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In vivo iodination of a misfolded proinsulin reveals co-localized signals for Bip binding and for degradation in the ER

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The signal for degradation of proteins in the endoplasmic reticulum (ER) is thought to be the exposure of internal domains which are buried when the protein has adopted its correct conformation and which are also exposed in assembly intermediates. This raises the question of why the intermediates are not degraded. We developed a system based on the peroxidase-catalyzed iodination of tyrosine residues which continuously monitors the exposure of internal domains of proinsulin. In CHO cells this system discriminated between assembly intermediates of wild type (wt) proinsulin and misfolded proinsulin, as shown by the exclusive iodination of a misfolded mutant which was finally degraded in the ER. Iodination *in vitro* showed that the assembly intermediates of wt proinsulin also exposed internal domains. This iodination was inhibited by the addition of the molecular chaperone Bip which was co-immunoprecipitated with proinsulin in CHO cells. The results obtained with the mutant proinsulin support the assumption that exposed internal domains represent the signal for degradation in the ER. Observations of wt proinsulin show that Bip masks internal domains of normal assembly intermediates during the entire assembly process, thereby suppressing their degradation. We propose that internal domains contain co-localized signals for Bip binding and for degradation.

Key words: Bip/endoplasmic reticulum/iodination/protein degradation/protein folding

Introduction

Biosynthesis and degradation of proteins take place simultaneously in the cytosol and the endoplasmic reticulum (ER). As yet, a general targeting signal for degradation, i.e. ubiquitination, has been identified only in cytosolic proteins (for review see Rechsteiner, 1987; Jentsch, 1992). Degradation in the ER has been intensively studied for the T cell antigen receptor (TCR). The α-subunit of TCR (TCRα) bears a signal for degradation which consists of the exposure of two basic amino acids in its transmembrane domain (TMD). These amino acids are masked after its assembly with CD3δ, resulting in the abrogation of rapid degradation (Bonifacino *et al.*, 1990). Recently it was shown that the TMD is unable to maintain TCRα integrated in the membrane unless it is assembled with other subunits of the TCR complex, suggesting that degradation might occur in the lumen of the ER (Shin *et al.*, 1993). An example of metabolically regulated degradation in the ER is hydroxymethylglutaryl-coenzyme A reductase. Its degradation, which also depends on the TMD (Gil *et al.*, 1985), is accelerated by its product mevalonic acid by an unknown mechanism (Correll and Edwards, 1994). Degradation of soluble proteins in the ER is exemplified by the unassembled immunoglobulin κ light chain (Gardner *et al.*, 1993) and the α_1-antitrypsin variant PiZ (Le *et al.*, 1992). In the case of IgM, a thiol group which is responsible for the assembly of the monomers (Alberini *et al.*, 1990) targets the IgM monomers for degradation when assembly does not occur (Fra *et al.*, 1993). General signals for degradation, however, are as yet unknown.

General signals for degradation of the many misfolded proteins have not yet been identified. It has been assumed that the exposure of domains which are buried in the correctly folded and assembled state might trigger recognition of a misfolded state by the degradation apparatus in the ER (Bonifacino and Lippincott-Schwartz, 1991). However, such structural motifs, which we will refer to as internal domains, are exposed not only in misfolded proteins, but in all folding and assembly intermediates. We therefore asked how the degradation apparatus that recognizes exposed internal domains is able to discriminate misfolded proteins from folding and assembly intermediates. This problem cannot be addressed directly since the proteases responsible for the degradation of misfolded proteins in the ER are, as yet, unknown. Only a family of cysteine proteases called ER60 which participate in the degradation of resident ER proteins has been isolated (Urade *et al.*, 1992). Unidentified serine proteases appear to operate in the degradation of unassembled Igκ light chains (Gardner *et al.*, 1993) and of unassembled H2 subunits of the asialoglycoprotein receptor (Wikström and Lodish, 1991).

Misfolded secretory proteins are often found in complexes with the heavy chain binding protein (Bip), the most abundant and best characterized molecular chaperone of the ER which belongs to the heat shock protein (hsp) 70 family (for review see Gething and Sambrook, 1992). Hsp70 chaperones are thought to operate mainly by preventing the incorrect intermolecular association and aggregation of nascent and unfolded polypeptide chains (Rothman, 1989; Hartl *et al.*, 1994). This is achieved by reversibly binding to exposed hydrophobic stretches in the protein backbone (Flynn *et al.*, 1991; Blond-Elguindi *et al.*, 1993) of nascent polypeptides (Gething *et al.*, 1986; Dorner *et al.*, 1987). An additional function of Bip might be the retrieval of misfolded proteins which have escaped from the ER (Hammond and Helenius, 1994).

We have developed a system based on the peroxidase-catalyzed iodination of tyrosine residues which allows

continuous monitoring of the exposure of internal domains of proinsulin. The peroxidase selectively iodinates a misfolded mutant proinsulin which is finally degraded in the ER leaving assembly intermediates of proinsulin unlabeled. We provide evidence for the inhibition of iodination of assembly intermediates by binding to Bip. These results suggest that internal domains contain co-localized signals for Bip binding and for degradation. This model is able to explain why assembly intermediates are not recognized by the degradation apparatus of the ER.

