Regulation of Mac-2BP secretion is mediated by its \( N \)-glycan binding to ERGIC-53

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The leguminous-type (L-type) lectin ER-Golgi intermediate compartment (ERGIC)-53, a homo-oligomeric endoplasmic reticulum (ER)-Golgi recycling protein, functions as a transport receptor for newly synthesized glycoproteins in the early secretory pathway. Although a limited subset of cargo glycoproteins transported by ERGIC-53, such as the coagulation factors V and VIII, cathepsin C and Z and \( \alpha \)-antitrypsin, has been identified, the exact role of the \( N \)-glycan binding of ERGIC-53 in the transport of secretory glycoproteins for ER exit has yet to be clarified.

By screening a cDNA library isolated from HepG2 cells via a green fluorescent protein fragment complementation assay, we assessed several candidate luminal ERGIC-53-interacting partners and identified Mac-2 binding protein (Mac-2BP) as a novel ERGIC-53-transported cargo glycoprotein. Using an \( N \)-glycan-binding-deficient mutant of ERGIC-53 (N156A) or treatment with \( N \)-glycosylation processing inhibitors, as well as the introduction of the ER-mis-targeting mutant (KKAA), we demonstrated that high-mannose-type \( N \)-glycans to malectin and calnexin activates the quality control of nascent protein synthesis, which facilitates the correct folding of the nascent polypeptide chain (Ware et al. 1995; Chen et al. 2011). \( N \)-Glycans on correctly folded polypeptides are then modified by ER-\( \alpha \)1,2-mannosidase I. Mannose trimming by ER-\( \alpha \)-mannosidase I generates the B isomer of Man\(_9\)GlcNAc\(_2\) \( N \)-glycans (M8B), which are recognized by another ER-resident lectin, ERGIC-53. As a member of the L-type lectin family, ERGIC-53 functions as a cargo receptor during the early secretion of nascent proteins and mediates the ER-Golgi transport of nascent proteins into the Golgi for further maturation, which is indispensable for final secretion.

The type I transmembrane protein ERGIC-53 was initially defined as a marker protein that predominantly localizes to the ER-Golgi intermediate compartment (ERGIC; Schweizer et al. 1988), and constitutively cycles between the ER and the Golgi (Hauri et al. 2000). The luminal portion of ERGIC-53 is composed of a carbohydrate recognition domain (CRD), which is highly homologous to leguminous lectins. In vivo experiments demonstrated that ERGIC-53 preferentially binds to D-mannose in a divalent cation-dependent manner (Pimpam et al. 1991; Arar et al. 1995). Recently, it was clarified that high-mannose-type \( N \)-glycans (Kamiya et al. 2008), especially M8B (Kawasaki et al. 2008), are crucial for ERGIC-53 binding. In addition, both pH and Ca\(^{2+}\) concentration regulate the sugar-binding activity of ERGIC-53 (Appenzeller-Herzog et al. 2004). Newly synthesized monomeric ERGIC-53 dimerizes rapidly and then finally forms a functional homohexamer stabilized by two conserved cysteine residues in the stalk domain (Nufer et al. 2003). Moreover, the C-terminal KKFF sequence induces the cycling of ERGIC-53 between the ER and the Golgi. The association of vesicle coating protein complex II (COPII) with the double phenylalanine (FF) motif induces exit from the ER and the anterograde transport of ERGIC-53 (Kappeler et al. 1997), whereas the interaction of COPI with the double lysine (KK) motif induces the retrograde transport of ERGIC-53, which mediates the ER retention of ERGIC-53 (Tisdale et al. 1997). Based on these characteristics, ERGIC-53 was considered a cargo synthesis. During the translocation of the newly synthesized polypeptides into the ER lumen, nascent polypeptides are tagged with Glc\(_3\)Man\(_8\)GlcNAc\(_2\) \( N \)-glycan (G3M9) on consensus asparagine residues. The G3M9 is subsequently trimmed by glucosidases I and II to generate Glc\(_2\)Man\(_9\)GlcNAc\(_2\) (G2M9) and Glc\(_3\)Man\(_9\)GlcNAc\(_2\) \( N \)-glycans (G1M9), which are captured by malectin and calnexin, respectively. Binding of \( N \)-glycans to malectin and calnexin activates the quality control of nascent protein synthesis, which facilitates the correct folding of the nascent polypeptide chain (Ware et al. 1995; Chen et al. 2011). \( N \)-Glycans on correctly folded polypeptides are then modified by ER-\( \alpha \)1,2-mannosidase I. Mannose trimming by ER-\( \alpha \)-mannosidase I generates the B isomer of Man\(_9\)GlcNAc\(_2\) \( N \)-glycans (M8B), which are recognized by another ER-resident lectin, ERGIC-53. As a member of the L-type lectin family, ERGIC-53 functions as a cargo receptor during the early secretion of nascent proteins and mediates the ER-Golgi transport of nascent proteins into the Golgi for further maturation, which is indispensable for final secretion.

