fig. 6).

DISCUSSION

Silkworm is one of the best characterized model organisms, due to its suitability for large scale culture, available genome/cDNA database (26, 27), and the use of various well established techniques, such as RNAi (39). Using the PSG, silkworm kinesins were cloned, some cargo molecules of BmKinesin-1 were identified (14), and the roles of the COPI complex in Golgi morphology and PSG tube expansion were unraveled (4), suggesting that the silkworm PSG is a model for studying intracellular trafficking and key developmental processes (14, 40). Here, we identify BmCREC as a novel cargo protein of COPI vesicles driven by BmKinesin-1 in PSG and reveal its role in ER/Golgi morphology maintenance and PSG tube expansion. This study further strengthens that the silkworm PSG would facilitate identification of...
cargo molecules of motor proteins and serve as a model for studying intracellular trafficking.

Our data show that BmCREC is localized mainly in the lumen of the ER, whereas it was previously showed to supercoil DNA according to an in vitro experiment (15, 16). It is possible that another BmCREC isoform produced by alternative splicing may enter the nucleus to supercoil DNA. This is supported by an early report that DmSCF (the BmCREC homologue in *Drosophila*) encodes two isoforms of about 30 and 45 kDa. The 30-kDa isoform is transported into the nucleus to exert its function as a DNA supercoiling factor (17). Therefore, it is possible that there is another alternatively spliced isoform of BmCREC, which enters the nucleus to supercoil DNA in silkworm. However, we did not amplify any more transcripts from the PSG cDNA, possibly due to its relatively limited mRNA expression or the lack of this isoform in PSG cells.

COPI vesicles are reported to be transported by the microtubule-based motor protein Kif5b (41), and COPI deficiency leads to disrupted ER/Golgi morphology (4). However, ER/Golgi morphology remain normal in Kif5b-knock-out mouse cells (42), suggesting that Kif5b is dispensable for the Golgi-to-ER transport. Recently, a report of the role of Kinesin-2 in COPI-mediated recycling (43) reconciled the controversy, indicating that Kif5b and Kinesin-2 might synergistically transport COPI vesicles. Here, we show that BmCREC is transported by the Kinesin-1-driven COPI vesicles, but whether Kinesin-2 is involved needs further investigation.

Previous studies showed that HDEF is served as an ER retention signal (34, 35), but it is believed to be inefficient (36). Our results indicate that the C-terminal tetrapeptide HDEF of BmCREC is necessary for its ER retention of BmCREC, suggesting that HDEF is sufficient to direct BmCREC to the ER. However, although HDEF resembles the consensus ER retention signal KDEL, it is unclear whether the HDEF motif is also recognized by KDEL or other unknown receptors. Here, we find that BmCREC is associated directly with KDEL, and this association is mediated by the HDEF motif of BmCREC, suggesting that HDEF is also recognized by the KDEL and plays an important role in retrieving proteins back to the ER.

The ER and Golgi apparatus are two major members of the endomembrane system, whose morphology and homeostasis are strictly regulated to guarantee the normal physiology of a cell. Our data show that BmCREC is crucial for maintaining ER/Golgi morphology. BmCREC belongs to the CREC protein family and contains seven EF-hand domains that usually bind calcium ions (18). In mammalian cells, CREC family members were reported to regulate calcium homeostasis by various pathways such as SERCA2 and SPCA1 (44, 45). Previous reports showed that an increase in cytosolic calcium ion concentration may induce Golgi fragmentation and vesiculation (46) as well as change in ER morphology (47). Also, the ER network formation is reported to be correlated with rapid ER calcium efflux (48). Therefore, it is possible that deficiency of ER resident BmCREC induces a disorder of calcium homeostasis, which results in disrupted ER/Golgi morphology. Nonetheless, all of these hypotheses require further investigation and verification.

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