Results

Peroxidase selectively iodinates insulin which exposes internal domains

Internal domains of a protein are buried when the protein has reached the correctly folded and assembled state. Because the tyrosine residues of insulin are exposed in all states except for the fully assembled hexamer (Baker *et al.*, 1988), they represent internal domains. We analyzed whether the iodination of tyrosine residues by peroxidase distinguished fully assembled insulin hexamers from assembly intermediates, i.e. unfolded, monomeric or dimeric insulin. Equimolar amounts of insulin were dissolved in acetate buffer, pH 4.0, or Tris buffer, pH 7.5 and iodinated by lactoperoxidase (LPO). The loss of activity of LPO at pH 4.0 was 40% as determined by use of aprotinin as a substrate (not shown) and was corrected for by the use of a correspondingly higher amount of enzyme. Gel filtration analysis (Figure 1A) showed that insulin monomers and dimers were stabilized by their dissolution in acetate buffer at pH 4.0, whereas in Tris buffer at pH 7.5 insulin existed as a hexamer or assemblies of hexamers. When iodination by LPO was performed under these conditions (Figure1B), insulin was not iodinated at pH 7.5, i.e. in the form of a fully assembled hexamer. In contrast, insulin monomers and dimers (pH 4.0) were iodinated. The minute iodine label on hexameric insulin resulted from non-enzymatic iodination as demonstrated by the same degree of iodination in the absence of LPO. Upon prolonged exposure of the autoradiograph, the small amounts of proinsulin present in the insulin preparation were seen to behave exactly like insulin (not shown). These results demonstrated that LPO selectively iodinated assembly intermediates of insulin and proinsulin which still exposed internal domains.

Misfolded B5Ser-proinsulin is retained and degraded in the ER

In order to use the peroxidase–insulin system in living cells we co-transfected Chinese hamster ovary cells (CHO cells) expressing human thyroid peroxidase (TPO) with the cDNA of either proinsulin or of the proinsulin mutant B5Ser-proinsulin. B5Ser-proinsulin carries an *N*-glycan and was expected to be retained and degraded in the ER because of misfolding. Immunoprecipitation of proinsulin and B5Ser-proinsulin from cell lysates and media showed that only proinsulin was secreted (see Figure 4, immunoblot). Accumulation of B5Ser-proinsulin was not detectable in the cell lysates, suggesting its intracellular degradation. On immunofluorescence microscopy, B5Ser-proinsulin showed a reticular staining pattern characteristic of proteins localized in the ER (Figure 2A). Double

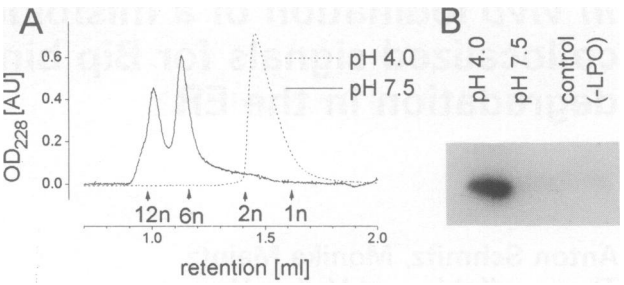

Fig. 1. Iodination of insulin at distinct states of assembly. (**A**) Gel filtration: insulin was loaded onto a Superdex 75 column and run in Tris buffer, pH 7.5 (——), or acetate buffer, pH 4.0 (- - -). At pH 7.5 insulin was eluted at retention volumes corresponding to hexamers (6*n*) or assemblies of hexamers whereas, when run at pH 4.0, it was eluted at retention volumes corresponding to monomeric (1*n*) and dimeric (2*n*) insulin. Thus insulin was stabilized in defined states of assembly depending on the dilution buffer. (**B**) Iodination: equimolar amounts of insulin were iodinated *in vitro* by LPO in Tris buffer, pH 7.5, or acetate buffer, pH 4.0, as described in Materials and methods and analyzed on SDS–tricine–PAGE. The autoradiograph showed that insulin was enzymatically iodinated only when dissolved in acetate buffer, pH 4.0, i.e. when present as monomers and dimers. The minute labeling of insulin hexamers resulted from non-enzymatic iodination, as it persisted in the absence of LPO. Thus peroxidase-catalyzed iodination *in vitro* distinguished between assembly intermediates and fully assembled insulin.

staining of B5Ser-proinsulin and of lysosomes prelabeled with wheat germ agglutinin (WGA) coupled to TRITC revealed that B5Ser-proinsulin was not localized in lysosomes (Figure 2B). In order to determine whether B5Ser-proinsulin was exported from the ER, the structure of its *N*-glycan was analyzed by digestion with endoglycosidase H (EndoH, Figure 2C). After treatment of cells with brefeldin A (BFA) the *N*-glycan of B5Ser-proinsulin became partly resistant to EndoH. Because BFA is known to induce relocation of Golgi enzymes into the ER (Lippincott-Schwartz *et al.*, 1989), this observation showed that the *N*-glycan of B5Ser-proinsulin was, in principle, a substrate for the processing glycosidases resident in the Golgi bodies. Nevertheless, in untreated cells the *N*-glycan was completely sensitive to EndoH indicating that B5Ser-proinsulin was not transported to the Golgi complex but retained in the ER. The amount of B5Ser-proinsulin, quantified by densitometry of the immunoprecipitates, was not elevated in the presence of BFA. The inability of BFA to cause accumulation of B5Ser-proinsulin further strengthened the conclusion that B5Ser-proinsulin was degraded in the ER.

To demonstrate that B5Ser-proinsulin was indeed misfolded we compared its conformation with that of proinsulin. In order to exclude conformational changes of the proteins due to the formation of originally absent disulfide bonds during isolation the proteins were carboxymethylated before cell lysis. B5Ser-proinsulin was deglycosylated by treatment with peptide-*N*-glycosidase F (PNGaseF). Figure 3 shows immunoblots of the electrophoretically separated proteins. On denaturing SDS–tricine–polyacrylamide gel electrophoresis (PAGE), deglycosylated B5Ser-proinsulin showed a migration pattern identical to that of proinsulin, demonstrating that glycosylation fully accounted for the higher molecular weight of B5Ser-proinsulin. However, on non-denaturing PAGE deglycosyl-

