Mechanism of the Facilitation of PC2 Maturation by 7B2: Involvement in ProPC2 Transport and Activation but Not Folding

Laurent Muller, Xiaorong Zhu, and Iris Lindberg

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, Louisiana 70112

Abstract. Among the members of the prohormone convertase (PC) family, PC2 has a unique maturation pattern: it is retained in the ER for a comparatively long time and its propeptide is cleaved in the TGN/secretory granules rather than in the ER. It is also unique by its association with the neuroendocrine protein 7B2. This interaction results in the facilitation of proPC2 maturation and in the production of activatable proPC2 from CHO cells. In the present study, we have investigated the mechanism of this interaction.

ProPC2 binds 7B2 in the ER, but exits this compartment much more slowly than 7B2. We found that proPC2 was also slow to acquire the capacity to bind 7B2, whereas 7B2 could bind proPC2 rapidly after synthesis. This indicated that proPC2 folding was the limiting step in the formation of the complex. Indeed, sensitivity of native proPC2 to N-glycanase F digestion and inhibition of proPC2 folding supported the notion that 7B2 is not involved in the early steps of proPC2 folding, and that proPC2 must fold before binding 7B2. Under experimental conditions that prevent propeptide cleavage, 7B2 expression increased proPC2 transport to the Golgi. This increase exhibited the same kinetics as the facilitation of the removal of the propeptide. Finally, proPC2 activation could be reconstituted in Golgi-enriched subcellular fractions. In vitro, 7B2 was required for proPC2 activation at an acidic pH.

Taken together, our results demonstrate that rather than promoting proPC2 folding, 7B2 acts as a helper protein involved in proPC2 transport and is required in the proPC2 activation process. We propose, therefore, that 7B2 stabilizes proPC2 in a conformation already competent for these two events.

The major posttranslational modifications that result in the production of active peptides are endoproteolytic cleavage of the precursor, followed by exoproteolytic cleavage, and NH2-terminal acetylation and/or COOH-terminal amidation. The enzymes responsible for endoproteolytic cleavage events have recently been cloned (for review see Seidah and Chretien, 1994; Rouille et al., 1995). These prohormone convertases (PC)1 are the mammalian homologues of the serine protease subtilisin. Their expression pattern is either ubiquitous, as for furin (Hatsuzawa et al., 1990) and PACE4 (Kiefer et al., 1991), or tissue specific, as for PC1 and PC2, which are restricted to neuroendocrine cells (Seidah et al., 1990, 1991; Smeekens et al., 1991), or PC4, which is restricted to the testis (Nakayama et al., 1992; Seidah et al., 1992). The PCs are synthesized as proenzymes which undergo activation by cleavage of the proregion. The role of this propeptide as an intramolecular chaperone has been established in the case of subtilisin (Zhu et al., 1989), and the same function has been proposed for the propeptides of other enzymes of this family, including the PCs (for review see Siezen et al., 1995). The removal of the proregion of PC1 (Benjannet et al., 1993; Zhou and Lindberg, 1993; Lindberg, 1994; Zhou and Mains, 1994), furin (Molloy et al., 1994; Vey et al., 1994) or PC5 (DeBie et al., 1996), occurs in the ER shortly after synthesis. PC2, however, displays a very different posttranslational processing pattern: it is retained in the ER much longer than the other PCs studied so far, and it is converted to mature PC2 in the TGN or in the secretory granules, and not in the ER (Guest et al., 1992; Benjannet et al., 1993; Shen et al., 1993; Zhou and Mains, 1994). This unique maturation pathway is correlated with the specific association of proPC2 with the neuroendocrine protein 7B2 first shown by Braks and Martens (1994).

7B2 is a secretory protein of 185 amino acids migrating at 27 kD on SDS-PAGE (Martens et al., 1989; Hsi et al., 1982). The 27-kD form is a precursor protein that is cleaved in the TGN (Ayoubi et al., 1990; Paquet et al., 1991, 1994) into two domains. The COOH-terminal domain (residues 155–185) of 7B2 (CT peptide) constitutes a specific inhibi-
tor of PC2 activity (Martens et al., 1994; Lindberg et al., 1995). The region of the C1 peptide responsible for this effect has been identified (Van Horsen et al., 1995), and a mechanism for the termination of the inhibition has been proposed (Zhu et al., 1996a). The 21-kD NH2-terminal domain binds to PC2 (Zhu and Lindberg, 1995). We have identified a proline-rich segment of this NH2-terminal domain that is responsible for 7B2 binding to proPC2 (Zhu et al., 1996b). We have already shown that binding of 7B2 to PC2 results in the facilitation of proPC2 maturation and that the NH2-terminal domain alone can mediate this effect (Zhu and Lindberg, 1995). We have also demonstrated that whereas proPC2 secreted from CHO cells could not be activated, coexpression of 7B2 resulted in the secretion of activatable proPC2 (Zhu and Lindberg, 1995). The mechanism of the facilitation of proPC2 maturation by 7B2, however, remains unknown. Based on immunoprecipitation experiments and on the homology of 7B2 with proteins of the chaperone 60 family, Braks and Martens (1994) have proposed that 7B2 is a molecular chaperone specific for PC2. These authors considered a chaperone as a protein that binds to and stabilizes another protein, which results in the facilitation of its correct fate in vivo, according to Hendrick and Hartl (1993). This definition, however, does not refer to a particular function but encompases such different functions as folding, oligomeric assembly, transport to a subcellular compartment, or switching between active/inactive conformations (for review see Hendrick and Hartl, 1993). In the present paper, we refer to molecular chaperones according to a more specific definition, however, does not refer to a particular function but encompasses such different functions as folding, oligomeric assembly, transport to a subcellular compartment, or switching between active/inactive conformations (for review see Hendrick and Hartl, 1993). In the present paper, we refer to molecular chaperones according to a more specific definition; i.e., as proteins that interact either transiently with folding intermediates, which results in the facilitation of folding, or more stably with unfolded or aggregated forms of other proteins (for review see Hurtley and Helenius, 1989; Rothman, 1989; Gething and Sambrook, 1992; Hartl, 1996).

7B2 binding to proPC2 can occur in the presence of brefeldin A, suggesting that the association of the two proteins is initiated in a preGolgi compartment (Benjannet et al., 1995; Braks et al., 1996). The demonstration of immunoprecipitation of sulfated 7B2 and proPC2 indicate that the two proteins are still associated in the TGN, before cleavage of 7B2 and activation of proPC2 (Benjannet et al., 1995). Thus, 7B2 and proPC2 are transported together from the ER to the TGN. The facilitation of proPC2 conversion by 7B2 in AtT-20 cells could, therefore, result from the action of 7B2 at different steps of the secretory pathway: (a) proPC2 folding in the ER, since folding is a limiting step in protein transport to the Golgi (for review see Lodish, 1988); (b) proPC2 transport from the ER to the Golgi, as both proteins are transported together within the secretory pathway; and (c) proPC2 conversion into mature PC2 in the TGN or in the secretory granules, as we have shown that 7B2 coexpression is required to obtain activatable proPC2 from CHO cell secretions medium (Zhu and Lindberg, 1995). In the present work, we have studied the interaction of proPC2 with 7B2 in these compartments of the secretory pathway by radiolabeling experiments in AtT-20 cells overexpressing either PC2 alone or PC2 and 7B2. We have also performed subcellular fractionation of CHO cells that overexpress proPC2 in order to reconstitute the activation of proPC2 in the presence of recombinant 7B2 in vitro. Our data establish that in AtT-20 cells, 7B2 is not involved in proPC2 folding but increases proPC2 transport to the Golgi. Additionally, using our in vitro system, we demonstrate that 7B2 is required for the productive cleavage of the propeptide, i.e., for the generation of active mature PC2.

