Degradation and Endoplasmic Reticulum Retention of Unassembled α- and β-Subunits of Na,K-ATPase Correlate with Interaction of BiP*  

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Assembly of α- and β-subunits in the endoplasmic reticulum is a prerequisite for the structural and functional maturation of oligomeric P-type ATPases. In Xenopus oocytes, overexpressed, unassembled α- and β-subunits of Xenopus Na,K-ATPase are retained in the endoplasmic reticulum (ER) and are degraded with different kinetics, while unassembled β-subunits of gastric H,K-ATPase leave the ER. In this study, we have investigated the role of the immunoglobulin-binding protein, BiP, in the folding, assembly, and ER retention of ATPase subunits. We determined the primary sequence of Xenopus BiP and used polyclonal antibodies to examine the interaction with BiP of various wild type and mutant α- and β-subunits overexpressed in Xenopus oocytes. Our results show that ER-retained, unassembled Na,K-ATPase β-subunits, but not transport-competent H,K-ATPase β-subunits, efficiently associate with BiP until assembly with α-subunits occurs. Furthermore, the kinetics of BiP interaction with unassembled wild type and mutant Na,K-ATPase β-subunits parallel their respective stability against cellular degradation. Finally, α-subunits that are overexpressed in oocytes and are readily degraded and endogenous oocyte α-subunits that are stably expressed as individual assembly-competent proteins also interact with oocyte or exogenous BiP, and the interaction time correlates with the protein’s stability. These data demonstrate for the first time that BiP might be involved in a long term maturation arrest and/or in the ER quality control of a multimembrane-spanning protein and lend support for a universal chaperone function of BiP.

The biosynthesis and maturation of secretory and integral membrane proteins involves multiple ordered steps: translation and translocation into the ER, co-translational modifications, additional conformational maturation and multimerization, and screening by various “quality control” mechanisms prior to transport to the Golgi apparatus (for review, see Ref. 1). Our model system to investigate these events is the αβ heterodimeric Na,K-ATPase of Xenopus expressed in Xenopus oocytes (for review, see Ref. 2). Part of the appeal of studying the Na,K-ATPase is that the two subunits are structurally and functionally distinct (for a review, see Ref. 3). The multimembrane-spanning α-subunit is the catalytic subunit, hydrolizing ATP and forming a phosphoinintermediate during a complex series of ion translocation events. Most of the ~100-kDa α-subunit is embedded within the membrane or is cytoplasmic. In contrast, the ~45-kDa β-subunit is a type II glycoprotein, which traverses the membrane once, and the large ectodomain is modified by both disulfide bridges and carbohydrate additions.

The Xenopus oocyte has proven to be a particularly useful expression system for the study of the structural and functional maturation of the Na,K-ATPase. The oocyte, like all animal cells, has functioning Na,K-ATPase αβ complexes in its plasma membrane. However, the oocyte also accumulates individual, stable α-subunits in the ER, which can be recruited to the plasma membrane as functional Na,K-ATPase by the sole injection of β-subunit cRNA (4, 5). On the other hand, exogenous α-subunits overexpressed in the oocyte by the injection of α-subunit cRNA without co-expression of β-subunits are rapidly degraded, probably in or close to the ER (6). Controlled proteolysis has shown that the α-subunit reaches its mature conformation only following αβ assembly (4, 7). Like the α-subunit, unassembled Xenopus Na,K-ATPase β-subunit overexpressed in the oocyte is degraded without leaving the ER (6). Both glycosylation and disulfide bond formation are important for β-subunit maturation (8, 9). Finally, the molecular requirements of the assembly sites in the α- and β-subunits have been partially characterized (10–14).

Little is known about the interactions of the Na,K-ATPase subunits with resident ER chaperones involved in protein biosynthesis, folding, and assembly. An abundant ER luminal chaperone is BiP (GRP78), a member of the highly conserved heat-shock 70-kDa family of stress proteins (Hsp70; for review, see Refs. 15 and 16). Like other members of this family, BiP binds ATP and has ATPase activity (17).

