Stable interaction of the cargo receptor VIP36 with molecular chaperone BiP

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VIP36 is an intracellular lectin that cycles between the endoplasmic reticulum (ER) and the Golgi apparatus, and is thought to act as a cargo receptor in the transport and sorting of glycoproteins. Here we sought to identify the proteins that interact with VIP36 during the quality control of secretory proteins. VIP36 was crosslinked and immunoprecipitated from HEK293 cells that expressed Myc-tagged VIP36. An ∼80 kDa protein coprecipitated with VIP36 and LC/MS/MS analysis revealed it to be immunoglobulin-binding protein (BiP), a major protein of the Hsp70 chaperone family. A VIP36 mutant with defective lectin activity was also proficient for the coimmunoprecipitation of an equivalent amount of BiP, indicating that the interaction between VIP36 and BiP was carbohydrate-independent. Immunoelectron microscopy experiment demonstrated that the interaction between VIP36 and BiP occurred in the ER. However, the VIP36 coprecipitated with BiP was resistant to endo β-N-acetylglucosaminidase H treatment. A pulse-chase experiment revealed that the amount of BiP interacting with VIP36 did not change over more than 2 h. These results suggest that the interaction of VIP36 and BiP is not due to chaperone–substrate complex. Surface plasmon resonance analysis using recombinant proteins confirmed these binding characteristics of VIP36 and BiP in vitro. The interaction between recombinant soluble VIP36 and BiP is dependent on divalent cations but not on ATP. This mode of interaction is also different from that observed between BiP and its chaperone substrates. These observations suggest a new role for VIP36 in the quality control of secretory proteins.

Keywords: BiP/cargo receptor/chaperone/interaction/VIP36

Introduction

Secretory proteins are cotranslationally translocated into the lumen of the ER, where they interact with ER-resident chaperones such as the immunoglobulin-binding protein (BiP), calnexin, and/or calreticulin. Only secretory proteins that fold correctly are transported through the Golgi apparatus to their final destinations. Several proteins are known to be transported by specific receptors. Such receptors may include the membrane proteins ERGIC-53, the p24 family, and Erv29p, which cycle between the ER and the Golgi apparatus (Nichols et al. 1998; Appenzeller et al. 1999; Muniz et al. 2000; Belden et al. 2001). ERGIC-53 bears homology to leguminous lectins and binds to mannose (Itin et al. 1996) and is therefore proposed to recognize the high mannose-type oligosaccharides attached to proteins and to transport these glycoproteins from the ER to the Golgi apparatus. Indeed, the lack of ERGIC-53 impairs the secretion of the procathepsin C and blood coagulation factors V (FV) and VIII (FVIII) glycoproteins (Nichols et al. 1998; Vollenweider et al. 1998). Chemical cross-linking studies have revealed that ERGIC-53 interacts with procathepsin Z in a mannose- and calcium-dependent manner (Appenzeller et al. 1999; Appenzeller-Herzog et al. 2005). However, ERGIC-53 and its mutant, which is unable to bind to mannose, both coimmunoprecipitate with FVIII, and treatment with tunicamycin does not reduce the interaction between ERGIC-53 and FVIII (Cunningham et al. 2003), which indicates that protein–protein interactions also contribute to this interaction. It is therefore possible that ERGIC-53 also acts as a molecular chaperone in addition to transporting glycoproteins.

The vesicular integral membrane protein VIP36 was originally identified as a component of apical post-Golgi vesicles in polarized Madin-Darby canine kidney cells (Fiedler et al. 1994). VIP36 shares significant homology with leguminous lectins as well as with ERGIC-53. Its ability to recognize high-mannose type glycans (Hara-Kuge et al. 1999; Kamiya et al. 2005) and its broad localization from the ER to the cis-Golgi (Fulkrug et al. 1999; Shimada et al. 2003a, 2003b) indicates that VIP36 also functions as a cargo receptor that facilitates the transport of various glycoproteins. In this study, we sought to identify the proteins that associate with VIP36. One of these was found to be BiP, an Hsp70 homologue, and the interaction between VIP36 and BiP occurred in the ER constitutively.