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Introduction

The binding of endoplasmic reticulum (ER)-resident lectins to their \( N \)-glycan ligands plays a pivotal role in glycoprotein

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receptor for the ER-Golgi transport of newly synthesized glycoproteins. Recent research revealed that ERGIC-53 was required for the efficient transport of a limited subset of glycoproteins, including blood coagulation factors V and VIII (Nichols et al. 1998), cathepsin C and Z (Vollenweider et al. 1998; Nyfeler et al. 2006) and α1-antitrypsin (Nyfeler et al. 2008). However, the involvement of the sugar-binding activity of ERGIC-53 and its association with MCFD2 for the transport and secretion of these proteins in the cell remained controversial.

To identify novel cargo glycoproteins transported by ERGIC-53, we performed prominent protein fragment complementation assay (PCA; Michnick et al. 2000)-based screening. Compared with traditional approaches, this strategy is significantly more efficient due to its capacity to monitor low affinity and transient luminal interactions of animal lectins with their cargo glycoproteins in living cells (Nyfeler et al. 2005). Using a previously established green fluorescent protein (GFP)-based PCA screening approach (Magliery et al. 2005) to screen ERGIC-53-interacting partners from a cDNA library isolated from HepG2 cells, we identified Mac-2 binding protein (Mac-2BP) as a novel cargo glycoprotein transported by ERGIC-53. We also show evidence that the N-glycan binding of ERGIC-53 is essential for the ER-Golgi transport of newly synthesized glycoproteins during early secretion.

Results
Screening for ERGIC-53-interacting partner molecules
The ER- and the ERGIC-distributed L-type lectin, ERGIC-53, have been reported to participate in the intracellular transport of a limited set of glycoproteins, such as α1-antitrypsin, cathepsin C and Z and coagulation factors V and VIII. To identify new cargo molecules transported by ERGIC-53, PCA based on a general cDNA library screening strategy was performed (Remy and Michnick 2004). In this strategy, human ERGIC-53 was N-terminally fused with the N-fragment of GFP (nGFP-ERGIC-53) and used as the bait in a screen of luminal partner molecules. In addition, a cDNA library isolated from human HepG2 cells was C-terminally fused with the C-fragment of GFP (cDNA-cGFP) and used as the prey in the screening (Figure 1A). BW5147 cells stably expressing nGFP-ERGIC-53 were established, and the cDNA-cGFP library was co-expressed in these cells using a retrovirus expression system. After several rounds of enrichment for GFP-positive cells using a fluorescence-activated cell sorter, 16.7% of the total cells showing GFP-positive signals were isolated (Figure 1B), and individual clones of the sorted cells were recovered. Based on GFP complementation, cells having relatively strong fluorescence were selected. Finally, genomic DNA was extracted from the cells and the cDNAs encoding the possible candidates of ERGIC-53-interacting partners were amplified by polymerase chain reaction (PCR). Based on nucleotide sequence validation, seven candidate ERGIC-53-interacting partners were identified: Mac-2BP, α2-HS glycoprotein (AHSG), Nodal modulator 2, poramin (transmembrane protein 123; TMEM123), protein disulfide isomerase (PDI) A3 (ERp57), apolipoprotein A-I and glucosidase II β-subunit.
Antibody. FLAG-tagged Mac-2BP significantly co-precipitated with endogenous ERGIC-53, even when ERGIC-53 was not overexpressed (Figure 2B, lane 3). Moreover, the co-expression of ERGIC-53 increased the amount of precipitated Mac-2BP (Figure 2B, lane 4). The results of both PCA and immunoprecipitation demonstrate a direct intracellular interaction between ERGIC-53 and Mac-2BP and identify Mac-2BP as a novel ERGIC-53-interacting partner.

**Table I. Cloned cDNAs encoding ERGIC-53-interacting candidates using PCA**

<table>
<thead>
<tr>
<th>Proteins (human)</th>
<th>Numbers of amino acid residues</th>
<th>Numbers of N-glycosylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cloned cDNA</td>
<td>Full-length cDNA</td>
</tr>
<tr>
<td>Apolipoprotein A-I (APOA1)</td>
<td>219</td>
<td>267</td>
</tr>
<tr>
<td>Apolipoprotein A-I (APOA1)</td>
<td>216</td>
<td>267</td>
</tr>
<tr>
<td>AHSG</td>
<td>280</td>
<td>367</td>
</tr>
<tr>
<td>Mac-2-binding protein (Mac-2BP)</td>
<td>462</td>
<td>585</td>
</tr>
<tr>
<td>Nodal modulator 2 (NOMO2)</td>
<td>370</td>
<td>1222</td>
</tr>
<tr>
<td>Porin (TMEM123)</td>
<td>128</td>
<td>208</td>
</tr>
<tr>
<td>PDI A3 (ERp57)</td>
<td>375</td>
<td>505</td>
</tr>
<tr>
<td>Glucosidase IIβ (GIIB)</td>
<td>247</td>
<td>528</td>
</tr>
<tr>
<td>Glucosidase IIβ (GIIB)*</td>
<td>422</td>
<td>528</td>
</tr>
</tbody>
</table>

All cloned cDNA contained an initiation codon and were fused in frame to cFP cDNA. *The cDNAs with different lengths were obtained.