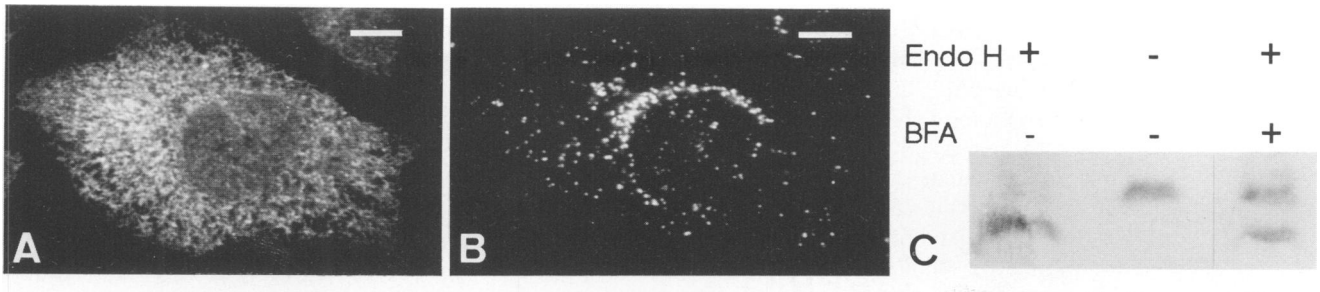

Fig. 2. Retention and degradation of B5Ser-proinsulin in the ER. (**A** and **B**) Immunofluorescence microscopy: after prelabeling of lysosomes with TRITC–WGA CHO cells co-expressing TPO and B5Ser-proinsulin were stained with guinea pig anti-insulin antiserum followed by DTAF-labeled goat anti-guinea pig antibody. B5Ser-proinsulin showed a reticular staining pattern characteristic of proteins localized in the ER (A, bar = 10 μm). The staining pattern of the lysosomes was distinct from that of B5Ser-proinsulin, ruling out a localization of B5Ser-proinsulin in lysosomes (B, bar ≐ 10 μm). (**C**) EndoH analysis: lysates of CHO cells expressing B5Ser-proinsulin were subjected to digestion by EndoH. The digests were separated on SDS–tricine–PAGE and B5Ser-proinsulin was identified by immunoblotting. When cells were preincubated with BFA, the *N*-glycan became partially resistant to EndoH indicating that it was, in principle, a substrate for Golgi-located processing glycosidases. In untreated cells the *N*-glycan was completely sensitive to EndoH indicating that B5Ser-proinsulin was retained in the ER. Note that the amount of B5Ser-proinsulin was not increased in the presence of BFA demonstrating that export from the ER was not required for its degradation.

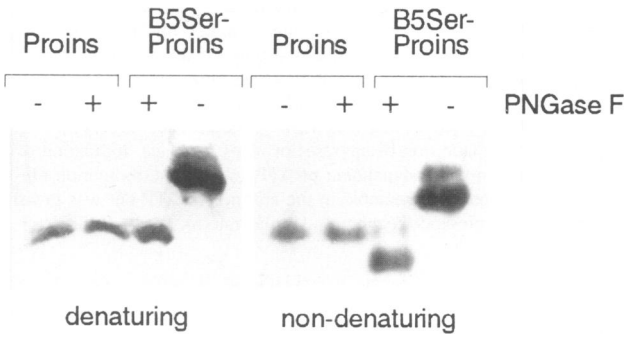

Fig. 3. Misfolding of B5Ser-proinsulin. Proinsulin or B5Ser-proinsulin expressing CHO cells was carboxymethylated in order to prevent the artificial formation of disulfide bonds during the following cell lysis and deglycosylation by PNGaseF. The proteins were analyzed on denaturing SDS–tricine–PAGE or non-denaturing PAGE according to Davis (1964) and visualized by immunoblot. The electrophoretic mobilities of proinsulin and deglycosylated B5Ser-proinsulin were identical on denaturing PAGE showing that glycosylation fully accounted for the higher molecular weight of B5Ser-proinsulin. Run on non-denaturing PAGE B5Ser-proinsulin had a higher mobility than proinsulin, suggesting that B5Ser-proinsulin was misfolded.

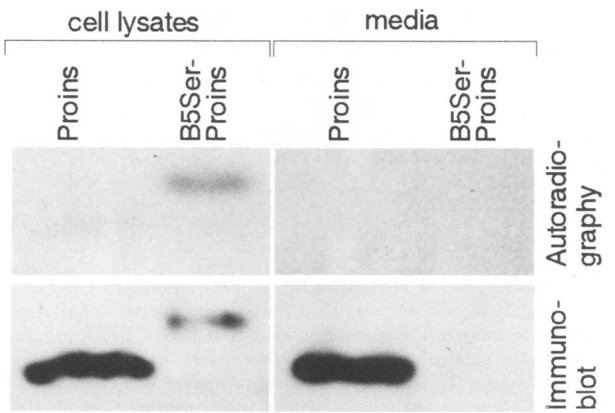

Fig. 4. Discrimination of misfolded B5Ser-proinsulin from proinsulin in CHO cells by peroxidase-catalyzed iodination. CHO cells co-expressing TPO and either proinsulin or B5Ser-proinsulin were incubated with Na[^{125}I] for 15 h. Proinsulin and B5Ser-proinsulin were immunoprecipitated from cell lysates and media and resolved on SDS–tricine–PAGE. On immunoblots of the media only proinsulin was detected, whereas both proinsulin and B5Ser-proinsulin were found in the cell lysates. The autoradiographs showed that only B5Ser-proinsulin was iodinated. Even in the medium where the proinsulin secreted during the labeling period accumulated no iodinated proinsulin was found. Thus, in CHO cells peroxidase discriminated misfolded B5Ser-proinsulin from normal proinsulin.

ated B5Ser-proinsulin migrated faster than proinsulin. The difference in electrophoretic mobility showed that the conformation of B5Ser-proinsulin was different from proinsulin. In summary these results showed that B5Ser-proinsulin represented a misfolded protein which was retained and degraded in the ER.