Results

BiP coimmunoprecipitates with VIP36

To search for VIP36-associated proteins, HEK293 cells transfected with a vector expressing Myc-tagged VIP36 were metabolically labeled for 3 h with [35S]methionine/cysteine, followed by immunoprecipitation with an anti-Myc antibody. The immunoprecipitated proteins were separated by SDS-PAGE and detected by autoradiography. As shown in Figure 1A, Myc-tagged VIP36 was copurified with a cellular protein that had a relative molecular mass of ∼80 kDa. This protein was absent from control immunoprecipitation reactions carried out using mock-transfected HEK293 cells.

The luminal region of VIP36 has a mannose-specific lectin domain (Hara-Kuge et al. 1999; Kamiya et al. 2005) that is structurally homologous to plant leguminous lectin. The key amino
agrees well with predicted molecular weight of BiP (78 kDa). kDa molecular weight of the protein estimated by SDS-PAGE analysis identified that the protein was BiP. Notably, the and analyzed by tandem mass spectrometry (LC/MS/MS). This fragments were separated by reverse-phase liquid chromatography and analyzed by tandem mass spectrometry (LC/MS/MS). This analysis identified that the protein was BiP. Notably, the ∼80 kDa molecular weight of the protein estimated by SDS-PAGE agrees well with predicted molecular weight of BiP (78 kDa).

To prepare a large amount of the ∼80 kDa protein, we performed an immunoprecipitation experiment on a large scale using HEK293 cells that stably express Myc-tagged VIP36. The protein thus obtained was treated with trypsin and the fragments were separated by reverse-phase liquid chromatography and analyzed by tandem mass spectrometry (LC/MS/MS). This analysis identified that the protein was BiP. Notably, the ∼80 kDa molecular weight of the protein estimated by SDS-PAGE agrees well with predicted molecular weight of BiP (78 kDa).

To confirm that the protein coprecipitating with VIP36 was BiP, the precipitated proteins were subjected to Western blotting using an anti-BiP antibody. HEK293 cells that stably expressed Myc-tagged VIP36 were lysed after having been exposed to the cross-linker DSP, which can penetrate cell membranes. The lysates were then incubated with an anti-Myc antibody and protein-G beads. To release any denatured proteins that may interact with BiP due to the lysis of the cells, we further incubated the immunocomplexes with 1 mM ATP after washing the beads. The coprecipitated proteins were blotted and stained with the anti-BiP antibody. BiP coprecipitated with Myc-tagged VIP36, whereas the BiP band was not detected when HEK293 cells were stably transfectected with the mock vector (Figure 1B). Next, we transiently expressed 3× FLAG-tagged BiP in HEK293 cells that stably expressed Myc-tagged VIP36. After treating the cells with DSP, we subjected the lysates to immunoprecipitation with an anti-FLAG antibody. The coprecipitated proteins were separated on a SDS-polyacrylamide gel, blotted onto a membrane, and detected with an anti-VIP36 antibody. As shown in Figure 1C, the 36 kDa Myc-tagged VIP36 protein was detected in the lysates of these cells (Figure 1C) but not in the lysates of cells that had been cotransfected with mock vector lacking the 3× FLAG-tagged BiP cDNA. To confirm that VIP36 interacts with BiP under the physiological condition, untransfected HEK293 cells were used for immunoprecipitation assay. After treatment with DSP, immunoprecipitation was performed with anti-VIP36, and the coprecipitated proteins were blotted and stained with the anti-BiP antibody. BiP was coprecipitated with VIP36 from untransfected cell lysates (Figure 1D), indicating that VIP36 interacts with BiP under physiological condition.