(Table I). To eliminate false-positive candidates, candidate proteins were confirmed by the co-expression of nGFP-ERGIC-53 and each isolated cDNA-cGFP in HEK293T cells (Figure 1C and D). Mac-2BP, AHSG, TMEM123 and ERp57 exhibited significant complementation with ERGIC-53 compared with MCDF2 (a constitutive ERGIC-53 binding partner) and albumin (an unglycosylated protein) as positive and negative controls, respectively. Both Mac-2BP and AHSG are secretory proteins that are highly glycosylated (Yoshioka et al. 1986; Koths et al. 1993), and the complementation of Mac-2BP by ERGIC-53 was markedly more robust than that of AHSG (Figure 1D). Based on these observations, the interaction of Mac-2BP with ERGIC-53 was investigated in detail.

**Mac-2BP is a novel ERGIC-53-associated cargo glycoprotein**

Because the Mac-2BP cDNA obtained from the screening using the PCA was partial (from 1 to 462 amino acids), full-length cDNA encoding Mac-2BP (585 amino acids) was generated by PCR from HeLa cell cDNAs and subsequently subcloned into a vector in which the C-fragment of GFP was C-terminally fused to the protein (pcDNA3.1-Mac-2BP-cGFP, Figure 1A). The constructed expression vector was then co-transfected with pcDNA3.1-nGFP-ERGIC-53 into HEK293T cells to monitor the interaction between ERGIC-53 and Mac-2BP. Expression of nGFP-ERGIC-53 or Mac-2BP-cGFP alone did not produce a significant GFP signal (Figure 2A). In contrast, when nGFP-ERGIC-53 and Mac-2BP-cGFP were co-expressed, 20.8% of the cells expressed GFP fluorescence (Figure 2A), indicating the intracellular interaction of ERGIC-53 with Mac-2BP. Next, this interaction was validated by immunoprecipitation. FLAG-tagged Mac-2BP was overexpressed in HeLa cells and the lysates were subjected to immunoprecipitation with an anti-ERGIC-53 antibody. FLAG-tagged Mac-2BP significantly co-precipitated with endogenous ERGIC-53, even when ERGIC-53 was not overexpressed (Figure 2B, lane 3). The results of both PCA and immunoprecipitation demonstrate a direct intracellular interaction between ERGIC-53 and Mac-2BP and identify Mac-2BP as a novel ERGIC-53-interacting partner.

**ERGIC-53 interacts with Mac-2BP via high-mannose-type N-glycans**

ERGIC-53 functions as a cargo receptor with a lectin domain that is highly homologous to the leguminous lectins in plants (Itin et al. 1996). Mac-2BP is a secreted glycoprotein containing seven N-glycosylation sites (Koths et al. 1993). Based on this, it was postulated that the sugar-binding activity of ERGIC-53 might be essential for its interaction with Mac-2BP. To verify this, the PCA was performed using an N-glycan-binding-deficient mutant of ERGIC-53, termed N156A (Itin et al. 1996). Expression of the N156A mutant significantly abolished the complementation of nGFP-ERGIC-53 by Mac-2BP cGFP (Figure 3A and B), indicating that the interaction of ERGIC-53 with Mac-2BP is N-glycan-dependent. Next, the complementation of ERGIC-53 by Mac-2BP in the presence or the absence of several N-glycan processing inhibitors was investigated. Treatment with the glucosidase inhibitor, castanospermine (CST), or the Golgi α-mannosidase II inhibitor, swainsonine (SW), preserved the complementation of ERGIC-53 by Mac-2BP; whereas treatment with the ER α-mannosidase I inhibitor, kifunensine (KIF), increased GFP fluorescence intensity by 1.5-fold (Figure 3C and D). KIF inhibits ER α-1,2-mannosidase I (Elbein et al. 1990) and induces the expression of Man₉GlcNAc₂ (M₉) and Man₉GlcNAc₂ (M₉) N-glycans in HeLaS3 cells (Kawasaki et al. 2007). Kawasaki et al. (2008) demonstrated that ERGIC-53 specifically binds to M₉B N-glycans. Thus, the observation that KIF treatment increased the complementation of ERGIC-53 by Mac-2BP suggests that the direct interaction of ERGIC-53 with Mac-2BP occurs via the binding of high-mannose-type glycans.