BiP activity is thought to be important at multiple stages during the biosynthesis and ER residency of secretory and transmembrane proteins. Genetic and biochemical data suggest that BiP participates in the translocation of nascent polypeptides into the ER lumen (18–22), possibly acting as a translocation motor (for review, see Ref. 23). Furthermore, early interaction of BiP with folding polypeptide chains is

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¶ This abbreviation was used: ER, endoplasmic reticulum; PCR, polymerase chain reaction; GST, glutathione S-transferase; endo-α, endogenous Na,K-ATPase α-subunit.
thought to protect the nascent chain from aggregation and to stabilize incompletely folded polypeptides in a folding or assembly-competent configuration (24). In agreement with this notion is that BiP associates preferentially with peptide stretches containing hydrophobic amino acid residues expected to be exposed only in unfolded, misfolded, or unassembled polypeptides (25). According to this prediction, BiP would interact transiently with any polypeptide that is transferred to the ER lumen. Typically, however, interaction of BiP has mainly been documented for wild type and mutant viral proteins (for review, see Ref. 26) and for soluble and type I or II subunits of oligomeric proteins that are overexpressed in cells without their partners (27–30). In these cases, BiP remains associated for a prolonged time, since the protein cannot reach without their partners (27–30). In these cases, BiP remains associated for a prolonged time, since the protein cannot reach

In this study, we determined the primary sequence of Xenopus BiP, and used a polyclonal BiP antibody to examine the association of BiP with various wild type and mutant β- and α-subunits of Na,K-ATPase overexpressed in Xenopus oocytes as well as with the oocyte’s stable α-subunits, which are expressed as individual subunits. We document that overexpressed α-subunits associate with BiP with an interaction time corresponding to the stability of the various β-proteins. In addition, by studying the interaction of oocyte or exogenous wild type and mutant BiP with α-subunits, we demonstrate for the first time that also large multimembrane-spanning proteins efficiently interact with BiP.

MATERIALS AND METHODS

Xenopus BiP Antiserum—A cDNA fragment encoding amino acids Ala129–Val575 of Xenopus BiP (see Fig. 1) was isolated from a Xenopus gastric cDNA library (43) by PCR using two degenerate oligonucleotides containing appropriate restriction site overhangs (CGTGGATCCG-CIAA(A/G)TTIGA(G/A)G(T)I(T)IT1AA(C/T)ATG and CAACGTC(C/T) TCI(A/G)CTICG(A/G)TTI(A/G)ATITCC, corresponding to the peptide sequence AKFEELMM and GINPDEAV, respectively). A glutathione S-transferase-BiP fusion protein (GST-BiP) was constructed by subcloning this domain (as a BamHI/BglII fragment) in frame with the glutathione S-transferase (GST) open reading frame of the vector pGEX-2T (Pharmacia Biotech Inc.). Bacterially produced GST-BiP was affinity-purified using glutathione-Sepharose 4B (Pharmacia), recovered by elution with glutathione, and injected subcutaneously into rabbits using standard protocols.

Xenopus BiP cDNA Isolation and Construction of BiP Mutants—A full-length Xenopus BiP cDNA was obtained from a cDNA library prepared with oligo(dT) priming of poly(A+) RNA isolated from A6 cells (derived from Xenopus kidney). The cDNA library was cloned into the vector pSPORT (Life Technologies, Inc.), and transformed into bacteria, and ifIIs were screened with a PCR-generated BiP cDNA fragment. The fragment was amplified between a 5′ sense, degenerate oligonucleotide GCGGATCCGGIGA(A/G)CTIGA(C/T)IT1GIA(C/T)ATG and a 3′ antisense, specific oligonucleotide containing a EcoR I site derived from the sequence obtained from the PCRamplified fragment used for the fusion protein, from a sequence corresponding to the peptide sequence Gly197–Tyr207 and a specific oligonucleotide (containing an EcoRI site) derived from the sequence obtained from the PCR fragment from the second primer were added and corresponding to the peptide sequence Gly129–Tyr139. A single, full-length clone was identified and sequenced by the dideoxynucleotide method on both strands. The cDNA was subcloned in the expression plasmid pSOD3 (31) for cDNA synthesis. The full-length Xenopus BiP cDNA, in the in vitro synthesized tRNA, and the protein products are referred to as BiPwt (see Fig. 1). The translational competence of BiPwt tRNA was confirmed in an in vitro translation in a rabbit reticulocyte lysate as described previously (13).

A truncated BiP (BiPtr; see Fig. 1) lacking the carboxyl-terminal five amino acids (EKDEL) was constructed employing PCR, an antisense, mutagenic oligonucleotide (CGGGATCTAACTCTCCCGGACACT, and a more 5′ specific oligonucleotide. The resulting PCR fragment was used to replace the 3′-end of the BiPwt cDNA downstream from the unique BglII site (located close to the middle of the BiPwt cDNA). A second mutant BiP containing an internal deletion of 48 amino acids (BiPd; see Fig. 1) was constructed using the three PstI sites within the BiPwt cDNA. BiPd cDNA was digested with PstI (releasing two fragments) and recircularized. The larger, 924-base pair PstI fragment was then reinserted, resulting in the deletion mutant missing the 144 base pairs encoding amino acids Ala139–Ala185. Finally, the double mutant (BiPΔtr) was constructed by combining the cDNAs encoding the 5′-end of BiP and the 3′-end of the BiP at the BglII site.