No folding and assembly intermediates of proinsulin are detected by TPO in CHO cells

Peroxidase iodinated insulin and proinsulin *in vitro* as long they exposed internal domains (see Figure1). Thus we expected peroxidase to detect folding and assembly intermediates of proinsulin as well as misfolded proinsulin in the ER of living cells. CHO cells co-expressing TPO and either proinsulin or B5Ser-proinsulin were incubated for 15 h with Na[^{125}I] (Figure 4). Iodinated proinsulin was not found in cell lysates or in the culture medium where the proinsulin synthesized during the labeling period accumulated. In contrast, B5Ser-proinsulin was iodinated, but not secreted. The same result was obtained after 30 min of iodination (not shown). As monomeric and dimeric proinsulin were iodinated *in vitro* (see Figure 1), this result suggested that their iodination in CHO cells was suppressed by a mechanism which, nevertheless, allowed iodination of B5Ser-proinsulin. In the following experiments we examined the underlying mechanisms.

TPO is not restricted to subcompartments of the ER

One possible explanation for the selective iodination of B5Ser-proinsulin by TPO is the restriction of TPO to subcompartments of the ER which were accessible to B5Ser-proinsulin, but not to proinsulin. We therefore examined the intracellular distribution of enzymatically active TPO cytochemically by the diaminobenzidine technique at the electron microscope level. The homogeneous distribution of the TPO reaction product throughout the entire ER, including the perinuclear cisterna (Figure 5),

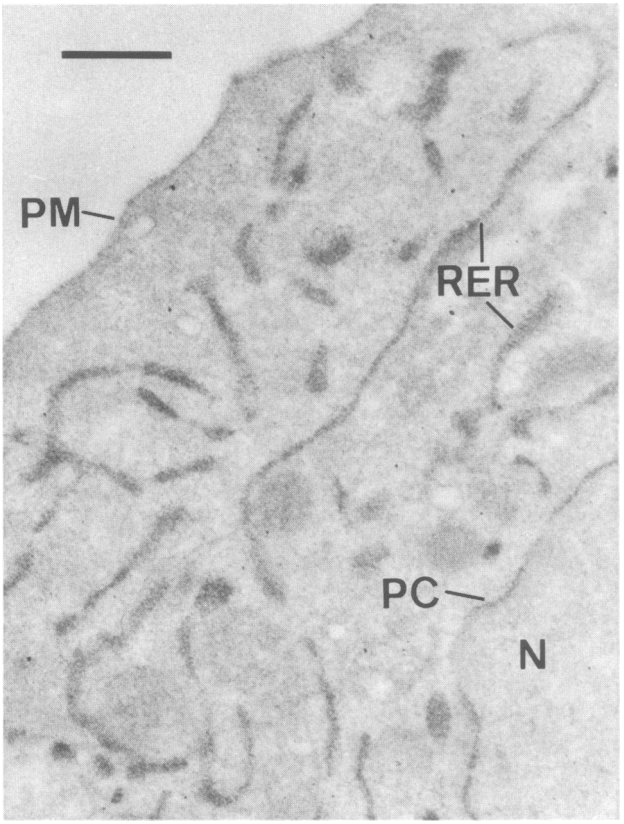

Fig. 5. Distribution of TPO in the ER. In TPO-transfected CHO cells enzymatically active TPO was localized electron microscopically using the DAB technique. The reaction product is visible in all ER cisternae including the perinuclear cisterna with no visible region of the ER being excluded. The homogeneous staining showed that enzymatically active TPO was not restricted to special subcompartments of the ER. Bar, 0.5 μm; N, nucleus; PC, perinuclear cisterna; RER, rough endoplasmic reticulum; PM, plasma membrane.

strongly argued against a subcompartmentalization of TPO in the ER.

Trapped assembly intermediates of proinsulin are protected from iodination by an ATP-sensitive factor

If proinsulin assembled into hexamers very rapidly, iodination by TPO could be too insensitive to detect very short-lived assembly intermediates. To exclude this possibility we trapped dimeric proinsulin in the ER by the combined addition of phenanthroline and BFA. Hexamerization of insulin is abolished by chelation of zinc ions (Kaarsholm *et al.*, 1989): this can be achieved in living cells by incubation with the membrane permeable zinc chelator phenanthroline (Harada *et al.*, 1993). BFA, which prevents export of proteins from the ER (Fujiwara *et al.*, 1988), inhibited secretion of proinsulin (not shown). Even under these conditions proinsulin was not iodinated, whereas B5Ser-proinsulin was (Figure 6A). Immunoblot analysis confirmed that equal amounts of proinsulin and B5Ser-proinsulin were immunoprecipitated (not shown). This result did not rule out the possibility that folding and assembly of monomers to dimers occurred too fast to be detected by iodination. However, as dimers of proinsulin were iodinated *in vitro* (see Figure 1) the lack of iodination in the presence of BFA and phenanthroline strongly suggested the protection of the trapped dimers of proinsulin

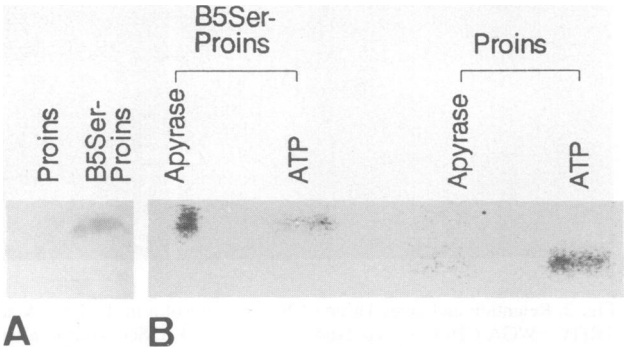

Fig. 6. Trapped assembly intermediates of proinsulin were protected from iodination by an ATP-sensitive factor. (**A**) Assembly intermediates of proinsulin were not iodinated in CHO cells: CHO cells co-expressing TPO and either proinsulin or B5Ser-proinsulin were preincubated for 30 min with phenanthroline to prevent hexamerization of proinsulin and with BFA to prevent export of the trapped intermediates from the ER. The cells were incubated for 30 min with Na[^{125}I] in the presence of the drugs. Proinsulin and B5Ser-proinsulin were immunoprecipitated and separated on SDS–tricine–PAGE. As shown on the autoradiograph only B5Ser-proinsulin was iodinated. (**B**) ATP restored iodination of assembly intermediates *in vitro*: proinsulin and B5Ser-proinsulin isolated from cells treated as described above were iodinated *in vitro* in the absence of ATP (achieved by the addition of apyrase) or in its presence. Iodination of B5Ser-proinsulin was independent of ATP. In contrast, iodination of proinsulin was barely detectable in the absence of ATP but was greatly increased in its presence reaching a similar rate to that of B5Ser-proinsulin.

in CHO cells by a compound which was lost during the purification process.