**Interaction between VIP36 and BiP is stable over time**

BiP is a molecular chaperone that transiently interacts with unfolded stretches of nascent polypeptides for as long as these regions remain unfolded. If VIP36 is a chaperone substrate of BiP, BiP should gradually release newly synthesized VIP36 after its initial binding. To test if this is the case, we performed pulse-chase label experiments in the presence of cycloheximide, which prevents new protein synthesis, and then immunoprecipitated the VIP36–BiP complexes. The ratio of BiP to VIP36 did not

**BiP interacts with an endo H-resistant form of VIP36**

VIP36 has a N-glycosylation site and is resistant to digestion with endo β-N-acetylglucosaminidase H (endo H) at steady state (Fiedler et al. 1996). A kinetic analysis of the maturation of the N-linked sugar chains showed that more than half of the newly synthesized VIP36 was already endo H-resistant after 60 min (Fullekrug et al. 1999). To know which form of VIP36 interacts with BiP under the physiological condition, we transiently expressed 3× FLAG-tagged BiP in HEK293 cells and stably transfected with the mock vector (Figure 1B). Next, we transiently expressed 3× FLAG-tagged BiP in HEK293 cells that stably expressed Myc-tagged VIP36. After treating the cells with DSP, we subjected the lysates to immunoprecipitation with an anti-FLAG antibody. The coprecipitated proteins were separated on a SDS-polyacrylamide gel, blotted onto a membrane, and detected with an anti-VIP36 antibody. As shown in Figure 1C, the 36 kDa Myc-tagged VIP36 protein was detected in the lysates of these cells (Figure 1C) but not in the lysates of cells that had been cotransfected with mock vector lacking the 3× FLAG-tagged BiP cDNA. To confirm that VIP36 interacts with BiP under the physiological condition, untransfected HEK293 cells were used for immunoprecipitation assay. After treatment with DSP, immunoprecipitation was performed with anti-VIP36, and the coprecipitated proteins were blotted and stained with the anti-BiP antibody. BiP was coprecipitated with VIP36 from untransfected cell lysates (Figure 1D), indicating that VIP36 interacts with BiP under physiological condition.

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Stable interaction between VIP36 and BiP

Fig. 2. BiP interacts with an endo H-resistant form of VIP36. (A) HEK293 cells stably expressing Myc-tagged VIP36 were treated with α1,2-mannosidase I inhibitor, DMJ. After immunoprecipitation with an anti-Myc antibody, the immunoprecipitants were incubated with endo H and subjected to Western blot analysis with an anti-VIP36 antibody. (B) Transfection and immunoprecipitation were performed in the same manner as Figure 1C. The immunoprecipitants were incubated with endo H and subjected to Western blot analysis with an anti-VIP36 antibody.

Table I. The number of VIP36 and BiP particles visible by immunoelectron microscopy

<table>
<thead>
<tr>
<th>ER</th>
<th>Golgi apparatus</th>
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<tr>
<td>VIP36</td>
<td>BiP</td>
</tr>
<tr>
<td>39.8 ± 26.0</td>
<td>28.6 ± 15.3</td>
</tr>
<tr>
<td>28.8 ± 13.1</td>
<td>5.1 ± 4.2</td>
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Gold particles (10-nm; VIP36, 15-nm; BiP) localized in the ER or in the Golgi apparatus were counted. The average and SD in 1 μm² area of 50 pictures in 293 cells expressing Myc-VIP36 were calculated.

change over more than 2 h (Figure 3), which suggests that BiP binds to VIP36 stably over long periods of time.

Colocalization of VIP36 and BiP in the ER

We used electron microscopy to investigate the localization of VIP36 and BiP in 293 cells expressing Myc-tagged VIP36. The number of gold particles of VIP36 and BiP per μm² is shown in Table I, and the representative electron micrograph is shown in Figure 4. These data reveal that the 10-nm gold particle-labeled VIP36 and the 15-nm particle-labeled BiP mainly colocalized in the ER. Although VIP36 was also detected in the Golgi apparatus, colocalization with BiP was not observed. This indicates that VIP36 localizes from the ER to the Golgi apparatus and interacts with BiP particularly in the ER.