Na,K-ATPase and H,K-ATPase Subunit cDNAs, Construction of Chimeras and Mutants, and Subunit-specific Antibodies—The expression in Xenopus oocytes of the cDNAs encoding the Xenopus laevis Na,K-ATPase α-subunit (α1; Ref. 32), β-subunit (β1; Ref. 32), and β3-subunit (β3; Ref. 33) have been described (4, 5, 34). In Ref. 13 we described the expression in Xenopus oocytes of the rabbit gastric H,K-ATPase β-subunit (b1H; Ref. 35) and the constitutive and expression of chimeras between Xenopus β1 and rabbit gastric βH. The two truncated Xenopus α1 constructs shown in Fig. 4 were the result of PCR errors; in both cases, a single point mutation introduced into the cDNA encoding α1 created a stop codon. Thus, the peptides encoded by α1R647 and α1R669 end with Gly370 in the first cytoplasmic loop between transmembrane spans M2 and M3 and Gin366 in the second cytoplasmic loop between transmembrane spans M4 and M5, respectively. Two C-terminally truncated β3, β3R550, and β3R510 were obtained as follows. The wild type β3 in the pSOD vector was cut with PstI or BamHI, respectively, in the open reading frame and with HindIII in the 3′-untranslated region. After blunting, the cDNAs were ligated.

Polyclonal antibodies to α1 or β1, or β3 (33) or monodonal antibodies against βH (13, 36) have been described.

Protein Expression in Xenopus Oocytes, Immunoprecipitations, and Western Blots—In vitro synthesized RNA (cRNA) was prepared according to Melton et al. (37) and injected (50 nl/oocyte) into stage V/VI oocytes isolated from Xenopus laevis as described previously (4). Oocytes were incubated at 19°C in modified Barth’s medium containing 0.6 M NaCl (38) and supplemented with 0.01 M HEPES, pH 7.4, and 2 μM 2′-azido-2′-deoxyadenosine (an inhibitor of reverse transcriptase). The medium was changed every 2 days with fresh medium. ATP (1 mM) and glucose (10 mM) were included to deplete ATP (38). Immunoprecipitations were performed at 4°C for 1 h (with BiP antiserum in the open complex between transmembrane spans M2 and M3, and Gin66 in the second cytoplasmic loop between transmembrane spans M4 and M5, respectively). Two C-terminally truncated β3, β3R550, and β3R510 were obtained as follows. The wild type β3 in the pSOD vector was cut with PstI or BamHI, respectively, in the open reading frame and with HindIII in the 3′-untranslated region. After blunting, the cDNAs were ligated.

RESULTS

Characterization of Xenopus BiP—A full-length cDNA encoding the Xenopus BiP protein was isolated from a cDNA library prepared from A6 cells (BiPwt; Fig. 1). As expected, given the remarkable conservation during evolution of BiP (39), the deduced amino acid sequence of the Xenopus protein is nearly identical (95% amino acid identity) to that of the rat (17). We found, however, sequence variations at the amino acid level between the full-length BiPwt cDNA and partial cDNA fragments obtained by PCR (as well as by conventional cloning), including the PCR fragment used to generate a fusion protein for the preparation of a BiP antibody (Fig. 1). These differences are probably not due to PCR artifact, because the changes were generally conservative and, in some cases, independent PCR yielded clones with identical sequences. Xenopus laevis is tetraploid, and either co-existence of different alleles or gene duplication could account for these differences. Winning et al. (40) have identified three variants of the BiP protein in Xenopus that are expressed in an ordered pattern during embryogenesis. While the significance of multiple BiP proteins in Xenopus remains unknown, our data suggest that they may be due to the expression of more than one BiP gene.
**Interaction of Na,K-ATPase Subunits with BiP**

To study the expression of Xenopus BiP and its interactions with the Xenopus Na,K-ATPase α1- and β1-subunits in oocytes, we prepared antibodies against a Xenopus BiP fusion protein (Fig. 1). On Western blots, the BiP antiserum recognized the we prepared antibodies against a sequence, BiP

The BiP sequence is the deduced amino acid sequence of the PCR product used to construct a GST-BiP. The residues that differ from those of the PCR product, the full-length Xenopus BiP are shown. The models of BiP shown in B show some of the functional domains and mutations discussed in the text. BiPΔ lacks the C-terminal EKDEL motif of BiP and BiPΔtr lacks both sequences.