**N-glycan binding of ERGIC-53 is necessary for the secretion of Mac-2BP**

Because ERGIC-53 was reported to function as a cargo receptor for the ER-Golgi transport of several secretory glycoproteins, the role of the N-glycan binding of ERGIC-53 in the ER-Golgi transport and the secretion of Mac-2BP was investigated. The secreted and intracellular levels of FLAG-tagged Mac-2BP were evaluated in HeLa cells co-transfected with vectors for the expression of ERGIC-53 and FLAG-tagged Mac-2BP. Following transfection, culture supernatants and cell lysates were collected and FLAG-tagged Mac-2BP in each fraction was monitored by immunoblotting with an anti-FLAG antibody. Mac-2BP was detected in the culture supernatant from cells transfected with FLAG-tagged Mac-2BP alone (Figure 4A, lane 2), and the overexpression of ERGIC-53 significantly enhanced the level of secreted Mac-2BP (Figure 4A, lane 3). These observations indicate that endogenous ERGIC-53 mediates the secretion of Mac-2BP, and this secretion was enhanced by the introduction of exogenous ERGIC-53. In addition, Mac-2BP secreted from ERGIC-53 overexpressing cells showed a smaller molecular weight compared with Mac-2BP secreted from mock vector.
transfected cells (Figure 4A, lanes 2 and 3). Mac-2BP from ERGIC-53 overexpressed cells was partially susceptible to endo-β-N-acetylglucosaminidase H (data not shown), indicating that the N-glycan processing of the protein was inadequate. One possible explanation for this is that the overexpression of ERGIC-53 accelerated the ER-Golgi transport of Mac-2BP, which might be too rapid to allow complete processing of the N-glycans on transported Mac-2BP. This inadequate glycan processing may perturb the maturation of Mac-2BP in the Golgi, which was reflected by the decreased molecular weight of the protein. Another possibility is that the prolonged association of Mac-2BP with ERGIC-53 in the Golgi might cause the inadequate sugar processing on Mac-2BP. Since ERGIC-53 is retrogradely transported by forming a COPI vesicle in association with COPI, the retrograde transport of ERGIC-53 might be retarded because of relatively smaller amount of COPI proteins under the condition of ERGIC-53 overexpression in the cell. When the N-glycan binding-deficient ERGIC-53 mutant, N156A, was overexpressed in the cells, the amount of secreted Mac-2BP in the culture supernatant was dramatically diminished to levels even lower than those of Mac-2BP secreted from mock vector-transfected cells (Figure 4A, lane 4). It has been demonstrated that the intermolecular disulfide bonds in the stalk domain of ERGIC-53 promote the homodimer and homohexamer formation and that only hexamerized ERGIC-53 efficiently mediates the ER-Golgi transport of cargo proteins (Nufer et al. 2003). Thus, the inhibition of
Mac-2BP secretion by the overexpression of the N156A mutant may be due to the hexamerization of wild-type ERGIC-53 with the N156A mutant, which resulted in preventing its binding to Mac-2BP. These results also confirmed the important role of the N-glycan binding of ERGIC-53 in Mac-2BP secretion.

Next, several Mac-2BP N-glycan deletion mutants were constructed by substituting Asn with Gln on N-glycosylation sites, and the effect of overexpressing ERGIC-53 on the secretion of these Mac-2BP mutants was evaluated. The partially N-glycosylated (ND2, ND5) or unglycosylated (ND7) mutants of Mac-2BP (Figure 5A) were expressed in HeLa cells along with ERGIC-53. When N-glycans of Mac-2BP were deleted, a slight decrease in the intracellular expression of Mac-2BP was observed (Figure 5C). Immunoprecipitation with an anti-ERGIC-53 antibody demonstrated that the binding of ERGIC-53 to Mac-2BP was significantly impaired by decreasing the number of N-glycans on Mac-2BP (Figure 5B, second panel). However, some binding of ERGIC-53 to Mac-2BP was still observed in cells overexpressing unglycosylated Mac-2BP (ND7), suggesting that other possible interactions in addition to N-glycan binding mediate the binding of ERGIC-53 to Mac-2BP. Moreover, the secretion of these Mac-2BP N-glycan deletion mutants was also examined. The results revealed an inverse correlation between the number of N-glycans that were deleted and the amount of Mac-2BP secreted into the culture supernatant (Figure 5B, third panel). In addition, the decrease in the level of Mac-2BP secretion paralleled the decrease in binding of Mac-2BP to ERGIC-53 (Figure 5D and E), and much more obvious than the decreased expression of...
intracellular Mac-2BP, intracellular ERGIC-53 and separated by SDS indicated. After 24 h, the culture supernatant and cell lysates were collected for immunoblotting with anti-FLAG, anti-ERGIC-53 and anti-β-actin antibodies, respectively. (B) The levels of secreted Mac-2BP were quantified by scanning densitometry. All data are representative of three independent experiments. Data represent the mean ± SD from three independent experiments. **P < 0.01 and *P < 0.05 (Student’s t-test).

intracellular Mac-2BP (Figure 5C). These observations support the conclusion that the secretion of Mac-2BP is dependent on the N-glycan binding of ERGIC-53.

**Secretion of Mac-2BP is dependent on the budding of ERGIC-53-mediated COPII vesicles from the ER**

During early secretion, newly synthesized glycoproteins are packaged into COPII-coated transport vesicles that bud from the rough ER and move to the Golgi (Schechter and Orci 1996). ERGIC-53 mediates the formation of budding vesicles via the binding of C-terminal FF residues to COPII (Kappeler et al. 1997). To confirm that ERGIC-53 induces the secretion of Mac-2BP via COPII vesicles, a vector encoding a distinct ERGIC-53 mutant termed KKAA was constructed by substituting COPII-binding FF residues with double alanines (AA), and the effect of this mutation on the secretion of Mac-2BP was investigated. First, the subcellular localization of KKAA was observed. Wild-type ERGIC-53 has been reported to be distributed around the ER and Golgi and to partially co-localize with the ER marker protein, CNX, and the Golgi marker protein, Golgi-58k (Qin et al. 2012). Immunofluorescence analysis demonstrated that ERGIC-53 in wild-type ERGIC-53-overexpressing cells merged with that of Golgi-58k (Figure 6A). In contrast, KKAA mutant-overexpressing cells displayed a completely different immunostaining pattern in which the KKAA mutant failed to co-localize with Golgi-58k (Figure 6A). This demonstrated that the mutation of the FF residues on ERGIC-53 resulted in the perturbation of ER exit and subsequent retention of ERGIC-53 in the ER, as reported previously (Vollenweider et al. 1998). However, despite the aberrant subcellular localization in cells expressing the KKAA mutant, immunoprecipitation analysis revealed that the binding of ERGIC-53 to Mac-2BP was not abrogated by the mutation of the FF residues (Figure 6B). Therefore, the level of secreted Mac-2BP was examined by immunoblottedting. Overexpression of the KKAA mutant dramatically diminished the amount of secreted Mac-2BP to a level lower than that of mock vector-transfected cells (Figure 6C). These observations indicate that the ERGIC-53-mediated formation of COPII vesicles is essential for the secretion of Mac-2BP.