Due to their ability to bind to folding and assembly intermediates of secretory proteins in the ER, molecular chaperones could be responsible for the protection of the trapped proinsulin dimers. Binding of molecular chaperones to their ligands can be disrupted *in vitro* by the addition of ATP (Suzuki *et al.*, 1991). We therefore tested whether proinsulin isolated from cells treated as described above could be iodinated *in vitro* in the absence or presence of ATP (Figure 6B). B5Ser-proinsulin was efficiently iodinated in the absence as well as in the presence of ATP. In contrast, the iodination of proinsulin was barely detectable when ATP was omitted but greatly increased in the presence of ATP, reaching a similar rate to that of B5Ser-proinsulin. This result suggested that molecular chaperones were responsible for the protection of proinsulin assembly intermediates.

Bip prevents iodination of proinsulin dimers

As Bip is the major chaperone of the ER we examined whether it was associated with proinsulin. On co-immunoprecipitation both proinsulin and B5Ser-proinsulin were found to be associated with Bip (Figure 7A). Surprisingly, proinsulin, not the misfolded B5Ser-proinsulin, showed a stronger association with Bip. Although only ~1% of the total cellular Bip was co-immunoprecipitated with proinsulin it had to be postulated that all proinsulin dimers were bound to Bip because dimeric proinsulin was not iodinated. To test this prediction cells were incubated with phenanthroline and BFA to trap dimeric and monomeric proinsulin in the ER. The cells were lysed in the presence of ATP in order to dissolve proinsulin–Bip complexes or in the presence of apyrase to stabilize the complexes

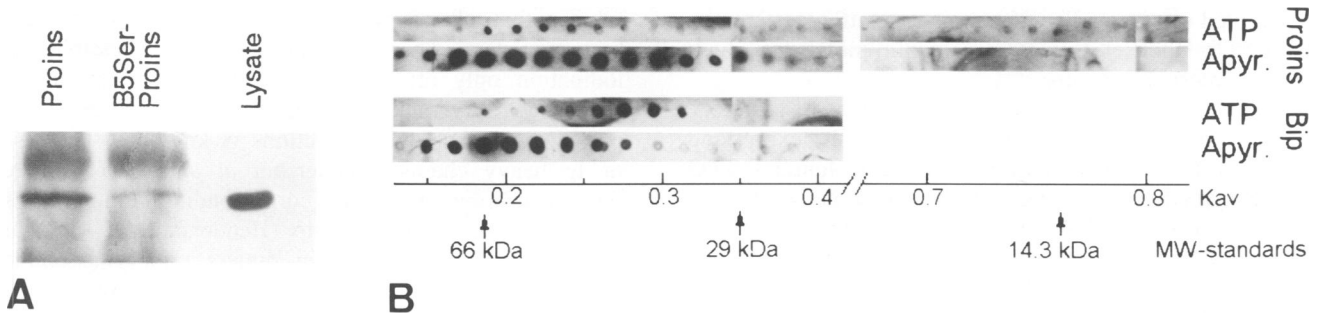

Fig. 7. Bip interacts with B5Ser-proinsulin and assembly intermediates of proinsulin in CHO cells. (**A**) Interaction of Bip and proinsulin in CHO cells: proinsulin was immunoprecipitated from lysates (625 μg protein) of either proinsulin or B5Ser-proinsulin-expressing cells. After gel electrophoretic separation of the immunoprecipitates and 5 μg of a total cell lysate (right lane) Bip was detected by an anti-Bip antiserum. The immunoblot showed that both proinsulin and B5Ser-proinsulin interacted with Bip. Surprisingly, Bip showed a stronger association with proinsulin than with the misfolded B5Ser-proinsulin. (**B**) All proinsulin dimers were bound to Bip: proinsulin monomers and dimers were trapped in the ER by treatment of cells with phenanthroline and BFA. Lysates were prepared in the presence of ATP or apyrase (Apyr.) which destroys the cellular ATP and loaded onto a gel filtration column equilibrated with or without ATP. Dot blots were performed using antibodies against proinsulin and Bip. Only the relevant fractions are shown. Arrows indicate the retention coefficient (K_{av}) of standard proteins. Free proinsulin monomers and dimers (9–18 kDa) were only detected in the presence of ATP whereas in the presence of apyrase proinsulin and Bip co-eluted in the high molecular weight range (90–100 kDa).

(Suzuki *et al.*, 1991). Lysates were analyzed by gel filtration in the presence or absence of ATP. Immunoblots of the fractions (Figure 7B) showed that dimeric and monomeric proinsulin (9–18 kDa) was present in the samples lysed in the presence of ATP, but was not detectable when lysed in the presence of apyrase. Instead, proinsulin and Bip co-eluted in the high molecular weight range (90–100 kDa). The complete disappearance of monomeric and dimeric proinsulin under conditions stabilizing Bip−ligand complexes demonstrated that all proinsulin monomers and dimers were bound to Bip. Quantitative binding of Bip to proinsulin assembly intermediates was further indicated by the finding that increasing the fraction of monomeric and dimeric proinsulin by treatment of cells with phenanthroline resulted in a 6-fold increase in co-immunoprecipitated Bip (not shown). In contrast, co-precipitation of Bip with B5Ser-proinsulin was increased only by a factor of 1.4 (not shown). To test whether Bip was able to inhibit iodination of insulin by peroxidase we iodinated dimeric insulin in the presence of Bip *in vitro*. Addition of an equimolar amount of Bip inhibited the iodination of insulin by ~50% (Figure 8A). Addition of BSA caused only a slight decrease in iodination demonstrating that the inhibition by Bip was not due to mere substrate competition or aggregation. The inhibitory effect of Bip was dose dependent (Figure 8B) reaching nearly full suppression when Bip was present in an amount five times greater than that of insulin. Taken together these experiments showed that Bip bound to proinsulin monomers and dimers and inhibited their iodination by peroxidase.