Materials and Methods

Antibodies

The antisem against PC2 (LSU18) was directed against a COOH-terminal peptide of mature PC2. The antisem against 7B2 (LSU13) was raised against residues 23-39 of 7B2. We have described the specificity of these two antisera elsewhere (Zhu and Lindberg, 1995). The monoclonal antibody MON102 was kindly provided by G. Martens (University of Nijmegen, Nijmegen, The Netherlands). It was directed against 7B2 amino acids 128-135 (Van Duijnhoven et al., 1991). The antisem directed against Birp/GRP78 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). It was directed against amino acids 24-43 of human Birp. Antiserum against calnexin was kindly provided by P. Arvan (Albert Einstein College of Medicine, New York, NY), and was directed against the COOH-terminal residues of murine calnexin (Kim et al., 1996).

Cell Culture, Site-directed Mutagenesis, and Transfection

All AtT-20 cells were cultured at 37°C in 5% CO2. They were maintained in DME containing 10% Nuserum (Becton Dickinson, Mountain View, CA), 2.5% fetal bovine serum ( Irvine Scientific, Santa Ana, CA) and the appropriate selection agents, 200 µg/ml G418 (Life Technologies Inc., Gaithersburg, MD) and/or 100 µg/ml hygromycin (Sigma Chemical Co., St. Louis, MO). Cells were transfected using Lipofectin (Life Technologies) as described (Zhu and Lindberg, 1995). The parental AtT-20 cells and those stably expressing PC2 were kindly provided by R. Mains (Johns Hopkins University School of Medicine, Baltimore, MD) (Zhou and Mains, 1994). The transfection of AtT-20/PC2 cells with a plasmid encoding rat 27-kD 7B2 has already been described (Zhu and Lindberg, 1995). The experiments in which we compared the kinetics of proPC2 maturation were performed with three different clones expressing 7B2 and PC2, using the parental cells as well as a transfected clone that did not overexpress 7B2 as controls. To obtain cell lines that expressed the 27-kD 7B2 and mutated proPC2, AtT-20 cells were first transfected with a pCEP4 plasmid (Invitrogen, Carlsbad, CA) encoding rat 27-kD 7B2. A high-expressing clone was selected and supertransfected with a plasmid pRC/CMV (Invitrogen) encoding PC2 mutated at the three glycosylation sites. The Asn of each glycosylation site was replaced by a Gin. This mutation was obtained by polymerase chain reaction-mediated methods already described (Zhu and Lindberg, 1995). The primers used were: 5'-GGGGCAACAGCTTCACTCCCCAAAGAGGGTG-3', 5'-GCACTACAACTCGTGTTGAG-3', 5'-CTCAACAGAGCTTTGAGGACAGTTCGAGG-3', 5'-GACCAGAAGAGAGACCTGAACATCCA-3', 5'-AGGTCTCCTCCTCGTGCTTGAGCAGTGTGAGG-3', 5'-AGGTCTCCTCCTCGTGCTTGAGCAGTGTGAGG-3', 5'-AGGTCTCCTCCTCGTGCTTGAGCAGTGTGAGG-3', 5'-AGGTCTCCTCCTCGTGCTTGAGCAGTGTGAGG-3', 5'-AGGTCTCCTCCTCGTGCTTGAGCAGTGTGAGG-3'. The study of the effect of this mutation was performed on two different clones.

The CHO cells stably expressing proPC2 that we used for the subcellular fractionation studies have already been described (Shen et al., 1993). This cell line was amplified for the expression of proPC2 using the dihydropyridine reductase method, but did not produce active enzyme.

Metabolic Labeling, Protein Extraction, and Immunoprecipitation

5 x 10^5 AtT-20 cells were labeled 40-48 h after seeding in six-well plates. They were labeled with 500 µCi/ml of [14C]methionine and cysteine (Promix, Amersham Corp., Arlington Heights, IL) for 10 min. After the pulse, they were extracted or chased in DME containing 2% fetal bovine serum. The sulfate-labeling experiments were performed in 12-well plates. Cells were labeled with 2 mCi/ml of [35S]sulfate (ICN Biomedicals, Inc., Irvine, CA) for 15 min and chased as previously described. In some experiments, cells were treated with 5 mM DTT during the pulse and the chase. They were then washed twice with PBS before a further chase without DTT. To
prevent the acidification of the pH in the TGN and the secretory granules, cells were incubated with 1 µm bafilomycin A1 (Kamiya Biomedical Co., Seattle, WA) during the chase.

Before extraction, cells were treated with 1 µl of 0.2 M iodoacetamide in PBS for 10 min on ice. All of the following steps of extraction and immunoprecipitation were performed at 4°C. For immunoprecipitation conducted under native conditions, proteins were extracted with a buffer containing 1% Triton X-100 in 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM iodoacetamide, 5 mM EDTA and 0.5 mM p-chloromercuriphenylsulfonic acid (Sigma Chemical Co.) and 1 mM phenylmethanesulfonyl fluoride (Boehringer Mannheim Corp., Indianapolis, IN). The extracts were centrifuged for 10 min at 15,000 g. Supernatants were diluted to 0.5% Triton X-100 and stored at -20°C before immunoprecipitation. For immunoprecipitation under denaturing conditions, proteins were extracted as previously described (Zhu and Lindberg, 1995).

Extracts were first incubated for 1 h in the presence of protein A coupled to Sepharose CL-4B (Pharmacia Biotech. Inc., Piscataway, NJ). The supernatants were then incubated for 4 h or overnight in the presence of antiserum directed against either PC2 or 7B2. To immunoprecipitate BiP/GRP78-bound PC2, BiPGRP78 was first immunoprecipitated under native conditions for 2 h. The immune complexes were boiled in 1% SDS for 5 min before dilution in immunoprecipitation buffer and PC2 immunoprecipitation. The immune complexes were precipitated with protein A coupled to Sepharose CL-4B and washed once with immunoprecipitation buffer, once with 0.5 M NaCl in PBS, and twice with PBS. They were then boiled in Laemmli sample buffer before separation by SDS-PAGE on 8.8 or 12% acrylamide gels. Gels were either treated for fluorography with Amplify (Amersham Corp.) or directly exposed to a phosphor screen and analyzed with a phosphorimager (Storm; Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics).