**MCFD2 is also involved in the secretion of Mac-2BP**

MCFD2 is a small soluble protein that forms an intact complex with ERGIC-53 via two EF-hand motifs on its C terminus (Zheng et al. 2010). The ERGIC-53–MCFD2 complex is indispensable for the ER–Golgi transport and the secretion of coagulation factors V and VIII (Zhang et al. 2005). A defect of either ERGIC-53 or MCFD2 results in the fatal coagulation disorder called combined deficiency of factors V and VIII (F5F8D; Nichols et al. 1998; Zhang et al. 2003). MCFD2 lacks subcellular signals but is localized to the ERGIC via its interaction with ERGIC-53 (Zhang et al. 2003). Of the limited number of cargo glycoproteins transported by ERGIC-53, MCFD2 is required for the transport of coagulation factors V and VIII (Zhang et al. 2005), but is dispensable for the transport of the lysosomal enzymes cathepsin C and Z (Nyfeler et al. 2006). To verify whether or not MCFD2 is involved in the secretion of Mac-2BP, secreted Mac-2BP in culture supernatant was evaluated by immunoblotting in the presence of elevated or suppressed MCFD2 expression. Overexpression of MCFD2 significantly enhanced the secretion of Mac-2BP, whereas the knockdown of MCFD2 by specific siRNAs successfully abrogated this increment (Figure 7A). Quantification of intracellular MCFD2 expression (Figure 7B) and secreted Mac-2BP (Figure 7C) indicated that the level of intracellular MCFD2 paralleled that of secreted Mac-2BP. These observations support the conclusion that both ERGIC-53 and MCFD2 are involved in the secretion of Mac-2BP. Next, we evaluated Mac-2BP secretion by the overexpression of ERGIC-53-binding-deficient MCFD2 mutants. ERGIC-53-binding-deficient mutants of MCFD2 were first found from F5F8D patients (Zhang et al. 2003). Investigated by BIAcore analysis, MCFD2 mutants (D129E, I136T) interacted with ERGIC-53 much weaker ($K_{a} = 1.4 \times 10^{4}, 1 \times 10^{5}$ M$^{-1}$) than wild-type MCFD2 ($K_{a} = 1.6 \times 10^{8}$ M$^{-1}$) (Kawasaki et al. 2008). The overexpression of wild-type MCFD2 significantly enhanced the secretion of Mac-2BP, whereas the overexpression of D129E or I136T mutants could not increase Mac-2BP secretion (Figure 7D and E). This observation suggests that the binding of MCFD2 to ERGIC-53 is
essential for the transport of Mac-2BP from the ER to the Golgi, which facilitates the early secretion of Mac-2BP.

Discussion

The fragment PCA has been proven to be a simple approach for studying protein–protein interactions in living cells. The PCA provides features such as the capacity to analyze cytosolic or luminal interactions in living cells and to easily monitor weak and transient protein–protein interactions in vivo (Piehler 2005). Utilizing the yellow fluorescent protein (YFP)-based PCA, α1-antitrypsin was successfully identified as a cargo protein transported by ERGIC-53 (Nyfeler et al. 2008) and VIP36 (Reiterer et al. 2010). In addition to YFP, another fluorescent protein, GFP, has also been utilized for PCA (Remy and Michnick 2004). In this study, we performed the GFP-based PCA to search for novel cargo proteins transported by ERGIC-53. Because human hepatocellular carcinoma HepG2 cells spontaneously synthesize numerous
secretory proteins and express relatively high levels of ERGIC-53, we screened a cDNA library from HepG2 cells using the GFP-based PCA. Compared with the PCA utilized by other research groups (Nyfeler et al. 2008), flow cytometry was used here to perform more efficient and accurate quantification of the GFP fluorescence induced by protein–protein complementation. Most of the candidate proteins obtained from the screening were either secreted or membrane-anchored glycoproteins that were N-glycosylated, though the cDNAs encoding these proteins were partial. This observation implied that the sugar-binding ability of ERGIC-53 plays a key role in the binding of ERGIC-53-interacting partners. Of the candidates obtained from the screening, Mac-2BP and AHSG displayed relatively high complementation with ERGIC-53 and were known to be highly glycosylated secretory proteins that are constitutively expressed in liver cells (Ullrich et al. 1994; Denecke et al. 2003). ERp57 was the only unglycosylated ERGIC-53-interacting partner identified in the screening. It has been postulated that ERGIC-53 might co-localize with this unglycosylated candidate because ERGIC-53 preferentially recognizes correctly folded glycoproteins that have escaped from the calnexin cycle (Appenzeller-Herzog et al. 2005), and ERp57 is a calnexin-associated PDI that regulates the folding of nascent glycoproteins in the ER lumen (Zapun et al. 1998). Intimate co-localization of ERGIC-53 and ERp57 may be a reasonable mechanism for facilitating the quality control and the transport of nascent glycoproteins.