In vitro binding of BiP to sVIP36 requires divalent cations

We used surface plasmon resonance (SPR) to analyze the in vitro interaction between recombinant sVIP36 and BiP prepared in Escherichia coli cells. Recombinant biotinylated VIP36 was immobilized on an SPR sensor chip and recombinant BiP was passed over it. In the presence of 20 mM HEPES (pH 7.4), 50 mM KCl, and 2 mM Mg2+, BiP bound to the VIP36-coated surface with high affinity (\( K_d = 3.62 \times 10^{-8} \) M, TableII) but did not bind to a control surface immobilized with Nkrp1c (Figure 5). Since recombinant proteins expressed in E. coli lack glycosylation, the interaction between recombinant sVIP36 and BiP

Table II. Kinetic constants for the interaction between VIP36 and BiP, and between P15 and BiP measured by SPR. VIP36 or P15 were immobilized onto SPR chips

<table>
<thead>
<tr>
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<th>( k_{on} ) (M⁻¹ s⁻¹)</th>
<th>( k_{off} ) (s⁻¹)</th>
<th>( K_d ) (M)</th>
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<tbody>
<tr>
<td>P15 and BiP</td>
<td>ATP (-)</td>
<td>5.95 × 10⁴</td>
<td>3.90 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>ATP (+)</td>
<td>2.30 × 10⁵</td>
<td>0.102</td>
</tr>
<tr>
<td>VIP36 and BiP</td>
<td>ATP (-)</td>
<td>4.12 × 10⁴</td>
<td>1.49 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>ATP (+)</td>
<td>1.44 × 10⁵</td>
<td>2.59 × 10⁻³</td>
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BiP was injected and the binding was followed over times as the change in RU. The \( k_{on} \) and \( k_{off} \) were determined from the association and dissociation phases, respectively. The apparent \( K_d \) is calculated from the ratio of \( k_{off}/k_{on} \).
Fig. 4. Colocalization of VIP36 and BiP in the ER in HEK293 cells expressing VIP36. (A) Immunoelectron micrograph of VIP36 (10-nm gold particles) and BiP (15-nm gold particles) in HEK293 cells showed colocalization of VIP36 and BiP in the ER, but not in the Golgi apparatus. Magnified images in the Golgi region (B) and the ER region (C) were shown. VIP36 and BiP were indicated in black arrows and white arrowheads, respectively. Bar = 0.1 µm. Ce, centriole.

Fig. 5. BiP specifically binds to sVIP36. Biotinylated VIP36 (or biotinylated Nkrp1c as a negative control) was immobilized on the surface of an SPR chip via streptavidin. BiP was passed over the chip at time zero (arrow), and the BiP solution was replaced by buffer at the time indicated by the second arrow.

supports our earlier observation that the interaction does not depend on the carbohydrate moieties of these proteins.

We next examined the effect of divalent cations on the interaction between sVIP36 and BiP. As divalent cations have not been reported to be required for the binding of BiP and chaperone substrates, we chose the P15 peptide, which mimics a BiP chaperone substrate (Misselwitz et al. 1998), as a negative control.

Peptide P15 was immobilized on a sensor chip and recombinant BiP was passed over it. The binding of BiP to peptide P15 was unchanged in the presence or absence of 2 mM Mg$^{2+}$ (Figure 6A). In contrast, the binding of sVIP36 to BiP was substantially reduced in the absence of divalent cations. Ca$^{2+}$ or Mn$^{2+}$ at 2 mM strengthened the binding to the same extent as Mg$^{2+}$ (Figure 6B).