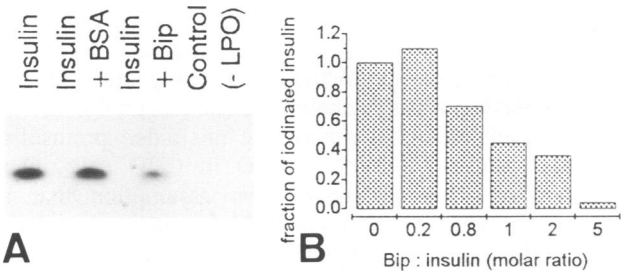

Fig. 8. Bip inhibits iodination of assembly intermediates *in vitro*. (**A**) Inhibition of the iodination of dimeric insulin *in vitro*: insulin was dissolved in acetate buffer, pH 4.0 and iodinated by LPO in the absence or presence of equimolar amounts of Bip or BSA. The iodination of insulin was decreased by ~50% in the presence of Bip. BSA inhibited the iodination of insulin only very slightly indicating that the inhibition by Bip is not due to mere substrate competition or aggregation. (**B**) Dose dependence of the inhibition: insulin was iodinated in the presence of increasing amounts of Bip as described in (A). Iodine incorporation was quantified by densitometry of the autoradiographs. Nearly complete suppression of iodination was reached when Bip was present at levels five times that of insulin.

Discussion

In this study we addressed the question of how the degradation apparatus in the ER which recognizes exposed internal domains is able to distinguish between misfolded proteins and normal assembly intermediates. To analyze the underlying mechanisms we have designed a monitoring system which detects exposed internal domains of proinsulin. This monitoring system consists of an iodinating peroxidase and insulin as a suitable substrate. TPO and LPO iodinate tyrosine residues, preferentially when present in the sequences DY, EY, EXY, SYS or TYS (Lamas *et al.*, 1989). Insulin and proinsulin contain four tyrosine residues, one of which is part of ENY (Bell *et al.*, 1979). The essential feature of the system *in vitro* is that only assembly intermediates of insulin and proinsulin become iodinated. This is explained by crystallographic data which show that the tyrosine residues which are in the same environment in the insulin and in the proinsulin molecule (Frank *et al.*, 1972) become successively buried as the assembly of insulin proceeds (Baker *et al.*, 1988). Of the four residues exposed in the insulin monomer, two are still exposed in the dimer. The only residue which remains on the surface in the hexamer is located in a hydrophobic pocket. As tyrosine residues are only exposed in unfolded, monomeric and dimeric insulin but not in the fully assembled hexamer, they represent internal domains, i.e. domains which are buried in the correctly folded and

assembled oligomer. Therefore this iodination system can be used to label selectively those insulin molecules which expose internal domains.

To study the exposure of internal domains of secretory proteins in living cells, we used CHO cells expressing human TPO. As shown by cytochemical techniques, TPO was enzymatically active in the ER. These cells were co-transfected with cDNA coding for either proinsulin or a glycosylated mutant (B5Ser-proinsulin). To our knowledge the TPO–proinsulin system is the first system which allows continuous monitoring of the exposure of internal domains of a protein in the ER. Since the ER is the place where proinsulin folds and assembles into hexamers internal domains should be exposed. We therefore expected TPO to iodinate newly synthesized proinsulin during folding and assembly. However, our results show that TPO does not detect any folding and assembly intermediates of proinsulin in CHO cells, although monomeric and dimeric proinsulin as assembly intermediates became iodinated *in vitro*. In contrast B5Ser-proinsulin, which is misfolded, retained and degraded in the ER, was readily iodinated by TPO in CHO cells, demonstrating the exposure of internal domains containing tyrosine residues. Apparently, exposed internal domains contain the signal for degradation of B5Ser-proinsulin. Although assembly intermediates and misfolded proinsulin show the same recognition motif, i.e. exposed internal domains, the misfolded proinsulin is selectively recognized by TPO in CHO cells. Our observations strongly support the assumption that a degradation apparatus based on the recognition of exposed internal domains is able to discriminate between misfolded proteins and assembly intermediates.

Subcompartmentalization of TPO in the ER as well as the rapidity with which hexamerization of proinsulin occurs have been excluded as factors which could explain the selective recognition of the misfolded mutant proinsulin by TPO. As TPO can only iodinate tyrosine residues if these are freely accessible, the lack of iodination of folding and assembly intermediates can be explained by the assumption that their tyrosine residues are permanently masked during the folding and assembly process. Molecular chaperones are ideal candidates for this purpose. Their function is defined as the prevention of incorrect interactions of exposed internal domains by their binding to those domains (Ellis, 1993). Indeed we found a greater amount of Bip coimmunoprecipitated with proinsulin than with B5Ser-proinsulin. This suggested that the binding of Bip to proinsulin was more stable than to B5Ser-proinsulin thereby preventing the iodination of proinsulin. The weak binding of B5Ser-proinsulin may be explained by the presence of the *N*-glycan in the neighborhood of one of the two potential Bip binding sites as predicted according to the scoring system of Blond-Elguindi *et al.* (1993).