**Glycosidase Digestions**

Immunoprecipitated PC2 was boiled in 2% SDS in 10 mM Tris, pH 7.4, for 5 min and diluted in the digestion buffer. Endoglycosidase H digestion was performed in 100 mM sodium acetate, pH 5.5, in the presence of 10 µU/ml endoglycosidase H (Boehringer Mannheim Corp.). N-glycanase F digestion was performed in 100 mM sodium phosphate, pH 7.4, containing 0.5% Nonidet-P40 in the presence of 4 U/ml N-glycanase F (Boehringer Mannheim Corp.). Control digestions were performed with either of these buffers without enzyme. Incubations were conducted overnight at 37°C. They were stopped by addition of Laemmli sample buffer and boiled 5 min before separation by SDS-PAGE. Digestion of PC2 with N-glycanase F under native conditions was performed directly after PC2 immunoprecipitation. The incubations were conducted in the presence of 8 U/ml enzyme at 37°C for 30–120 min, with either time period providing the same results.

**Sucrose Gradient Centrifugation**

We investigated PC2 aggregation in the ER by sucrose gradient centrifugation according to Simons and collaborators (1995). Briefly, AtT-20 cells were radiolabeled and proteins were extracted as described previously for native immunoprecipitation and loaded on the top of a continuous sucrose gradient (10–40%) containing 0.1% Triton X-100. The gradients were centrifuged for 7 h at 4°C in a rotor (SW50.1; Beckman Instrs., Fullerton, CA) at 45,000 rpm. PC2 was immunoprecipitated from the fractions and separated by SDS-PAGE. The radioactivity of the gels was analyzed with a phosphorimager.

**Subcellular Fractionation and Marker Assays**

CHO/PC2 cells were grown in four 850 cm² rollers for each membrane preparation. Rollers were washed with calcium-free PBS on ice. All of the following steps were performed at 4°C. Cells were scraped in calcium-free PBS and pelleted. They were resuspended in 0.25 M sucrose in 10 mM Tris, pH 7.4, and homogenized with a stainless steel ball-bearing homogenizer. The homogenate was centrifuged for 20 min at 7,500 g. The supernatant was pelleted at 56,000 rpm in a rotor (TL 100.4; Beckman Instrs.) for 35 min. Pellets were resuspended in 1.15 M sucrose and loaded on the bottom of a discontinuous sucrose gradient composed of 1.5 ml of 0.86 M sucrose and 1.5 ml of 0.25 M sucrose. The gradient was centrifuged at 46,000 rpm in a rotor (TL 100.4; Beckman Instrs.) for 140 min. Five fractions were collected.

Galactosyl transferase activity was used as a marker for the Golgi membranes. The activity was measured according to Chaney et al. (1989), using [3H]UDP-galactose as a substrate (Amersham Corp.). The molecular chaperone BiP/GRP78 was used as a marker for the ER. The content of each fraction was assayed by immunoblotting.

**Immunoblotting**

Proteins were separated by SDS-PAGE on 8.8% gels. They were then transferred to nitrocellulose membranes that were incubated with antiserum as described previously (Shen et al., 1993). Alkaline phosphatase activity was detected using either nitroblue tetrazolium and 5-bromo-4chloro-3-indolyl phosphate as substrate or the Vistra Western blot substrate (Amersham Corp.). Membranes were analyzed with a phosphorimager (Storm; Molecular Dynamics) and quantitation of the results was performed with ImageQuant software (Molecular Dynamics).

**Enzyme Assays**

PC2 activity was measured using 200 µM Pyr-Glu-Arg-Thr-Lys-Arg-amimethylcoumarin (AMC) as a substrate as previously described (Lamango et al., 1996). The assay was performed in 100 mM sodium acetate, pH 5, containing 5 mM CaCl₂, 1% Triton X-100 in the presence of a trypsin inhibitor cocktail composed of 1 µM pepstatin, 0.28 mM tosylphenylalanine chloromethyl ketone (TPCK), 1 µM trans-epoxysuccinic acid (E64), and 0.14 mM tosyllysyl chloromethyl ketone (TLCK). All incubations were conducted at 37°C for time periods ranging from 30 min to 4 h. In some experiments, the activity was also measured in the presence of 1 µM 7B2 CT peptide, a specific inhibitor of PC2 activity (Lindberg et al., 1995). The activity was measured in triplicate and assays were reproduced with three different subcellular fraction preparations. Fluorescence of AMC was measured with a fluorometer (Cambridge Technology, Inc., Cambridge, MA). The amount of released product was calculated by reference to the fluorescence of free AMC standard.

**Results**

**7B2 Is Secreted More Slowly Than 7B2 Due to Its Slower Transport to the TGN**

Kinetic studies of the release of newly synthesized 7B2 and PC2 show that 7B2 is secreted more rapidly than PC2 when both proteins are stably coexpressed in AtT-20 cells (Zhu and Lindberg, 1995; Table I). In constitutively-secreting cells, the limiting factor in the secretion of proteins is the time required for their folding in the ER (for review see Lodish, 1988), but in neuroendocrine cells, different kinetics of secretion can also be indicative of differences in sorting at the exit from the TGN (for review see Kelly, 1985). To distinguish between these two possibilities, we studied the release of sulfated PC2 and 7B2, as sulfation is a posttranslational modification that specifically takes place in the trans-Golgi (Baueuerle and Huttner, 1987). Sulfated 7B2 and PC2 were released at the same rate into the medium (Table I). The difference between the release of newly synthesized and sulfated 7B2 and PC2 indicates, therefore, that the limiting step of proPC2 secretion is the exit from the ER. On the other hand, newly synthesized 7B2 exits from this compartment more rapidly than PC2. This is in agreement with the difference in half-life between 7B2 and proPC2, which are both cleaved in the TGN/secretory granules: the 7B2 precursor half-life is 15 min, whereas the proPC2 half-life is ~100 min in AtT-20 cells that overexpress both proteins (Zhu and Lindberg, 1995). This result is also consistent with the long retention of proPC2 in the ER already described in different cell models (Guest et al., 1992; Benjannet et al., 1993; Shen et al., 1993; Zhou and Mains, 1994), and with the subcellular localization of PC2 in transfected AtT-20 cells (Mains et al.,
Table I. Secretion of Newly Synthesized and Sulfated 7B2 and ProPC2

| Time (min) | Percent secreted | 30 min | 60 min |
|-----------|------------------|--------|--------|
| Methionine| 7B2              | 10     | 27     |
|           | PC2              | 0.3    | 15     |
| Sulfate   | 7B2              | 29     | —      |
|           | PC2              | 31     | —      |

AtT-20 cells were labeled for 10 min with [35S]methionine and cystine (Methionine), or for 15 min with [35S]sulfate (Sulfate). They were then chased for the indicated time periods. 7B2 and PC2 were immunoprecipitated and separated by SDS-PAGE. The radioactivity in the gels was analyzed with a phosphorimager. The percent of 7B2 and PC2 released per total labeled 7B2 and PC2, respectively, was calculated in duplicate and the experiments were reproduced twice.