**ATP does not affect the interaction between VIP36 and BiP**

It has been reported that BiP releases its chaperone substrate in the presence of ATP (Wei et al. 1995; Bukau et al. 1998), and that the binding of BiP to peptide P15 is markedly reduced in the presence of 1 mM ATP (Misselwitz et al. 1998). This was confirmed by SPR analysis using recombinant BiP and peptide P15 (Figure 7A), as the $K_d$ was increased by three orders of magnitude in the presence of ATP (Table II). Next, we examined the effect of ATP on the interaction between VIP36 and BiP. As shown in Figure 7B, the interaction between VIP36 and BiP is ATP-independent since the $K_d$ values of the interaction between VIP36 and BiP were almost the same in the presence or absence of ATP (Table II). Moreover, while the on and off rates of the binding of BiP to both VIP36 and peptide P15 were higher in the presence of ATP, the binding affinity of BiP and VIP36 was not altered by ATP, whereas ATP reduced the affinity between BiP and peptide P15 (Table II).
Stable interaction between VIP36 and BiP

Fig. 6. The interaction between VIP36 and BiP is dependent on divalent cations. (A) Peptide P15, which mimics BiP substrates, was immobilized on the surface of an SPR chip via its C-terminal cysteine, and BiP was passed over it in the presence or absence of Mg\(^{2+}\). (B) BiP was passed over a chip bearing immobilized VIP36 in the presence or absence of divalent cations.

Fig. 7. The interaction between VIP36 and BiP is not affected by ATP. BiP was passed over the chip bearing immobilized peptide P15 (A) or sVIP36 (B) in the presence or absence of 1 mM ATP.

Discussion

The ER has a quality control system for proofreading newly synthesized proteins in eukaryotic cells. If a protein does not attain a correctly folded conformation, it is not transported to its final destination and is instead degraded. The molecular chaperones and folding sensors that are involved in these processes often have the dual functions of assisting the folding process and dispatching improperly folded proteins for destruction in a process called ER-associated degradation (McCracken et al. 1996). Calnexin, calreticulin, UDP-glucose glucosyltransferase (UGGT), protein disulfide isomerase (PDI), ERp57, BiP, GRP94, and ER-degradation-enhancing α1,2-mannosidase-like protein (EDEM) all participate in this quality control process and sometimes function together in complexes (Kleizen et al. 2004). For example, calnexin, calreticulin, and ERp57 form a complex (Corbett et al. 1999; Oliver et al. 1999), as do calnexin and EDEM (Molinari et al. 2003; Oda et al. 2003). These proteins have common inducible characteristics under several ER-stress conditions.

Cargo receptors participate in secondary quality control, which refers to various selective mechanisms that regulate the export of individual protein species or protein families in the secretory pathway (Ellgaard et al. 2003). These cargo receptors are considered to participate not only in the export of folded proteins from the ER but also in the retrieval of misfolded proteins from the Golgi to the ER (Ellgaard et al. 1999; Arvan et al. 2002). ERGIC-53 and VIP36 are induced by ER-stress conditions (Nyfeler et al. 2003), and ERGIC-53 forms a complex with MCFD2, which is also induced by ER-stress conditions (Spatuzza et al. 2004), but VIP36 has not been found to be a complex so far. To investigate the possibility that VIP36 also forms a complex with other molecules, we searched for proteins that interact with the cargo receptor VIP36.

To identify the proteins that associate with VIP36, cell lysates were prepared from HEK293 cells expressing Myc-tagged VIP36 and an anti-Myc antibody was used for immunoprecipitation. LC/MS/MS analysis of the trypsin-digested fragments of the coprecipitated ∼80 kDa protein revealed that the molecular chaperone BiP is a VIP36-interacting protein. Coprecipitation of VIP36 with BiP was also confirmed from untransfected cell lysates, indicating that VIP36 interacts with BiP under the physiological condition. BiP is a major protein of the Hsp70 family. It mainly localizes in the ER (Haas 1994), where it binds to various nascent and newly synthesized proteins and assists their folding as a chaperone. BiP consists of an ATPase domain, a peptide-binding pocket, and a lid domain covering the peptide-binding pocket (Bukau et al. 1998). ATP/ADP bound to the ATPase domain regulates reversible peptide binding, and hydrolysis of the ATP bound to BiP enhances the binding of peptide chains in its immediate vicinity. There was the possibility that BiP associated with VIP36 still in the process of folding. However, coimmunoprecipitated VIP36 with BiP was resistant to endo H, strongly indicating that the interaction is not the chaperone–substrate association. Several kinds of proteins, including thyroglobulin (Kim et al. 1992), human immunodeficiency virus type 1 envelope glycoprotein gp160 (Knarr et al. 1999), and blood coagulation factor VIII (Dorner et al. 1987) have been reported to be BiP chaperone substrates. These proteins associate with BiP in a time-limited manner with association half-lives of about 10, 30, and 60 min, respectively. To test whether newly synthesized...
VIP36 also binds transiently to BiP, we performed a time-course pulse-chased experiment. In contrast to what is seen with BiP chaperone substrates, the amount of BiP that coprecipitated with Myc-tagged VIP36 did not change over 120 min, which indicates that VIP36 associates with BiP in a stable manner. It is reported that J proteins also associate with BiP constitutively and function as cochaperones to enhance the ATPase activity of BiP (Bukau et al. 1998). However, the ATPase activity of BiP did not change in the presence or absence of VIP36 (data not shown), which indicates that VIP36 does not function as a cochaperone.