![Fig. 1. Primary structure of Xenopus BiP.](image)

**Fig. 2. Characterization of BiP antiserum and overexpression of BiP in Xenopus oocytes following cRNA injection.**

A. Characterization of the BiP antiserum by Western blot analysis. Purified bacterially produced proteins or total cellular proteins were sized by SDS-PAGE, transferred to nitrocellulose, and probed with BiP antiserum raised against a GST-BiP (Fig. 1 and "Materials and Methods"). The following were loaded onto the gel: GST (lane 1), GST-BiP (lane 2), GST-BiP digested with thrombin to release the BiP peptide (lane 3), 20 mg of total protein extracted from the Xenopus kidney cell line A6 (lane 4), and 20 mg of a murine L cell detergent extract (lane 5). B. Tunicamycin induction of BiP in A6 cells. Twenty mg of total protein was sized by SDS-PAGE, transferred to nitrocellulose, and probed with BiP antiserum. In lane 2, A6 cells were incubated for 4 h at 28°C with 3 mg/ml tunicamycin prior to extraction, C. Expression of BiP following cRNA injection into Xenopus oocytes. Oocytes were injected with BiPwt cRNA as indicated and metabolically labeled with 2 μCi/ml [35S]methionine for 4 h. Detergent extracts were prepared following labeling and a 24-h chase and subjected to immunoprecipitation after SDS denaturation (lanes 2-5). Lane 1 shows an immunoprecipitation from an in vitro translation of BiPwt cRNA in a reticulocyte lysate. Immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. The mobility of proteins of known molecular mass is indicated.

**Table 1.**

| Peptide-binding domain | Signal sequence | Translated product |
|------------------------|----------------|--------------------|
| **BiP wt**             | -17 1          | 624 496 633 638    |
| **BiP tr**             | -17 1          | 624 496 633 638    |
| **BiP Δ**              | -17 1          | 624 496 633 638    |
| **BiP Δtr**            | -17 1          | 624 496 633 638    |

**Fig. 2.**

**A.** Antibodies were raised against a GST-BiP (Fig. 1 and "Materials and Methods"). The following were loaded onto the gel: GST (lane 1), GST-BiP (lane 2), GST-BiP digested with thrombin to release the BiP peptide (lane 3), 20 mg of total protein extracted from the Xenopus kidney cell line A6 (lane 4), and 20 mg of a murine L cell detergent extract (lane 5).

**B.** Tunicamycin induction of BiP in A6 cells. Twenty mg of total protein was sized by SDS-PAGE, transferred to nitrocellulose, and probed with BiP antiserum. In lane 2, A6 cells were incubated for 4 h at 28°C with 3 mg/ml tunicamycin prior to extraction, C. Expression of BiP following cRNA injection into Xenopus oocytes. Oocytes were injected with BiPwt cRNA as indicated and metabolically labeled with 2 μCi/ml [35S]methionine for 4 h. Detergent extracts were prepared following labeling and a 24-h chase and subjected to immunoprecipitation after SDS denaturation (lanes 2-5). Lane 1 shows an immunoprecipitation from an in vitro translation of BiPwt cRNA in a reticulocyte lysate. Immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. The mobility of proteins of known molecular mass is indicated.

**Fig. 3.**

Interaction of Na,K-ATPase Subunits with BiP—The BiP antiserum not only immunoprecipitated BiP protein following denaturation, but it also recognized native BiP in nondenaturating conditions and co-precipitated associated proteins. Fig. 3A shows the recognition of the endogenous, oocyte BiP (lanes 1 and 2) and its interaction with Na,K-ATPase subunits (lanes 3-8) in oocytes labeled for 6 h and following a 24-h chase. Six hours after α1 cRNA injection, the BiP antiserum co-precipitated a protein corresponding in size to the α1-subunit (Fig. 3A, lane 3). Co-precipitation of α1 was no longer apparent after a 24-h chase (Fig. 3A, lane 4), which correlates with the degradation of the α1-subunit when expressed without a β-subunit (Ref. 6; Fig. 3B, lanes 3 and 4). Like α1, the core-glycosylated ER form of β1 was co-precipitated with BiP from the pulse-labeled oocytes (Fig. 3A, lane 5) and to a lesser extent from...
Both subunits co-precipitated with BiP but to a lesser extent.