1995; Creemers et al., 1996), and in anterior pituitary lactotrophs (Muller et al., 1997).

**Newly Synthesized 7B2 and ProPC2 Do Not Acquire Binding Capacity at the Same Rate**

Previous studies have proposed that the binding of 7B2 to proPC2 occurs in the ER (Benjannet et al., 1995; Braks et al., 1996) and that the two proteins are transported as a complex from the ER to the trans-Golgi (Benjannet et al., 1995). According to this hypothesis, 7B2 and proPC2 should exit from the ER at the same rate. This is in apparent contradiction with the differences in transport from the ER to the TGN that we have described above. Both hypotheses could be reconciled if 7B2 and proPC2 did not acquire the capacity to bind each other at the same rate before they exit the ER, i.e., if 7B2 could bind PC2 rapidly after synthesis, but proPC2 required a longer time before it could bind 7B2. To determine if this is the case, we studied the kinetics of association of 7B2 and proPC2. The same radiolabeled cell extracts were immunoprecipitated with antisera directed against 7B2 and PC2 (Fig. 1). We also observed the same kinetics of processing of 7B2 in AtT-20 cells expressing 7B2 in the absence of proPC2 (results not shown). Therefore, binding of proPC2 has no effect on 7B2 transport kinetics to the TGN. Taken together, these data support the idea that newly synthesized 7B2 binds only to unlabeled proPC2 synthesized before the pulse and accumulated in the ER, and that 7B2 exits this compartment sooner after synthesis than proPC2. Thus, proPC2 requires a longer time period in the ER environment before it can bind to 7B2. This suggested that proPC2 must fold before it can bind to 7B2, whereas 7B2 folding is apparently not a limiting step in the formation of the complex.

**7B2 Is Not Involved in ProPC2 Folding**

To investigate the chaperone activity of 7B2 on proPC2 folding, several strategies were attempted to establish a proPC2 folding assay. First we used digestion of immunoprecipitated proPC2 by N-glycanase F under native conditions to study proPC2 conformational changes (Fig. 2). The rationale of this experiment is that the conformation changes that occur during folding modify the access of the proPC2 coimmunoprecipitated by the antiserum directed against 7B2 continues to increase beyond 60 min (see Fig. 8).

Unlike the 7B2 antiserum, the antiserum directed against proPC2 could coimmunoprecipitate radiolabeled 7B2 immediately after the pulse (Fig. 1). This result implied that, unlike PC2, newly synthesized 7B2 acquires proPC2 binding capacity very soon after synthesis. The amount of 7B2 coimmunoprecipitated did not increase with time (Fig. 1).

The experiments presented in Fig. 1 also show that 7B2 is cleaved with the same kinetics whether it is bound to proPC2 or not. Cleavage of 7B2 is a marker for 7B2 transport from the ER to the trans-Golgi, as it occurs after sulfation (Paquet et al., 1994). Therefore, proPC2-bound 7B2 is transported to the TGN at the same rate as total 7B2 (Fig. 1). We also observed the same kinetics of processing of 7B2 in AtT-20 cells expressing 7B2 in the absence of proPC2 (results not shown). Therefore, binding of proPC2 has no effect on 7B2 transport kinetics to the TGN. Taken together, these data support the idea that newly synthesized 7B2 binds only to unlabeled proPC2 synthesized before the pulse and accumulated in the ER, and that 7B2 exits this compartment sooner after synthesis than proPC2. Thus, proPC2 requires a longer time period in the ER environment before it can bind to 7B2. This suggested that proPC2 must fold before it can bind to 7B2, whereas 7B2 folding is apparently not a limiting step in the formation of the complex.
ProPC2 Must Fold Before It Can Bind 7B2

To provide more direct evidence for the requirement of proPC2 folding before 7B2 binding, we studied the effect of the prevention of proPC2 folding on 7B2 binding. ProPC2 folding was impaired using two different approaches: treatment of the cells with DTT, and mutation of PC2 glycosylation sites.

DTT treatment prevents the formation of disulfides in newly synthesized proteins (Braakman et al., 1992) but does not affect the secretory pathway (Lodish and Kong, 1993). In AtT-20 cells treated with DTT, proPC2 conversion into PC2 was inhibited (Fig. 4a). ProPC2 was not secreted, but was degraded within the cells. On the other hand, DTT had no effect on the cleavage nor the secretion of 7B2 (Fig. 4b). When cells were incubated 30 min in the presence of DTT, the binding of proPC2 to 7B2 was inhibited (Fig. 4c). 7B2 binding was restored, however, 30 min after the washout of DTT from the medium (Fig. 4c). Therefore, formation of the disulfide bridges is required before association of 7B2 with proPC2.

To more specifically prevent proPC2 folding, we mutated its three N-glycosylation sites. The involvement of core-glycosylation in the folding of proteins in the ER has been proposed and mutation of the glycosylation sites of some secreted proteins results in their misfolding (for review see Helenius, 1994). The unfolded state of the nonglycosylated proPC2 was demonstrated by its increased binding to the molecular chaperone BiP/GRP78 (Fig. 5a), which binds to unfolded proteins and folding intermediates (Gething and Sambrook, 1992; Ou et al., 1993). If 7B2 were a chaperone, we might expect that its coexpression with proPC2 would modify the pattern of digestion of native proPC2 by N-glycanase F. AtT-20 cells that express PC2 alone or also 7B2 were labeled for 10 min and directly extracted or chased for 30 min before extraction. ProPC2 was immunoprecipitated under native conditions and digested with N-glycanase F as described in Materials and Methods. The reaction was stopped by addition of Laemmli sample buffer and boiled for 5 min before separation of proteins by SDS-PAGE.

Figure 2. 7B2 does not modify the pattern of digestion of native proPC2 by N-glycanase F. AtT-20 cells that express PC2 alone or also 7B2 were labeled for 10 min and directly extracted or chased for 30 min before extraction. ProPC2 was immunoprecipitated under native conditions and digested with N-glycanase F as described in Materials and Methods. The reaction was stopped by addition of Laemmli sample buffer and boiled for 5 min before separation of proteins by SDS-PAGE.

Figure 3. 7B2 does not modify the association of proPC2 with calnexin. AtT-20 cells that express PC2 alone (A/P) or also 7B2 (A/P/7) were labeled for 10 min and directly extracted or chased for 30 min before extraction. Calnexin was immunoprecipitated under native conditions. The immune complexes were boiled in 1% SDS for 5 min. They were then diluted in immunoprecipitation buffer and PC2 was immunoprecipitated.
Mutant proPC2 binding to BiP was stable over a 30-min chase (Fig. 5 a). This mutant did not bind to calnexin, which is in agreement with the lectin binding capacity of this chaperone (Ou et al., 1993). Unglycosylated proPC2 did not form more aggregates in the ER than wild-type proPC2, as assayed by centrifugation on 10–40% sucrose gradient (not shown). The unglycosylated mutant proPC2 could not be converted to mature PC2, as assayed by centrifugation on 10–40% sucrose gradient (not shown). The unglycosylated mutant proPC2 could not be converted to mature PC2 (Fig. 5 b). Two bands of approximately the same mol wt are present in the mutant lane (3NQ); the lower one corresponds to unglycosylated proPC2 and the upper one corresponds to BiP, as the immunoprecipitation was done under native conditions. Mutated proPC2 was not released in the medium but was degraded in the cells. This unfolded mutant form of proPC2 was also unable to bind 7B2 (Fig. 5 c).