After the screening of candidate ERGIC-53-interacting partners by the PCA, we performed immunoprecipitation assays and identified Mac-2BP as a novel ERGIC-53-transported cargo glycoprotein. Mac-2BP was first purified from the supernatant of human breast cancer cell line, SK-BR-3, and characterized as a 90-kDa Mac-2-binding protein (Koths et al. 1993). Later, Mac-2BP was found to be the novel ligand for galectin-3 that was involved in the cell–cell adhesion during tumor metastasis (Inohara et al. 1996). Immunohistochemical study and gene expression analysis showed that the higher expression of Mac-2BP was observed in various types of human malignant cells (Ullrich et al. 1994). Since elevated serum or tissue levels of Mac-2BP were related to short survival and metastasis in a variety of human cancers (Grassadonia et al. 2004), Mac-2BP was served as a candidate of the prognostic

Fig. 6 ERGIC-53 mediates the secretion of Mac-2BP via the formation of COPII budding vesicles. (A) A vector expressing wild-type ERGIC-53 (WT) or its ER-mis-targeting mutant (KKAA) was transfected into HeLa cells. After 24 h, the cells were fixed and the intracellular distribution of the Golgi marker (green) or ERGIC-53 (red) was visualized by immunostaining with anti-Golgi 58k and anti-ERGIC-53 antibodies, respectively. Nuclei (blue) were stained with 4′,6-diamidino-2-phenylindole and a merged image is shown in the right panel. White scale bars: 10 µm. (B) WT ERGIC-53 or the KKAA mutant (KKAA) was co-expressed with FLAG-tagged Mac-2BP in HeLa cells as indicated. After 24 h, cell lysates and culture supernatants were collected and subjected to immunoprecipitation using an anti-ERGIC-53 antibody. Precipitated Mac-2BP and ERGIC-53 were detected by immunoblotting using an anti-FLAG, anti-ERGIC-53 and anti-β-actin antibodies, respectively.
indicator for cancer diagnosis. Nevertheless, the function of Mac-2BP in cancer has not been understood yet. Here, we clarified that Mac-2BP is one of the major glycoproteins spontaneously synthesized and secreted by human hepatocellular carcinoma HepG2 cells, and ERGIC-53 regulated its early secretion. Because Mac-2BP was specifically expressed in several cancer cells, so mutations in the genes encoding ERGIC-53 or MCFD2 may not affect the physiological function of Mac-2BP under healthy conditions. However, it might be helpful for understanding Mac-2BP function to compare...
the metastatic phenotypes between F5F8D patients and other individuals.

We also demonstrated that ERGIC-53, as a cargo receptor, was necessary for the ER-Golgi transport and the subsequent secretion of Mac-2BP via N-glycan binding. However, some issues remained to be investigated. First, despite of the important role of N-glycan binding in the interaction between ERGIC-53 and Mac-2BP, binding of ERGIC-53 to unglycosylated Mac-2BP was not completely abolished, as revealed by immunoprecipitation (Figure 5B). This observation implied that not only N-glycan binding but also a direct protein–protein interaction may be involved in the association of ERGIC-53 and Mac-2BP. Previous research by other groups has demonstrated that the binding of ERGIC-53 to coagulation factor VIII is mediated by both sugar-binding and protein–protein interactions (Cunningham et al. 2003). Furthermore, it was reported that the recognition of both N-glycan and polypeptide conformation by ERGIC-53 are necessary for capturing procathepsin Z (Appenzeller-Herzog et al. 2005). These observations suggest that the recognition of protein conformation is another requirement for the efficient capture of newly synthesized glycoproteins by ERGIC-53 for ER exit. The dual mechanisms by which ERGIC-53 recognizes its cargo provides a good explanation why ERGIC-53 transports a limited subset of glycoproteins other than all N-glycosylated glycoproteins. Second, the overexpression of ERGIC-53 enhanced the secretion of Mac-2BP, and the alteration of the N-glycan structures on secreted Mac-2BP was observed (Figure 4A). The ER and Golgi provide a constitutive environment for the N-glycan processing of nascent glycoproteins during synthesis and maturation. ER α1,2-mannosidase I catalyzes the partial demannosylation of M9 high-mannose-type N-glycans, which accelerates the ER exit of correctly folded nascent proteins. Golgi α1,2-mannosidase I elicits further demannosylation, which is essential for complex-type N-glycan formation and protein maturation (Moremen et al. 1994). It was demonstrated that the overexpression of ERGIC-53 alters its localization pattern from a broad ER-Golgi distribution to a compact distribution in the Golgi (Qin et al. 2012). Thus, we postulated that the redistribution of ERGIC-53 triggered by its overexpression accelerates the ER-Golgi transport of Mac-2BP and that this accelerated transport might result in the inadequate demannosylation of high-mannose-type N-glycans on Mac-2BP. The observation that endo-β-N-acetylglucosaminidase H partially cleaved N-glycans on secreted Mac-2BP derived from ERGIC-53 overexpressing cells (data not shown) supported this conclusion. Third, we demonstrated that MCFD2 was involved in the regulation of Mac-2BP secretion. However, how this molecule coordinates with ERGIC-53 in the ER-Golgi transport of glycoproteins is still obscure. The EF-hand domain of MCFD2 interacts with the first β-sheet of the CRD on ERGIC-53 to form a stable complex, the formation of which is essential for the normal secretion of coagulation factors V and VIII (Zheng et al. 2010). We previously evaluated the binding of purified ERGIC-53 to N-glycans presented on the cell membrane and demonstrated that the interaction of MCFD2 with ERGIC-53 enhanced the sugar-binding ability of ERGIC-53 (Kawasaki et al. 2008). However, Nishio et al. (2010) performed X-ray crystallographic analysis of the ERGIC-53–MCFD2 complex and proved that the interaction with ERGIC-53 altered the active conformation of MCFD2, which may facilitate the capture of cargo glycoproteins by MCFD2, rather than influence the sugar-binding activity of ERGIC-53. Despite these different conclusions on the role of MCFD2 in ERGIC-53-mediated cargo transport, we confirmed that MCFD2 was involved in the secretion of Mac-2BP by the overexpression of ERGIC-53-binding-deficient mutants of MCFD2 or the knockdown of MCFD2 expression (Figure 7). Interestingly, although the overexpression of MCFD2 alone enhanced the secretion of Mac-2BP, it did not trigger alteration of the N-glycan structures on secreted Mac-2BP (Figure 7A), unlike the significant alteration of N-glycan structures that was observed in wild-type ERGIC-53 overexpressing cells (Figure 4A). It could be speculated that the overexpression of ERGIC-53 facilitated the secretion of Mac-2BP by accelerating the ER-Golgi transport of Mac-2BP, whereas the overexpression of MCFD2 facilitated Mac-2BP secretion by up-regulating the amount of Mac-2BP captured by ERGIC-53 for ER-Golgi transport during the early secretion step. These observations implied a possible role for MCFD2 in facilitating the ERGIC-53-mediated ER-Golgi transport of newly synthesized glycoproteins.