We prepared recombinant BiP and soluble VIP36 proteins in E. coli cells and examined their direct interaction by SPR analysis. The binding of VIP36 to BiP differs significantly from the binding between BiP and its unfolded ligands with regard to the effect of ADP/ATP and the requirement for divalent cations. With respect to the effect of ADP/ATP, our SPR binding analysis showed that unfolded ligands bind to BiP faster and are also released faster in the presence of ATP than in the presence of ADP: $K_d$ values between BiP and p15 peptide in the presence of ATP and ADP were $4.41 \times 10^{-8}$ and $6.6 \times 10^{-8}$ M, respectively (Table II). This is in good agreement with previous reports that suggest the binding of ATP to BiP opens up its binding site for unfolded ligands so that they can rapidly enter and exit the binding pocket, resulting in a high $K_d$ (Palleros et al. 1993; Schmid et al. 1994). When ADP is present, this peptide exchange is much slower and the affinity is higher. In contrast, the $K_d$ for the interaction between VIP36 and BiP did not change in the presence of ATP and ADP ($1.13 \times 10^{-8}$ and $3.62 \times 10^{-8}$ M, respectively), which indicates that BiP interacts with VIP36 in a stable manner and probably not via a peptide-binding pocket. With regard to the requirement for divalent cations, we showed that the binding between BiP and VIP36 is dependent on the presence of $Ca^{2+}$, $Mg^{2+}$, or $Mn^{2+}$ (Figure 6B). In contrast, BiP binds to its chaperone substrates without the need for divalent cations (Figure 6A) (Palleros et al. 1991). These two findings strongly indicate that VIP36 does not interact with BiP in the same way as it binds to unfolded ligands.

VIP36 has sugar-binding activity that is specific for N-linked high mannose-type sugar chains, especially higher molecular weight chains (Hara-Kuge et al. 1999; Kamiya et al. 2005). Some target glycoproteins may interact with VIP36 in carbohydrate-binding manner in the cells. However, major bands considered to be target glycoproteins were not detected (Figure 1A). If many kinds of target glycoproteins are coprecipitated with VIP36, the amount of each glycoprotein may be much smaller than BiP. This may be explained also by the fact that the $K_d$ values of VIP36 are three or four orders of magnitude lower for sugar ligands ($\sim 10^4$ M$^{-1}$, Kamiya et al. 2005) than BiP.

VIP36 cycles between the ER and the Golgi apparatus (Fullekrug et al. 1999) and has been suggested to be involved in the retrieval of misfolded proteins (Hauri et al. 2000). In our experiment, VIP36 coimmunoprecipitated with BiP was found to have endo H-resistant sugar chains. This indicates that VIP36 associated with BiP had already transported to the medial Golgi, in which sugar moieties on VIP36 were processed from endo H-sensitive high mannose type to endo H-resistant complex-type glycans. Immunoelectron microscopy experiment using gold-labeled anti-VIP36 and anti-BiP demonstrated that colocalization of VIP36 and BiP occurred especially in the ER but not in the Golgi (Figure 4). These observations may indicate that VIP36 interacted with BiP in the ER is a cargo receptor that has been retrotransported with misfolded glycoproteins to effectively deliver such proteins to ER-resident molecular chaperone BiP.