Taken together, these experiments demonstrate that prevention of the folding of proPC2 results in the loss of 7B2 binding capacity and, thus, that proPC2 must fold before it can bind 7B2. These data are in agreement with the lack of involvement of 7B2 in the early steps of proPC2 folding.

**7B2 Increases the Transport Rate of ProPC2 from the ER to the Golgi**

We have already shown that the half-life for the conversion of proPC2 decreases considerably in the presence of 7B2 (Zhu and Lindberg, 1995). The results presented above supported the notion that this effect is not mediated by an increase in the proPC2 folding rate. Thus, 7B2 could act by increasing either the rate of transport, or the rate of propeptide cleavage, or both. To study proPC2 transport to the Golgi apparatus, we investigated the acquisition of endoglycosidase H resistance. It should be noted that because of carbohydrate heterogeneity, PC2 never becomes completely resistant to endoglycosidase H digestion. The demonstration of the presence of an endoglycosidase H-resistant form of proPC2/PC2 requires, therefore, a control N-glycanase F digestion (Bennett et al., 1992). Even after release into the medium, only one or two of the three potential glycosylation sites have acquired endoglycosidase H resistance, whereas the others remain sensitive (Mains et al., 1995; Creemers et al., 1996). As the goal of this experiment was to differentiate between 7B2 effects on the transport of proPC2 and on its conversion into PC2, we needed to work under experimental conditions that prevent the cleavage of the PC2 propeptide. This was necessary since previous studies have shown that the conversion of proPC2 into PC2 is very closely related in time to the acquisition of endoglycosidase H-resistance: i.e., these studies could not identify any proPC2 partially resistant to endoglycosidase H digestion. The demonstration of the presence of an endoglycosidase H-resistant form of proPC2/PC2 requires, therefore, a control N-glycanase F digestion (Bennett et al., 1992). Even after release into the medium, only one or two of the three potential glycosylation sites have acquired endoglycosidase H resistance, whereas the others remain sensitive (Mains et al., 1995; Creemers et al., 1996). As the goal of this experiment was to differentiate between 7B2 effects on the transport of proPC2 and on its conversion into PC2, we needed to work under experimental conditions that prevent the cleavage of the PC2 propeptide. This was necessary since previous studies have shown that the conversion of proPC2 into PC2 is very closely related in time to the acquisition of endoglycosidase H-resistance: i.e., these studies could not identify any proPC2 partially resistant to endoglycosidase H nor any mature PC2 completely sensitive to endoglycosidase H (Mains et al., 1995; Creemers et al., 1996; Muller, L., and I. Lindberg, unpublished results).

ProPC2 conversion requires an acidic pH, which corresponds to the TGN and the secretory granules (Shennan et al., 1995; Lamango et al., 1996). To block proPC2 cleavage, we prevented the acidification of the pH in these intracellular compartments with bafilomycin A1 treatment. Bafilomycin A1 is a specific inhibitor of the vacuolar H+ -ATPase responsible for the acidification of pH in the TGN and secretory granules (Bowman et al., 1988). This treatment completely inhibited the conversion of proPC2 in
cells that express proPC2 alone and exhibit a low rate of conversion, as well as in cells that coexpress PC2 and 7B2 and, therefore, possess a much higher rate of processing (Fig. 6 a). This drug did not affect 7B2 cleavage (Fig. 6 b), which is in agreement with the proposed cleavage of 7B2 by furin (Paquet et al., 1994), an enzyme active at neutral pH (Bresnahan et al., 1990; Hatazawa et al., 1992; Molloy et al., 1992). Bafilomycin A1 had no effect on the binding of 7B2 to proPC2 (Fig. 6 b). It also did not prevent proPC2 transport from the ER to the Golgi, as demonstrated by the acquisition of endoglycosidase H resistance (Fig. 6 c). After a 2-h chase in the presence of bafilomycin A1, endoglycosidase H–digested PC2 migrated as a doublet (Fig. 6 c). The lower band comigrated with N-glycanase F–digested PC2 and, therefore, corresponded to endoglycosidase H–sensitive PC2. The upper band corresponded to PC2 partially resistant to endoglycosidase H. This result demonstrated that some of the newly synthesized PC2 had acquired complex carbohydrates in the Golgi in the presence of bafilomycin A1. This is in agreement with previous studies that have shown that bafilomycin A1 does not impede intracellular transport to the Golgi, but only between the TGN and the plasma membrane (Umata et al., 1990; Yoshimori et al., 1991).

These experimental conditions allowed us to study the effect of 7B2 on proPC2 transport to the Golgi. Cells expressing PC2 alone or PC2 and 7B2 were treated with bafilomycin A1. ProPC2 was immunoprecipitated, denatured, and digested with endoglycosidase H. Fig. 7 shows that the coexpression of 7B2 with PC2 increased proPC2 acquisition of endoglycosidase H resistance. Acquisition of endoglycosidase H resistance was very low (≈20% after 120 min) in cells that express PC2 alone, in agreement with the low maturation rate of proPC2 in these cells (Fig. 6 a) (Mains et al., 1995; Zhu and Lindberg, 1995). Coexpression of 7B2 increased the level of endoglycosidase H–resistant PC2 to ≈65% after a 120-min chase. The kinetics of acquisition of endoglycosidase H resistance paralleled those of proPC2 maturation that we have already described in these cells (Fig. 6 a; Zhu and Lindberg, 1995).

We also investigated the kinetics of acquisition of endoglycosidase H resistance of 7B2-bound proPC2 (Fig. 8). ProPC2 was immunoprecipitated under native conditions with the antiserum directed against 7B2, and denatured before digestion with endoglycosidase H. After a 30-min chase, 7B2-bound proPC2 migrated as a faint doublet corresponding to equal amounts of endoglycosidase H–sensitive and –resistant proPC2s. If the 7B2–proPC2 complexes were accumulated in the ER, the endoglycosidase H–sensitive 7B2-bound proPC2 should increase with time. If, on the other hand, the 7B2–proPC2 complexes were transported to the Golgi rapidly after their formation, the endoglycosidase H–resistant 7B2-bound proPC2 should increase. The data in Fig. 8 support the latter hypothesis. Indeed, the endoglycosidase H–resistant 7B2-bound proPC2 (Fig. 8) increased with time in parallel with the endoglycosidase H–resistant total PC2 immunoprecipitated with the PC2 antiserum (Fig. 8). This strongly suggests that the effect observed in Fig. 7 was mediated by the binding of proPC2 to 7B2. This result also demonstrates that proPC2 binds 7B2 in the ER and is then rapidly transported in the Golgi apparatus where it acquires complex oligosaccharides. Thus, the facilitation of proPC2 conversion by 7B2 occurs at least in part through an increase of its transport from the ER to the Golgi.