The interaction of Na,K-ATPase subunits with BiP is of interest, since we have previously shown that, unlike Xenopus Na/K and β, it is not retained within the ER of the oocyte when expressed without an α-subunit (Ref. 35). The β-subunit of mammalian gastric H,K-ATPase, β3 is expressed at low levels in Xenopus oocytes (13), during Xenopus early development, and in the adult brain (33), whereas β1 is the more broadly expressed isoform in adult Xenopus tissues (32). Like β1, β3 is retained in the ER in a core-glycosylated form when expressed in oocytes without an α-subunit (Ref. 13; Fig. 5B; lanes 1-4). While the expression of β1 and β3 was similar during the pulse-labeling period (Fig. 5B, compare lanes 1 and 3; ratio β1/β3, 1.2), fewer β3 than β1 molecules co-precipitated with BiP (Fig. 5A; lanes 1 and 3; ratio BiP/β1, 0.5; BiP/β3, 0.1). Following a 30-h chase, β1 continued to co-precipitate with BiP (Fig. 5A; lane 2), whereas β3 co-precipitation was hardly apparent (Fig. 5A, lane 4), corresponding to the more rapid turnover of β3 in the oocyte (Fig. 5B, compare lanes 2 and 4).

The study of the association of BiP with the mammalian gastric H,K-ATPase β-subunit (βHK; Ref. 35) was of interest, since we have previously shown that, unlike Xenopus Na/K and β, it is not retained within the ER of the oocyte when expressed without an α-subunit (13, 42, 43). Indeed, much of the βHK expressed in oocytes matures into the fully glycosylated form during the chase period (Fig. 5B; lanes 5 and 6). Little βHK co-precipitated with BiP following pulse labeling (Fig. 5A; lane 5), and none was apparent following chase (Fig. 5A; lane 6). Two chimeras between β1 and βHK (βNK/HK and βHK/NK), which can partly act as surrogates for β1 in the formation of functional Na,K-ATPase complexes (13) were expressed in oocytes to begin to characterize the structural domains within β1 that interact with BiP. As previously reported (13) βNK/HK, consisting of the cytoplasmic and transmembrane domains of β1 linked to the ectodomain of βHK, exits the ER without an

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**Fig. 3.** Expression of the α1- and β1-subunits of Xenopus Na,K-ATPase in oocytes and their association with BiP. Noninjected oocytes or oocytes injected with α1 cRNA (7 ng/oocyte), β1 cRNA (0.2 ng/oocyte), or α1 and β1 cRNA were metabolically labeled for 6 h in 1.8 mCi/ml [35S]methionine. Microsomal membranes were prepared after labeling and after a 24-h chase and extracted in digitonin. A, the extracts were subjected to immunoprecipitation with BiP antiserum in nondenaturing conditions and eluted in SDS sample buffer. B, aliquots of the same extracts were subjected to immunoprecipitation after SDS denaturation (lanes 1-10) or to immunoprecipitation under nondenaturing conditions (lanes 11 and 12) with the indicated antibody. Both A and B show SDS-PAGE of immunoprecipitated proteins followed by fluorography. The bands corresponding to α1, BiP, core-glycosylated β1 (β1cog), and fully glycosylated β1 (β1fog) are indicated. *, artifactual band probably consisting of actin (13), which is frequently observed in nondenaturing immunoprecipitations and which runs in front of the antibody heavy chain. One of four similar experiments is shown.

**Fig. 4.** BiP-co-precipitated proteins are the expressed α1- and β1-subunits of the Na,K-ATPase. Noninjected oocytes or oocytes injected with α1 cRNA (7 ng/oocyte), α1trch (5 ng/oocyte), α1trch (5 ng/oocyte), or β1 cRNA (0.2 ng/oocyte) were metabolically labeled for 24 h, and microsomal membranes were prepared and extracted in digitonin. The extracts were subjected to immunoprecipitation in nondenaturing conditions with BiP antiserum and eluted in SDS. One-half of each sample was then reimmunoprecipitated with either α1 (lanes 6-9) or β1 (lane 10) antisera. The primary (lanes 1-5) and the secondary immunoprecipitations (lanes 6-10) were analyzed by SDS-PAGE and fluorography. Protein standards of molecular mass 94, 67, 43, and 30 kDa are shown (st).