**Materials and methods**

**Recombinant DNAs**

To express VIP36 in eukaryotic cells, a human VIP36 cDNA lacking a signal sequence was cloned into pRC/CMV (Invitrogen, Carlsbad, CA), which was modified to include an N-terminal Myc-tag sequence after the CD8 signal sequence. Site-directed mutagenesis was performed by using a QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. To express recombinant VIP36 (sVIP36) in E. coli cells, a cDNA encoding the luminal domain of VIP36 (amino acids 45–322) was cloned into a pET3c vector (Studier et al. 1990) that was modified to include a C-terminal enzymatic biotinylation signal (Schatz 1993). To express BiP in eukaryotic cells, a human BiP cDNA lacking a signal sequence was cloned into p3xFLAG-CMV-8 (Sigma, St. Louis, MO). For E. coli expression of recombinant BiP, a cDNA encoding BiP without its signal sequence was cloned into pET21b (Studier et al. 1990), which contains a hexahistidine tag.

**Protein purification**

sVIP36 was expressed in the BL21(DE3)pLysS strain of E. coli and obtained as inclusion bodies. It was refolded by dilution as described (Matsumoto et al. 2001) and then purified by sequential anion exchange and gel filtration chromatography steps and biotinylated with BirA biotin ligase (Avidity, Denver, CO). BiP was expressed in the BL21(DE3)pLysS strain of E. coli and then purified by sequential Ni$^{2+}$-affinity and anion exchange chromatography steps.

**Cell culture and transfection**

Human embryonic kidney cell line HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 µg/mL penicillin, and 100 µM streptomycin. The cells were transfected by using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

**Metabolic labeling, crosslinking of proteins, and immunoprecipitation**

Prior to the metabolic labeling of the newly synthesized proteins, the cells were washed with labeling medium (methionine/cysteine-free RPMI-1640 medium supplemented with 10% heat-inactivated and dialyzed FCS, 2 mM glutamine, 100 µg/mL penicillin, and 100 U/mL streptomycin) (Sigma). Twenty-four hours after transfection, the cells were metabolically labeled with 100 µCi of $[^{35}S]$methionine/cysteine (Amer sham Biosciences Corp., Piscataway, NJ) for 3 h in labeling medium. The labeled cells were washed with cold PBS and then incubated at 4°C for 30 min with 2 mM diithiothreitol (Sigma) (DSP, Pierce) in PBS. Cells were subsequently washed twice with PBS containing 0.04% (w/v) EDTA before
further processing. For immunoprecipitation, $^{35}$S-labeled cells were lysed by incubation on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and 1 µg/mL leupeptin). The lysates were centrifuged at 12,000 × g for 30 min at 4°C to remove insoluble materials and then incubated with an anti-Myc antibody (9E10) (American Type Culture Collection, Manassas, VA). Immunocomplexes were precipitated with protein G-Sepharose (Amersham Pharmacia Biotech), washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF), and resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The labeled proteins were then visualized with a BAS 5000 system (Fuji Film, Kanagawa, Japan).

**Pulse-chase analysis**

Cells were metabolically labeled as described above. The pulse was ended by adding prewarmed RPMI-1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL cycloheximide. Cycloheximide is an inhibitor of protein translation. After various chase periods of various lengths, the cells were transferred to ice and washed with cold PBS, followed by immunoprecipitation with an anti-Myc antibody. The immunoprecipitated proteins were separated by SDS-PAGE and the gels were exposed to an imaging plate. Quantification of the autoradiograms was carried out with the BAS 5000 system.