**7B2 Induces the Conversion and the Activation of ProPC2 from Golgi-enriched Subcellular Fractions**

We have already shown that the coexpression of PC2 with the 21-kD NH2-terminal domain of 7B2 is required for the production of activatable proPC2 by CHO cells (Zhu and Lindberg, 1995). To study this requirement, we reconstituted proPC2 activation by adding recombinant 7B2 to subcellular fractions from CHO cells that express proPC2 alone. This system allowed us to study in vitro the effect of 7B2 on both the maturation and the activity of PC2.

The homogenate was first centrifuged at 7,500 *g* for 20
The supernatant (S1) was pelleted and the pellet separated by flotation on a sucrose gradient. We characterized the Golgi fraction using galactosyl transferase activity as a marker and the ER fraction using BiP/GRP78 as a marker (Table II). The Golgi fraction contained a small quantity of BiP which could be relevant to a small amount of contamination by ER membranes as well as to the cycling of BiP between the cis-Golgi and the ER (for review see Pelham, 1991; Hammond and Helenius, 1994). The proPC2 content of these fractions was quantitated by immunoblotting (Table II), using proPC2 purified from CHO cell medium as a standard (Lamango et al., 1996). ProPC2 was slightly more concentrated in the Golgi membranes, but the total content was higher in the ER fraction.

We analyzed the molecular forms of PC2 present in each fraction after an incubation at 37°C at pH 5 in the presence of 5 mM calcium. In the absence of recombinant 21-kD 7B2, proPC2 was detected in the three fractions, and a very small amount of the 71- and the 66-kD PC2 forms could also be detected in the Golgi fraction (Fig. 9a). When recombinant 21-kD 7B2 was added, proPC2 was converted into mature PC2 only in the Golgi fraction. This was not accompanied by any increase of the 71-kD form. There was no detectable mature PC2 in the S1 and ER fractions. PC2 activity was measured by incubating membranes under the same conditions using Pyr-Glu-Arg-Thr-Lys-Arg-AMC as a substrate. The activity was measured with 1 μg of total protein, and results were normalized to the proPC2 content of each fraction (Fig. 9b). No significant activity could be detected in any fraction in the absence of recombinant 21-kD 7B2, even in the Golgi fraction where a very small amount of 66-kD PC2 was detected by immunoblotting. However, 7B2 could induce enzyme activity in the different fractions, even in the S1 and ER, in which we could not detect mature PC2 by immunoblotting. The activity was highly enriched in the Golgi fraction compared to the S1 fraction, and very low in the ER fraction. When ER and Golgi fractions were mixed and the amount of total proteins doubled, the activity detected was inhibited by ~35% as compared with Golgi membranes alone (not shown). This result potentially implied the presence of an inhibitory factor in the ER fraction. This inhibition was not detected if the Golgi membranes were preactivated at an acidic pH at 37°C in the presence of calcium, thus indicating that it is the activation of proPC2 that is inhibited and not PC2 activity. Indeed, immunoblotting the mixed membranes showed that proPC2 conversion was partially inhibited (not shown). Conversion of proPC2 in the ER fraction could not be detected, and total PC2 activity in the ER fraction was only 10% of the total Golgi PC2 activity. Thus, even if the amount of proPC2 contained in the ER fraction and activated in the presence of 7B2 is underestimated due to the presence of an inhibitory factor, the main source of activatable proPC2 is the Golgi fraction.

When membranes were incubated in the absence of detergent, proPC2 could be cleaved but the activity detected was inhibited by 75% (not shown). This indicated that the activity is located within membrane-bound compartments. The identity of this activity as PC2 was demonstrated by its complete inhibition by the 7B2 CT peptide (Fig. 9b), a PC2-specific inhibitor (Lindberg et al., 1995). This peptide could not, however, prevent proPC2 conversion in Golgi membranes (Fig. 9a). As a control, we also performed the same experiments with CHO cells that do not overexpress PC2. Recombinant 21-kD 7B2 could not induce enzyme activity in any subcellular fraction from these cells (data not shown).
not shown), thus confirming that the activity measured corresponds to PC2 and not to an activity endogenous to CHO cell Golgi membranes or to lysosomal contamination.

**7B2-induced Activation of PC2 is pH and Calcium dependent**

We investigated the pH dependency of proPC2 conversion and PC2 activity measurable in the presence of 7B2 in the Golgi fraction. ProPC2 maturation was maximal at pH 5. It was much lower at pH 5.5 and was not detected when the pH was higher than this (Fig. 10 a). This correlated with the enzymatic activity which exhibited a pH optimum of 5 (Fig. 10 a). When the Golgi fraction was incubated in the absence of calcium and in the presence of EDTA, proPC2 maturation was partly inhibited, and PC2 activity was completely inhibited (Fig. 10 b). These results are consistent with the data available concerning PC2 activity (Bennett et al., 1992; Lindberg et al., 1995; Shennan et al., 1995; Lamango et al., 1996).

**7B2 Protects ProPC2 from Inactivation at Low pH**

In the experiments described above, the conversion of proPC2 was not complete: only 20% of the proPC2 present in the Golgi fraction was converted into mature PC2 (Fig. 9 a). This proportion could not be increased by extending the incubation period (not shown). We studied, therefore, the role of 7B2 and low pH independently by preincubating the Golgi fraction under different conditions and in the presence of detergent. When the membranes were preincubated at pH 5 in the absence of 7B2, no mature PC2 was formed (Fig. 11 a). Subsequent addi-

### Table II. Characterization of Subcellular Fractions

|          | S1 | Golgi | ER |
|----------|----|-------|----|
| Galactosyl transferase (cpm/µg) | 72 | 260 | 44 |
| BiP (A.U.) | 130 | 51 | 136 |
| proPC2 + PC2 (µg/mg protein) | 19 | 35 | 29 |
| proPC2 + PC2 (µg) | 93 | 14 | 30 |

Subcellular fractions were assayed for galactosyl transferase activity as described in Materials and Methods. Proteins of the fractions were separated by SDS-PAGE and transferred to nitrocellulose. BiP and PC2 immunoreactivity was detected using the Vistra Western blot substrate and quantitated with a phosphorimager. BiP immunoreactivity is expressed in arbitrary units (A.U.). The total PC2 content was calculated using PC2 purified from CHO cell medium as a reference.
tion of 7B2 to these membranes did not result in the production of mature PC2 (Fig. 11 b), nor of PC2 activity (Fig. 11 c). We hypothesize that exposure of proPC2 to an acidic pH results in its irreversible inactivation, and that 7B2 has a protective role on proPC2 activatable conformation. We have previously observed that 7B2 can protect PC2 from thermal denaturation (Lamango et al., 1996).