Interaction of Na,K-ATPase Subunits with BiP

**Fig. 5. Differential association of Na,K-ATPase β1- and β3-subunits and gastric H,K-ATPase β-subunit with BiP.** Oocytes were injected with cRNA encoding Xenopus Na,K-ATPase β1 (0.2 ng/oocyte), β3 (0.6 ng/oocyte), or two truncated mutants of β3 (β3tr200, β3tr250, 0.6 ng/oocyte), rabbit gastric H,K-ATPase β-subunit (βHK; 2.0 ng/glycoocyte), a chimera consisting of the cytoplasmic/transmembrane domains of Xenopus Na,K-ATPase β1 linked to the ectodomain of rabbit gastric H,K-ATPase β-subunit (βHK; 2.0 ng/oocyte), or the reciprocal chimera consisting of the cytoplasmic/transmembrane domains of βHK and the ectodomain of β1 (βHK/β1; 0.2 ng/oocyte). Microsomal membranes were prepared from oocytes following a 9-h pulse and 30-h chase. A and C, extracts were immunoprecipitated in non-denaturing conditions with BiP antiserum. B and D, an aliquot of the same extracts was immunoprecipitated after SDS denaturation with the indicated antibody. The core-glycosylated β-subunits (βcg) and the fully glycosylated β-subunits (βfg) are indicated. One out of two to four similar experiments is shown.

α-subunit and becomes fully glycosylated (Fig. 5B, lanes 7 and 8), similar to βHK. On the other hand, βHK/NK, in which the cytoplasmic and transmembrane domains of βHK are joined to the ectodomain of β1, is retained in the ER in its core-glycosylated form (Fig. 5B, lanes 9 and 10) similar to the wild type β1. Accordingly, βHK/NK associated poorly with BiP (Fig. 5A, lanes 7 and 8), similar to βHK, while βHK/NK associated efficiently with BiP (Fig. 5A, lanes 9 and 10), similar to β1. Thus from these data, we can conclude that different β-subunits interact with BiP, but the efficiency or kinetics of association is variable. The association is best with β-subunits that are retained in the ER, and it correlates with the respective stability of the retained polypeptides. In agreement with this observation is the finding that truncated, misfolded β3-subunits (β3tr200 and β3tr250), which cannot stabilize α-subunits (data not shown) and are retained in the ER with a half-life that is longer than that of β3 (Fig. 5D), associated much more efficiently and for a more prolonged time with BiP than did the wild type β3 (Fig. 5C).

In an attempt to further examine the role of BiP in Na,K-ATPase subunit stability and transport, we constructed a truncated BiP (BiPtr; see Fig. 1), which lacks the carboxyl-terminal ER retention signal KDEL (44). BiPwt and BiPtr were expressed in order to characterize the secretion of BiPtr from oocytes. BiPwt and BiPtr were expressed to a similar level (Fig. 6A, lanes 1 and 4) and were stable during a 48-h chase (Fig. 6A, lanes 2 and 5). While BiPwt was not immunoprecipitated from either the labeling media or chase media (Fig. 6A, lanes 7 and 8), BiPtr was immunoprecipitated both from the labeling media and, to a greater extent, from the chase media (Fig. 6A, lanes 9 and 10). However, the secretion of BiPtr was inefficient, and most of the protein remained in the oocyte. Removal of the vitellin layer, which encloses the oocyte and might be a potential barrier for BiP secretion, did not reduce the amount of BiPwt or BiPtr recovered from the oocyte extracts (Fig. 6A, lanes 3 and 6). Since one of our goals was to investigate the effect of BiPtr (and other BiP mutants) on Na,K-ATPase single-subunit trafficking and/or stability, it was important to assess the ratio between the endogenous oocyte BiP and the exogenous BiP expressed by cRNA injection. Although cRNA injection increased the amount of newly synthesized BiP by more than 30-fold (see Fig. 2C, lanes 2 and 4), the effect on the total BiP pool was much less pronounced. Western blot analysis revealed that the oocytes injected with BiPwt (Fig. 6B, lane 2) or BiPtr (Fig. 6B, lane 3) cRNA expressed only about 2 times more BiP than did the wild type BiP (Fig. 5C). The data presented so far confirmed previous observations that interaction of BiP with wild type and misfolded, assembly-incompetent subunits with BiP were different (44).