**Coimmunoprecipitation assay**

Twenty-four hours after transfection, HEK293 cells were harvested, lysed in lysis buffer, and clarified by centrifugation at 12,000 × g for 30 min at 4°C. After crosslinking of proteins by 2 mM DSP, the soluble supernatant was incubated with either an anti-Myc antibody or a mouse monoclonal anti-FLAG antibody (M2) (Sigma) for 2 h at 4°C. When using untransfected HEK293 cells, the immunoprecipitation was performed with anti-VIP36. The immunocomplexes were then precipitated with protein G-Sepharose and then washed three times with wash buffer containing 1 mM ATP to remove unexpected denatured proteins bound to BiP. The products were resolved with 12.5% SDS-PAGE under reducing conditions and detected by Western blotting with either a rabbit polyclonal anti-BiP antibody (9E10) (American Type Culture Collection, Manassas, VA) or an anti-VIP36 antibody. The anti-VIP36 antibody was raised against synthetic peptides conjugated with (ET-21) (Sigma) or an anti-VIP36 antibody. The anti-VIP36 was immobilized on the chip through its interaction with streptavidin. Biotinylated Nkn1c, an NK receptor, was immobilized on the chip via streptavidin as a control. Peptide P15 (ALLL-SAPRRGAGKKC) was immobilized by using a thiol coupling kit (Biacore International AB) as described previously (Mieselwitz et al. 1998). The experiments were performed at 25°C at a flow rate of 5–10 µL/mL of a running buffer containing 20 mM HEPES (pH 7.4), 50 mM KCl, and the indicated divalent cation (2 mM) or 1 mM ATP. To test the effect of pH on the interaction between sVIP36 and BiP, we used 20 mM MES (pH 7.0, 6.5, or 6.0) containing 50 mM KCl and 2 mM CaCl$_2$ as the running buffer (Grabe et al. 2001). The sensorgrams were analyzed using BlAevaluation 3.0 software (Biacore International AB). Kinetic constants were obtained by fitting curves to a single-site binding model.

**Immunoelectron microscopy**

Cells on a cover glass were fixed by 5% acrolein in 1/15 M phosphate buffer (PB), pH 7.4 for 15 min, washed well with PB for 30 min three times, and then postfixed in 1% osmium tetroxide for 2 h at 4°C and 7% sucrose in PB for 2 h at 4°C. The cells were dehydrated with a graded ethanol series at 0°C, embedded in Lowicryl K4M (Polysciences Inc., Niles, IL) and cured under ultraviolet light for 3 days at −35°C. For quenching any remaining aldehyde, the ultrathin sections were treated with 3% hydrogen peroxide for 10 min at 15°C, washed three times with water and then treated with 0.1% ammonium chloride for 10 min at 15°C. The ultrathin sections were washed thoroughly with PBS, treated with 190% normal goat serum (NGS) (Sigma) in PBS for 1 h at room temperature and then incubated with rabbit anti-VIP36 antibody (0.8 µg/mL) in PBS containing 1% bovine serum albumin (BSA) for 24 h at 4°C. The sections were rinsed thoroughly with PBS, incubated for 1 h at 20°C with 10-nm colloidal gold-labeled anti-rabbit IgG (1:200) (British Biocell International Ltd., London, UK), and then rinsed with PBS followed by water. For double-staining immunoelectron microscopy, the reverse side of the thin section was treated with hydrogen peroxide and ammonium chloride as described above.
and then treated with NGS under the same conditions. We did not stain on the same side with a cocktail of antibodies in order to interferences of antibodies. The section was incubated with mouse anti-BiP/GRP78 antibody (1:500) (BD Biosciences, San Jose, CA) in PBS containing 1% BSA for 24 h at 4°C, washed, and then incubated with 15-nm gold-labeled anti-mouse IgG (British Biocell International Ltd.) for 1 h at 20°C. The sections were stained with uranyl acetate for 5 min and stained with lead citrate for 1 min, then observed by an electron microscope (H7500, Hitachi, Tokyo, Japan).

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**Conflict of interest statement**

None declared.

**Abbreviations**

BiP, immunoglobulin-binding protein; SPR, surface plasmon resonance; VIP36, 36-kDa vesicular integral membrane protein.

**References**


Stable interaction between VIP36 and BiP