In an *in vitro* assay, we have shown that Bip inhibited the iodination of insulin and proinsulin monomers and dimers in a dose-dependent manner. As proinsulin monomers and dimers are assembly intermediates of the proinsulin hexamer, this finding strongly supports the idea that the binding of Bip to internal domains prevents iodination of intermediates in living cells. This view is strongly supported by the observation that proinsulin isolated from CHO cells is iodinated *in vitro* only in the presence of ATP. This is explained by the finding that all proinsulin monomers and dimers are bound to Bip. They therefore become accessible for iodination only after ATP-induced dissociation of the complexes. Reversible binding of Bip to surfaces which participate in subunit interactions is known in the case of Ig heavy chains (Hendershot *et al.*, 1987), where the association with the corresponding light chains results in the release of Bip (Hendershot, 1990). Our results imply that proinsulin dimers rapidly assemble into hexamers after release from Bip, thereby burying their internal domains. We cannot rule out the participation of other chaperones or other proteins operating in the protection of proinsulin. However, the experiments reported here (see Figure 7B) present evidence that Bip is the essential factor. In contrast, B5Ser-proinsulin must exist at least for some time without being bound to Bip. The finding that Bip protects proinsulin from iodination suggests that Bip might provide a functional equivalent to the toroid of the hsp60 chaperones, although there is no structural similarity between these two classes of chaperones. According to the present view, hsp60 chaperones supply the folding intermediates with a protected environment in which they can fold without the risk of incorrect interactions with other folding proteins (for recent reviews see Ellis, 1994; Hartl *et al.*, 1994). Folding and assembly are thought to occur inside the chaperone cage after release from the walls of the chaperone (Martin *et al.*, 1993; Saibil *et al.*, 1993). If folding is not complete the protein is rebound to the chaperone molecule and the cycle starts again. For Bip it has been shown that the half-life of unsecreted Ig light chains corresponds to their release from the chaperone (Knittler and Haas, 1992). However, it is not known how the cycle of binding and release is interrupted and how the misfolded proteins are targeted for degradation.

Iodination by TPO revealed that the misfolded B5Ser-proinsulin exposed its internal domains containing the signal for degradation. In contrast, the assembly intermediates of proinsulin were not iodinated because their internal domains were masked by the binding of Bip. Based on this finding we propose a model of co-localized signals for Bip binding and for degradation. This model does not require identity of the signals but suggests that they are both localized in internal domains which are buried in the interior of the correctly folded protein. Bip masks these domains during folding and assembly by cycles of binding and release. As Bip is an abundant resident of the ER, there is only a slight chance of the degradation apparatus gaining access to folding and assembly intermediates. If the protein is definitely unable to achieve a correct conformation the probability of degradation increases as the cyclic binding and release continues and periods during which the internal domains are accessible to the degradation apparatus accumulate. With binding of the misfolded protein to the degradation apparatus further cycles of binding to and release from Bip are interrupted. The model of co-localized signals for Bip binding and degradation in the ER explains why assembly intermediates are not degraded although they expose the same internal domains as their misfolded counterparts.

Materials and methods

Cell culture

CHO cells were cultured in nutrient mixture F-12/ HAM (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in 5% CO_2.

Plasmid construction

The cDNA of *Macaca fascicularis* preproinsulin (obtained from Dr Habermann, Hoechst AG, Frankfurt/Main, Germany) was cloned into the eukaryotic expression vector pRc/CMV (Invitrogen, Leek, The Netherlands). The consensus sequence for N-glycosylation of B5Ser-proinsulin was generated by substituting serine for the histidine at position 5 by site directed mutagenesis using the mutagenesis primer GAACCGCACAGACTCTGGTTAACA. The sequence of recombinant DNA was verified by sequencing.

Stable transfection

TPO-expressing CHO cells at 40% confluency (obtained from Dr Magnusson, Mount Sinai Medical School, NY, USA) were co-transfected with 20 μg plasmid DNA coding for either proinsulin or B5Ser-proinsulin and 2 μg pSVpac (Vara *et al.*, 1986) coding for puromycin resistance, using liposomes (DOTAP, Boehringer Mannheim). Two days after transfection selection medium containing 1 μg/ml puromycin (Sigma, Deisenhofen) was given to the cells. Puromycin concentrations were raised to 5 μg/ml within 6 days. After an additional 24 h the cells were trypsinized and placed in new selection medium containing 30 μg/ml puromycin. Puromycin-resistant colonies were picked after 6 days.

Immunofluorescence microscopy

For immunofluorescence studies, doubly transfected CHO cells were fixed in 4% paraformaldehyde, permeabilized in ethanol for 2 min and incubated for 90 min at 37°C with guinea pig anti-insulin antiserum (Dako, Hamburg) followed by DTAF-labeled goat anti-guinea pig antibody (Jackson Immunoresearch Inc., West Grove, USA) for 1 h at 37°C. For staining of lysosomes, doubly transfected cells were incubated with 10 μg/ml TRITC-labeled WGA (Vector laboratories Inc., Burlington, USA) for 1 h at 4°C to adsorb the WGA to the plasma membrane. The cells were then chased for 3 h at 37°C to allow endocytosis. After washing with 50 mM *N*-acetylglucosamine (Sigma) cells were fixed and stained for proinsulin. Cells were viewed with a confocal laser scanning microscope (TCS D4; Leica, Bensheim, Germany) using an argon/ krypton mixed-gas laser at 488 and 568 nm exicitation. Scans at a resolution of 1024×1024 pixels and at a pinhole setting of 60 were taken in the line averaging mode. Micrographs were taken on Kodak TMax films (Eastman Kodak Co., Rochester, NY, USA) using a hardcopy device (Focus Graphics, Oberau, Germany).

Electron microscopy

Doubly transfected CHO cells were fixed in 3% paraformaldehyde, 0.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2 and stained with diaminobenzidine (Strum and Karnovsky, 1970). The cells were postfixed in 1% unbuffered OsO_4 and embedded in Epon. Sections were stained with lead citrate and examined in a Philips CM 120 electron microscope (Philips Electron Optics, Eindhoven, Netherlands).