**Fig. 6. Intracellular accumulation and secretion of BiPtr following cRNA injection.** A, oocytes injected with BiPwt or BiPtr cRNA (2 ng/oocyte) were metabolically labeled for 25 h, and detergent extracts were prepared from one-third of the oocytes (lanes 1 and 4). Following a 48-h chase, the remaining oocytes were either extracted (lanes 2 and 5) or hand-stripped of their vitellin membrane, incubated in modified Barth’s medium containing 2 mM MgCl₂ and 1 mM ATP for 10 min at room temperature, and then extracted (lanes 3 and 6). Extracts were SDS-denatured and BiP-immunoprecipitated and sized by SDS-PAGE. In addition to the oocyte extracts, the labeling media (lanes 7 and 9) and chase media (lanes 8 and 10) were analyzed for BiP. An equal volume per oocyte of the medium was SDS-treated and immunoprecipitated. The BiP signal from oocyte extracts and growth media was obtained with 0.5 and 5 oocytes, respectively. B, Western blot with BiP antiserum in which 40 mg of yolk-depleted oocyte proteins were sized by SDS-PAGE. Lane 1, noninjected oocytes; lane 2, BiPwt cRNA-injected oocytes; lane 3, BiPtr cRNA-injected oocytes. 5 ng/oocyte of cRNA was injected, and oocytes were extracted 48 h after cRNA injection.

The data presented so far confirmed previous observations that interaction of BiP with wild type and misfolded, assembly-incompetent subunits with BiP were different (44).
Specificity of BiP interaction with endogenous Xenopus oocytes was confirmed by the immunoprecipitation of BiP with antisera in denaturing conditions (A) or an antisera in non-denaturing conditions (B). Oocytes were injected with BiP wt, BiP wt, or BiP Δ RNA (2 ng/oocyte) as indicated and metabolically labeled for 16 h. For one-half of each set of oocytes, digitonin extracts were prepared from microsomal membranes using the standard protocol (lanes 1, 3, and 5). For the remaining oocytes, hexokinase and glucose were omitted, while 3.0 mM MgCl₂ and 1.5 mM ATP (MgATP) were included during extraction and immunoprecipitation (lanes 2, 4, and 5). Both sets of samples were immunoprecipitated with a antisera in non-denaturing conditions, and sized by SDS-PAGE. In A, the immunoprecipitated BiP is indicated, while in B and C, endogenous Xenopus endo-α and the co-precipitated BiP are labeled.

In conclusion, overexpression of wild type and mutant BiP in Xenopus oocytes permitted us to reveal an association of BiP with endo-α as well as α-subunits of Na,K-ATPase with BiP and supports the notion that BiP not only interacts with soluble or type I and II glycoproteins but also with large multimembrane-spanning proteins. The documented interactions of BiP with wild type and mutant β- and α-subunits are qualitatively and quantitatively distinct and correlate with the stability of the proteins.

DISCUSSION

In the living cell, folding of multidomain proteins begins during synthesis and permits subunits of multimeric proteins to assemble into oligomers at the level of the ER. The process proceeds efficiently because of the assistance of helper proteins. According to this model, the best characterized ER molecular chaperone, the binding protein or BiP has been suggested to bind transiently to any polypeptide that emerges in the ER lumen (Ref. 1, and references therein). Typically, however, BiP interaction is mainly documented for soluble, or type I and II cellular, membrane proteins or for viral proteins. In this study, we have examined the interaction of BiP with α- and β-subunits of Na,K-ATPase and shown that not only the type II β, but also the multimembrane-spanning α, binds to BiP before subunit assembly. The kinetics of interaction is different for wild type unassembled or mutant assembly-incompetent subunits and correlate with the respective stability of the proteins.

What conclusions can be drawn from these data on the role of BiP-protein interaction in general and of BiP interaction with Na,K-ATPase subunits in particular? Based on experimental evidence, which shows that BiP interaction is more transient with some exportable proteins than with unassembled subunits of oligomers and more stable with misfolded, mutant proteins than with unassembled subunits, two alternate BiP functions have been proposed: 1) BiP acts as a catalyst (chaperone) for correct folding and prevents improper intra- and intermolecular interactions, and 2) BiP acts as part of the quality control system and prevents the transport of misfolded proteins out of the ER. The difference in the two views is more apparent than real, since both functions can be reconciled if it is assumed that BiP interacts with all proteins during translocation and remains tightly associated until proper folding through subunit assembly occurs or the protein is degraded.

An extension of this model predicts that BiP participates in the folding or assembly of normal proteins or, alternatively, in the ER retention of unassembled subunits as a result of aggregation of BiP-associated complexes.