In summary, this study provides evidence that ERGIC-53 mediates the ER-Golgi transport of a novel cargo protein, Mac-2BP, via N-glycan binding. In addition, MCFD2 was shown to be involved in regulating Mac-2BP secretion. The results reveal an N-glycan-dependent cargo receptor function for ERGIC-53 during the early secretion of newly synthesized glycoproteins.

Materials and methods

Cells

HEK293T and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 2 mM glutamine and 25 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid-NaOH (pH 7.4). BW5147 cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO). The monoclonal anti-ERGIC-53 antibody (ER3) was generated and purified as described previously (Qin et al. 2012).

Antibodies

Monoclonal anti-FLAG antibody (M2), cyanine 3 (Cy3)-conjugated polyclonal anti-ERGIC-53 antibody and monoclonal anti-β-actin antibody were purchased from Sigma-Aldrich (St Louis, MO). The monoclonal anti-ERGIC-53 antibody (ER3) was generated and purified as described previously (Qin et al. 2012).

PCR and nucleotide sequencing

PCR was performed using KOD-Plus DNA polymerase (Toyobo, Japan). Oligonucleotide primers were purchased from Sigma Genosys (Woodlands, TX) or Greiner Bio-One (Solingen, Germany). DNA sequence analysis was performed on a PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster, CA).
Screening of the cDNA library of HeLa cells

Human ERGIC-53 cDNA lacking its signal sequence was subcloned into a pMXs expressing vector containing cDNA encoding a GFP N-fragment (pMXs-nGFP-ERGIC-53). In addition, a cDNA library isolated from HeLa cells was amplified using the Superscript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning kit (Invitrogen) and subcloned into a pMXs expression vector containing cDNA encoding a GFP C-fragment (pMXs-cDNA-cGFP; Figure 1A). Using the Plat-E retrovirus transfection system, the pMXs-nGFP-ERGIC-53 vectors were transfected into BW5147 cells. After cells stably expressing nGFP-ERGIC-53 were obtained by selective culture in the presence of gypsy-myc, the pMXs-cDNA-cGFP plasmids were then transfected into the BW5147 cells. After 48 h, the cells were harvested and subjected to flow cytometry. Protein complementation between ERGIC-53 and interacting molecules was evaluated by monitoring the intensity of GFP fluorescence. Cells showing GFP-positive signals were sorted using a FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA) and harvested. The genomic DNAs of sorted cells from a single clone were extracted and genotyped by PCR then validated by nucleotide sequencing. Based on the results from the sequencing analysis, ERGIC-53 interacting candidates were identified.

GFP fragment complementation assay

Each nGFP-ERGIC-53 and Mac-2BP-cGFP (Figure 1A) cDNA was inserted into pcDNA3.1(-)bygms (Invitrogen) and co-transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. cDNAs encoding human MCDF2-cGFP and serum albumin cGFP were used as positive and negative controls, respectively. Transfected cells were cultured in the presence or the absence of CST (1 mM, Wako, Japan), KIF (8.6 µM, Calbiochem, Darmstadt, Germany) or SW (58 µM, Calbiochem). After 24 h, the cells were harvested and subjected to flow cytometry. Binding of ERGIC-53 to Mac-2BP, MCFD2 and serum albumin was evaluated based on the intensity of GFP fluorescence induced by complementation between nGFP-ERGIC-53 and Mac-2BP cGFP, MCFD2 cGFP and serum albumin cGFP, respectively.