Immunoprecipitation/co-immunoprecipitation

Doubly transfected CHO cells were lysed in lysis buffer (50 mM Tris−HCl, pH 7.5, containing 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA and 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain and 10 μg/ml pepstatin) for 30 min on ice. Lysates containing 500 μg protein were incubated with 5 μl guinea pig anti-insulin antiserum overnight followed by 25 μl protein A−Sepharose (Sigma) for 1 h on a rotation device at 4°C. Immunoprecipitates were washed in lysis buffer and 10 mM Tris−HCl, pH 7.5 and prepared for SDS−tricine−PAGE. For co-immunoprecipitation of Bip EDTA was omitted, but 30 U/ml apyrase (Sigma)was added.

Gel electrophoresis and immunoblotting

Insulin, proinsulin and B5ser-proinsulin were separated by discontinuous SDS−tricine−PAGE on a 10/16.5% polyacrylamide step gel (Schägger and von Jagow, 1987). Bip was resolved on a 10% polyacrylamide gel (Laemmli, 1970). The conformation of proinsulin and B5ser-proinsulin was analyzed on non-denaturing PAGE with an 18% resolving gel (Davis, 1964). After gel electrophoresis proteins were transferred onto nitrocellulose (Schleicher and Schuell, Dassel, Germany) according to the method of Kyhse-Anderson (1984). Samples were probed either with guinea pig anti-insulin antiserum (Linco Research Inc., St Louis, USA) or rabbit anti-GRP78 antibody (Affinity Bioreagents, Neshanic Station, USA) for 90 min. After incubation with secondary antibodies coupled to horseradish peroxidase the antigens were visualized by chemiluminescence (ECL, Amersham, Braunschweig, Germany).

Digestion with EndoH

Doubly transfected CHO cells were treated with or without 0.5 μg/ml BFA (Sigma) for 6 h, lysed and boiled for 5 min in 1% SDS. Digestion was performed in 0.1% SDS, 125 mM citrate buffer, pH 5.5, 40 μM PMSF and 100 U/ml EndoH (Boehringer Mannheim) for 15 h at 37°C. Samples were analyzed by SDS−tricine−PAGE and immunoblotting.

Conformation analysis of B5Ser-proinsulin

Doubly transfected CHO cells were carboxymethylated with 50 mM iodoacetamide for 10 min on ice. Lysates were digested in 20 μl lysis buffer containing protease inhibitors (see immunoprecipitation), 10 mM EDTA, 0.05% NaN_3 and 0.2 U PNGaseF (Boehringer Mannheim). After separation by SDS−tricine−PAGE or non-denaturing PAGE (Davis, 1964) proinsulin was identified by immunoblotting.

In vivo iodination

Doubly transfected CHO cells at 70% confluency were washed twice with medium without FCS and incubated with 250 μCi/ml Na[^{125}I] in medium without FCS for 15 h or 30 min at 37°C and 5% CO_2. For trapping monomeric and dimeric proinsulin in the ER cells were preincubated for 30 min with 0.5 μg/ml BFA and 0.6 mM phenanthroline (Sigma) and iodinated in the presence of these drugs. Proinsulin and B5ser-proinsulin were immunoprecipitated and visualized by autoradiography.

In vitro iodination

Iodination of purified insulin. Recombinant human insulin (0.33 mM; Sigma) was dissolved in a total volume of 50 μl in 50 mM Tris−HCl, pH 7.5, 200 μM $ZnCl_2$ or 50 mM acetate buffer, pH 4.0, without zinc. Both buffers contained 33 μM NaI, 0.2 μCi Na[^{125}I] and 200 mU/ml LPO (Sigma). The reaction was started by the addition of 30 μM H_2O_2 and stopped after 1 min by the addition of 5 μl 100 mM NaI, 150 mM NaN_3. For the inhibition studies recombinant hamster Bip (Stressgen, Victoria, Canada) was added in different molar ratios to insulin before the iodination was started. All samples were separated by SDS−tricine−PAGE and visualized by autoradiography.

Iodination of proinsulin isolated from CHO cells. After incubation for 1 h in the presence of 0.6 mM phenanthroline and 0.5 μg/ml BFA cells were permeabilized with 30 μg/ml digitonin (Sigma) to remove cytosolic proteins from the particulate fraction containing the undestroyed ER (Plutner *et al.*, 1992). The pellets were lysed in lysis buffer without EDTA. To the cleared lysates was added (final concentration): 8.25 nM NaI, 10 μCi Na[^{125}I], 125 mU LPO, 30 μM H_2O_2. The reaction was stopped after 10 min and proinsulin was immunoprecipitated and visualized by autoradiography. Autoradiographs were quantified by densitometry (C-R4AX Chromatopac, Shimadzu, Duisburg, Germany).

Gel filtration

Gel filtration of purified insulin. Recombinant human insulin (0.33 mM) was loaded upon a Superdex 75 PC 3.2/30 column (Pharmacia, Freiburg, Germany) in a Smart-System (Pharmacia) and run at a flow rate of 40 μl/min in 50 mM Tris−HCl, pH 7.5, 200 μM $ZnCl_2$ or 50 mM acetate buffer, pH 4.0.

Gel filtration of cell lysates. CHO cells expressing proinsulin were incubated for 1 h in the presence of 0.6 mM phenanthroline and 0.5 μg/ml BFA. Lysates were prepared either in lysis buffer without EDTA supplemented with 30 U/ml apyrase and loaded upon a Superdex 75 column equlilibrated in running buffer (50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 0.6 mM phenanthroline) or they were prepared in the same lysis buffer supplemented with 1 mM ATP, 2 mM $MgCl_2$ and loaded upon the same column equilibrated in running buffer containing 1 mM ATP, 2 mM $MgCl_2$. Proinsulin and Bip were detected by immunoblotting.

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