The studies on the interaction of BiP with the α-subunit and various β-subunits of oligomeric P-type ATPases support the dual role of BiP although our data do not permit prediction of a precise function of BiP in Na,K-ATPase processing. Significantly, β1 and β3 of Xenopus Na,K-ATPase but not βηHK of rabbit gastric H,K-ATPase are retained in the ER when expressed without α in Xenopus oocytes. Accordingly, only Na,K-ATPase β but not ηHK efficiently interacts with BiP over a longer time period. These data indicate a role of BiP in the ER retention of the Na,K-ATPase β. It is likely that ηHK rapidly folds and adopts a more correct, transport-competent conformation than Na,K-ATPase β, excluding the demonstration of the initial interaction with BiP under our experimental conditions. On the other hand, the fact that β1 subunits of Xenopus Na,K-ATPase are efficiently associated with BiP supports a role of BiP in protein folding. Indeed, until they are finally degraded, these unassembled, BiP-associated β-subunits are not grossly misfolded but have adopted and maintain an assembly-competent conformation, possibly through BiP interaction. Finally, the observation that mutated β-subunits that are assembly-incompetent and thus misfolded bind even more efficiently to BiP than unassembled β subunits, indicates the involvement of BiP in the ER quality control mechanism.

As mentioned above, only few data exist on the role and the interaction of BiP with multimembrane-spanning proteins. Other than our observation on BiP interaction with the α-subunit of Na,K-ATPase, a large membrane protein with 10 putative transmembrane segments, only the multimembrane-spanning subunits of the acetylcholine receptor have been shown to bind to BiP. The kinetics of association of acetylcholine receptor α-subunits suggests that BiP does not play a role in the folding or the early maturation of the protein but is associated with an unassembled, misfolded form, which is slowly degraded. On the other hand, another polytopic protein, the cystic fibrosis transmembrane conductance regulator does not...
form a detectable complex with BiP (50). Finally, and most significantly, mutation of KAR2, encoding the yeast homolog of BiP does not affect the folding and intracellular transport of the polytopic plasma membrane H-ATPase of yeast, a monomeric member of the P-type ATPase family (51). In this study we show that the catalytic \( \alpha \)-subunit of Na,K-ATPase, another member of the P-type ATPase family definitely associates with BiP. The difference in the behavior of these two related subunits might rely on the fact that the H-ATPase \( \alpha \)-subunit can mature and be transported to the plasma membrane without a \( \beta \)-subunit, while the Na,K-ATPase \( \alpha \)-subunit needs assembly with a \( \beta \)-subunit for its structural and functional maturation (2). Indeed, in contrast to H-ATPase \( \alpha \)-subunit, newly synthesized, individual Na,K-ATPase \( \alpha \)-subunits are structurally immature proteins, as reflected by their high trypsin sensitivity. Interaction with BiP and possibly other molecular chaperones, which assure the maintenance of an assembly-competent conformation might thus be important for Na,K-ATPase but not for H-ATPase \( \alpha \)-subunits. This hypothesis is supported by the observation that the endogenous, oocyte endo-

This latter possibility raises the question of the nature of BiP interaction sites in Na,K-ATPase subunits. Reversible binding of BiP is generally achieved through interaction with hydrophobic stretches in the protein backbone (25, 53). This might explain why mutant, misfolded \( \beta \)-subunits in which hydrophobic domains are expected to remain exposed interact more efficiently with BiP. An explanation for the observed, differential interaction kinetics of BiP with different wild type \( \beta \)-subunits is more difficult. A main difference between the studied Na,K-ATPase \( \beta 1 \) and \( \beta 3 \) or \( \beta HK \) is the presence of three (4), four (4), or six or seven (42) glycosylation sites, respectively. BiP association is inversely correlated with the existing number of sugar chains. It is possible that the co-translational addition of N-glycans facilitates correct folding, leading to a more transient interaction of BiP with heavily glycosylated proteins. A role for sugar chains in the initial folding could also explain why \( \beta HK \) acquires a more correct conformation, which permits its exit from the ER. The efficient interaction of BiP with the Na,K-ATPase \( \alpha \) is even more intriguing. Indeed, most of the mass of the mature \( \alpha \)-subunit is intramembrane or cytoplasmic, and the longest luminal loop consists of about 40 amino acid residues. It is, however, possible that the immature \( \alpha \)-subunit, which is not associated with \( \beta \)-subunit, does not yet have the correct membrane topology and exposes an important part of its C terminus to the luminal side, rendering it accessible for BiP interaction. On the other hand, it is not obvious why a truncated \( \alpha \)-subunit that only contains the first transmembrane segment remained in association with BiP.

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