When membranes were preincubated in the presence of the 21-kD 7B2 at pH 7.4, no mature PC2 was formed (Fig. 11 a). Lowering the pH of the incubation medium to 5, however resulted in the conversion of proPC2 into PC2 (Fig. 11 b), and in the detection of PC2 activity (Fig. 11 c). Such preincubation of the membranes for longer periods up to 2 h could not significantly increase the PC2 activity detected during the subsequent incubation, however (data not shown). This suggests that 7B2 does not per se confer an activatable conformation to proPC2, but rather interacts with a conformation that proPC2 has already achieved and that is competent for activation.

**Discussion**

The neuroendocrine protein 7B2 is a PC2-specific binding protein that facilitates proPC2 maturation in vivo (Zhu and Lindberg, 1995) as well as in vitro (Braks and Martens, 1995) and is required for the expression of PC2 activity in the medium of CHO cells (Zhu and Lindberg, 1995). The mechanism of these interactions has, however, not yet been determined. 7B2 could be involved in several steps of proPC2 posttranslational processing: folding in the ER, transport to the Golgi, or conversion into mature PC2.

**Binding of 7B2 to ProPC2 Is a Late ER Event**

7B2 and proPC2 bind within the secretory pathway (Braks and Martens, 1994). Both proteins are subject to proteolytic processing after their sulfation in the TGN (Benjannet et al., 1993; Paquet et al., 1994). There is, however, some discrepancy as to how these cleavages affect the concominoprecipitation of the two proteins (Fig. 1; Benjannet et al., 1995; Braks et al., 1996). These studies all agree on the binding of the 27-kD form of 7B2 to proPC2. Experiments using brefledin A have demonstrated that this binding occurred before the molecules reach the TGN (Benjannet et al., 1995; Braks et al., 1996). As this fungal metabolite blocks intracellular transport out of the ER and fuses the Golgi sacules with the ER cisternae, it does not constitute an appropriate tool to distinguish ER from Golgi events (see Klausner et al., 1992). The coimmunoprecipitation of 7B2 with endoglycosidase H–sensitive proPC2 that we describe here more directly demonstrates that the complex is actually formed in the ER. Sulfation experiments suggest that 7B2 and proPC2 are transported to the TGN as a complex (Benjannet et al., 1995), which is in agreement with our analysis of the endoglycosidase H resistance of the 7B2-bound proPC2. However, the kinetics of cleavage and secretion of 7B2 and PC2 are very different from each other (Guest et al., 1992; Benjannet et al., 1993; Shen et al., 1993; Paquet et al., 1994; Zhou and Mains, 1994; Zhu and Lindberg, 1995). These kinetics are at apparent variance with the hypothesis of the binding of 7B2 to proPC2 in the ER followed by transport of the complex to the Golgi. The comparison between the release of newly synthesized and sulfated 7B2 and PC2 demonstrate that 7B2 and PC2 are not transported from the ER to the Golgi at the same rate after their syntheses. These two proteins, therefore, do not spend the same amount of time in the ER before they can bind to each other. Indeed, our kinetic experiments demonstrate that whereas newly synthesized 7B2 could bind to proPC2 soon after synthesis, newly synthesized proPC2 could not bind to 7B2 before a further chase. The 7B2–proPC2 complex is formed, therefore, in the ER with 7B2 and proPC2 molecules that have not been synthesized at the same time: proPC2 is older than the 7B2 to which it is bound. Finally, analysis of 7B2-bound proPC2 acquisition of endoglycosidase H resistance demonstrated that the complex is not accumulated in the ER but is rapidly transported to the Golgi. Therefore, the association of 7B2 with proPC2 represents a late event in the time period that proPC2 spends in the ER.

**7B2 Is Not Involved in ProPC2 Folding**

Previous studies of PC2 and 7B2 biosynthesis provided evidence for another important difference between 7B2 and proPC2 exit from the ER: whereas radiolabeled 7B2 exits the ER as a homogenous pool within a short time span, the exit of a radiolabeled population of proPC2 molecules is widely spread in time (Guest et al., 1992; Benjannet et al., 1993; Shen et al., 1993; Paquet et al., 1994; Zhou and Mains, 1994; Zhu and Lindberg, 1995). In this paper we have also shown that whereas proPC2 secretion is sensitive to DTT treatment, 7B2 secretion is not, even though both proteins contain disulfide bridges. These results suggest differing requirements for 7B2 and proPC2 folding in the ER. The time spent in the ER is directly related to the folding of the proteins (for review see Lodish, 1988), and
the long period that proPC2 requires in the ER environment could reflect the complex folding of this particular protein.

Like other members of the subtilisin-like convertase family, PC2 is synthesized as a proenzyme. Though the primary structure of the propeptide is poorly conserved between the PCs, its general organization is similar. In the case of subtilisin, the propeptide acts as an intramolecular chaperone (Zhu et al., 1989). This chaperone activity of the propeptide has been extended to the other subtilisin-like enzymes based on structural homologies (Siezen et al., 1995). Because PC2 is retained in the ER longer than the other PCs, it could be hypothesized that it may require some specific intermolecular chaperone activities. 7B2 could fulfill such a function, even though it is a secretory protein. Whereas the molecular chaperones present within the secretory pathway were at first restricted to ER-resident proteins, this notion has been extended, as members of the heat shock protein family cycle between the Golgi and the ER (Hammond and Helenius, 1994; Satoh et al., 1996) and as other secretory proteins, such as the receptor-associated protein (RAP), have been shown to exhibit chaperone activity in the ER (Bu and Rennke, 1996; Willnow et al., 1996). However, our experiments indicate that 7B2 does not modify the aggregation state of proPC2 in the ER nor the sensitivity of native proPC2 to N-glycanase F digestion. 7B2 does not modify the pattern of association of proPC2 with traditional chaperones like BiP/GRP78 or calnexin. We could also demonstrate that prevention of proper proPC2 folding suppresses rather than enhances 7B2 binding. Two additional lines of evidence are in agreement with the lack of involvement of 7B2 in proPC2 folding. First, preincubating subcellular membranes with recombinant 7B2 for increasing periods did not result in an increase of proPC2 activation, suggesting that 7B2 alone could not provide a proper conformation, but could only act on already properly folded proPC2. Second, swapping the PC2 proregion with furin and PC1 propeptide demonstrated that the PC2 propeptide is required for the binding of proPC2 to 7B2, but is not sufficient to confer 7B2 binding to PC1 (Zhu, X., unpublished results), thus suggesting that the propeptide is required for the proper folding of proPC2 (Siezen et al., 1995) rather than directly for 7B2 binding. Taken together, these various lines of evidence lead to the conclusion that 7B2 is not involved in the early steps of proPC2 folding and cannot be considered a bona fide folding chaperone.