Immunoprecipitation

A cDNA encoding human Mac-2BP was amplified by PCR using cDNA from HeLa cells as a template and the following primers:

Forward primer: 5′-TAGAATTCAAGGCCATGACCCCT-3′;
Reverse primer: 5′-ATGGATCCGTCACACTGAGGAG-3′.

cDNA encoding the N-glycosylation-deficient variants of Mac-2BP (ND2, ND5 and ND7), in which the Asn residues in the N-glycosylation site were substituted with Gln (Figure 5A), were generated by the PCR-based mutagenesis of Mac-2BP cDNA using the KOD-Plus Mutagenesis Kit (Toyobo). Each wild-type and mutated Mac-2BP cDNA was ligated into a p3XFLAG-CMV-9 vector (Sigma-Aldrich). HeLa cells were co-transfected with pcDNA3.1(+)–ERGIC-53 and p3XFLAG-CMV9-Mac-2BP, -ND2, -ND5 or -ND7 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

After 24 h, the transfected cells were harvested using a cell scraper and then lysed in a solution of 50 mM Tris–HCl (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/mL leupeptin. The cDNA encoding human MCDF2 was amplified by PCR using U937 cell cDNA as described previously (Kawasaki et al. 2008). The siRNAs for the knockdown of MCDF2 were introduced into pSuper.retro.Puro vector (Oligoengine, Seattle, WA) according to the manufacturer’s protocol. Two kinds of siRNA plasmids for MCDF2 were constructed and used for the experiment.

Anti-ERGIC-53 antibodies were coupled to protein G-Sepharose 4 Fast Flow beads (GE Healthcare, Buckinghamshire, UK) by gentle rotation at 4°C for 1 h. Cell lysates were precleared with protein G-Sepharose 4 Fast Flow beads by gentle rotation at 4°C for 30 min and then subjected to immunoprecipitation with anti-ERGIC-53 antibody-coupled beads for 4 h at 4°C with gentle rotation. The beads were then washed three times with a solution of 50 mM Tris–HCl (pH 7.5), 0.1% (v/v) Triton X-100, 150 mM NaCl, 1 mM CaCl2, 1 mM PMSF and 1 µg/mL leupeptin. The immunoprecipitates were recovered by boiling in a buffer containing 100 mM Tris–HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol and 0.004% (w/v) bromophenol blue for immunoblotting analysis.

Polyacrylamide gel electrophoresis and immunoblotting

Precipitated proteins were separated by SDS–polyacrylamide gel electrophoresis according to the method of Laemmli (Laemmli 1970) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) at 15 V for 45 min using a semi-dry blotting system (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with 20 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, 0.005% Tween-20 (TBS-T) and 3% bovine serum albumin for 1 h, and then incubated with anti-FLAG or anti-ERGIC-53 antibodies for 1 h at 25°C followed by washing three times with TBS-T for 15 min each. Next, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma-Aldrich) or donkey anti-rabbit antibody (Sigma-Aldrich) for 1 h at 25°C, followed by washing three times for 15 min each with TBS-T. Finally, the membrane was processed using enhanced chemiluminescence (GE Healthcare) and exposed to ECL films (GE Healthcare).

Immunocytochemistry

HeLa cells were cultured in 8-well culture slides (BD Biosciences) coated with 20 µg/mL fibronectin. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min, washed three times with PBS and permeabilized with 0.1% Triton X-100/PBS for 60 min. After blocking with 5% goat serum (Sigma-Aldrich) for 60 min, the cells were incubated with 5 µg/mL of Cy3-conjugated anti-ERGIC-53 antibody (Sigma-Aldrich). For staining of the Golgi apparatus, the cells were incubated with 5 µg/mL of anti-Golgi 58k protein antibody (Sigma-Aldrich) followed by incubation with 5 µg/mL of Alexa Fluor 488-labeled anti-mouse IgG antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole. The coverslips were mounted in
immersion oil (Leica Microsystems, Wetzlar, Germany) and the cells were observed under a confocal laser scanning microscope LSM510 (Carl Zeiss, Goettingen, Germany) with LSM image browser software (Carl Zeiss).

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Conflict of interest
None declared.

Abbreviations
AA, double alamines; AHSG, α2-HS glycoprotein; COP, coating protein complex; CRD, carbohydrate recognition domain; CST, castanospermine; Cy3, cyanine 3; ER, endoplasmic reticulum; ERGIC, ER and Golgi intermediate compartment; FBS, fetal bovine serum; FF, double phenylalanine; FR, endoplasmic reticulum; ERGIC, ER and Golgi intermediate compartment; FBS, fetal bovine serum; FF, double phenylalanine; GFP, green fluorescent protein; KIF, kifunensine; KK, double lysine; L-type, leguminous-type; Mac-2BP, Mac-2 binding protein; PCA, protein fragment complementation assay; PCR, polymerase chain reaction; PDI, protein disulfide isomerase; SW, swainsone; YFP, yellow fluorescent protein.

References