7B2 Increases ProPC2 Transport to the Golgi

As 7B2 binds proPC2 in the ER, we investigated the effect of this binding on the exit from the ER and transport to the Golgi under experimental conditions that prevent proPC2 cleavage. Coexpression of 7B2 resulted in increased proPC2 acquisition of endoglycosidase H resistance, which paralleled the facilitation of proPC2 maturation (Zhu and Lindberg, 1995). This effect on proPC2 transport to the Golgi occurred through the binding of proPC2 to 7B2. 7B2 could act by stabilizing proPC2 in a conformation that is more transport competent. Indeed, we could demonstrate that 7B2-bound proPC2 does not accumulate in the ER but exits rapidly after the formation of the complex. Though potential differences in affinity between the antiserum directed against PC2 and that directed against 7B2 do not permit us to quantitate the proportion of proPC2 that reaches the Golgi bound to 7B2, our immunoprecipitation data indicate that the same amounts of total proPC2 and 7B2-bound proPC2 acquire endoglycosidase H resistance. These data imply that in AtT-20 cells, proPC2 reaches the Golgi mainly as a complex with 7B2, which could explain the high efficiency of 7B2 in promoting proPC2 maturation in AtT-20 cells. Studies using several constitutive cell lines that lack 7B2 have demonstrated, however, that proPC2 can, in fact, exit the ER in the absence of 7B2 (Benjannet et al., 1993; Shen et al., 1993). Rather than actually providing a transport-competent conformation to proPC2, which would be indicative of a folding activity, or providing transport competence to proPC2, which is apparently not required in constitutive cell lines, we propose that 7B2 stabilizes a transport-competent conformation of proPC2. This stabilization results in an increase in transport to the Golgi, which would nevertheless occur, albeit at a slower rate, without 7B2.

7B2 Is Required for ProPC2 Activation

Apart from the facilitation of proPC2 maturation, which could be explained by the increased transport of proPC2 only, 7B2 is also required to obtain activatable proPC2 from

Figure 12. Schematic representation of the steps of proPC2 posttranslational maturation in which 7B2 is involved. (1) 7B2 binds to unfolded proPC2. (2) The proPC2–7B2 complex formed in the ER is transported to the Golgi at a higher rate than proPC2 is alone (double arrow). (3) 7B2-bound proPC2 can undergo activation when it reaches the TGN/secretory granules which possess the requisite low pH and high calcium environment. PC2 propeptide is represented by the VV IV VV only two of the three potential glycosylation sites of PC2 are represented by the Y. The acquisition of endoglycosidase H resistance by one of the two oligosaccharides is indicated by C.
in this folding, 7B2 does not seem to be actively involved
docrine secretory pathway (Fig. 12). ProPC2 folding is a
also autocatalytic, or if another enzyme is involved.
tide that results in the production of an inactive enzyme, is
active cleavage of proPC2, i.e., the cleavage of the propep-
tive cleavage, i.e., the cleavage of the propeptide. We could observe, however, the forma-
tion of some 66-kD mature PC2 without detecting PC2 ac-
tivity when membranes were incubated in the absence of
turmode, or inactive form. The cleavage site of proPC2 propeptide
ever, does not permit us to determine the mechanistic dif-
parison of the propeptide. We could observe, however, the forma-
the presence of 7B2 in the mem-
brane compartments that contain proPC2 (not shown).
ese experimental conditions resemble a physiological situa-
tion in which proPC2 reaches an acidic pH in the
TG with removal of the propeptide in the absence of
7B2. Indeed, proPC2 is released as a mixture of proPC2 and processed PC2 from constitutive cells lacking 7B2 (Benjannet et al., 1993; Shen et al., 1993). This released pro-
PC2/PC2 is neither active nor activatable, however (Shen et al., 1993). In fact, 7B2 coexpression did not modify the kinetics of proPC2 secretion from CHO cells, but in-
creased the ratio of proPC2/PC2 present in the secretion
medium (Liu, J., I. Lindberg, and L. Muller, unpublished observations). The same increased ratio of proPC2/PC2 in the
presence of 7B2 has been described in BSC40 cells (Benjannet et al., 1995). We have also recently identified a point mutation in the PC2 catalytic domain that uncouples removal of the propeptide from actual activation, i.e., ac-
quision of enzymatic activity: this mutation results in an
increased rate of propeptide removal, but also a lack of
7B2 binding and of enzyme activity (Zhu, X., unpublished observations). Taken together, these data demonstrate that whereas the PC2 propeptide can be cleaved from proPC2 in the absence of 7B2, this cleavage only results in
the formation of inactive enzyme. These data support a di-
rect link between 7B2 and proPC2 activation.

Our experimental system of in vitro activation of the pro-
PC2 present in the Golgi membranes of CHO cells, how-
never, does not permit us to determine the mechanistic dif-
ference between the conversion of proPC2 to an active
or inactive form. The cleavage site of proPC2 propeptide
(RKKR) is a consensus site for several PCs, including fur-
in, which is responsible for the cleavage of proproteins in
the constitutive pathway. The autocatalytic conversion of
proPC2 has been proposed (Matthews et al., 1994), and is
by analogy with furin, PC1 and subtilisin most probably in-
tramolecular, but it is not known whether the unproduc-
tive cleavage of proPC2, i.e., the cleavage of the propep-
tide that results in the production of an inactive enzyme, is
also autocatalytic, or if another enzyme is involved.

Taken together, our results provide a general scheme of
the interaction of 7B2 with proPC2 within the neuroen-
docrine secretory pathway (Fig. 12). ProPC2 folding is a
slow process. Whereas PC2 propeptide might be involved in
this folding, 7B2 does not seem to be actively involved in
the early steps. This process could consist in the cycling
of proPC2 between partially folded intermediates and
an unstable folded transport-competent form which could
be stabilized by 7B2. This stabilization results in the in-
crease of proPC2 transport to the Golgi. Once the com-
plex has reached the Golgi, proPC2 oligosaccharides un-
dergo their maturation before processing of proPC2 in the
TGN/secretory granules. The low pH and high calcium of
these intracellular compartments triggers the conversion
of proPC2 into mature PC2. The proPC2 bound to 7B2
will undergo an activation process, whereas the free proPC2
will also be processed, most probably in the same subcellu-
lar compartments, but will not thereby be activated. Our
experiments demonstrate, therefore, that 7B2, which is
neither a folding chaperone nor a subunit of the enzymatic
complex, can actually induce the activation of proPC2 ac-
cording to a unique pathway. Our experimental system of
proPC2 maturation in vitro from subcellular fractions
should permit us to study more precisely the molecular
mechanism of PC2 activation in the presence of 7B2.

We thank J. Finley (Louisiana State University Medical Center) for her
expertise with cell culture. We acknowledge J. Liu (Louisiana State Uni-
versity Medical Center) and Y. Fortenberry (Louisiana State University Medical Center) who initiated some of these experiments. We also wish to
thank P. Arvan (Albert Einstein College of Medicine) and G. Martens
(University of Nijmegen) for gifts of antisera, and E. Etienne (Collège de
France, Paris, France) for photographic work.

This work was supported by National Institutes of Health Grants (DA05864 and DK49703) to I. Lindberg, who was supported by a Re-
search Scientist Development Award (K02 DA0204) from National Insti-
tute of Drug Abuse (NIDA). X. Zhu was supported by a postdoctoral fel-
lowship (F32 DA05700) from NIDA.

Received for publication 17 June 1997 and in revised form 9 September 1997.

References

Ayoubi, T.A.Y., H.L.P. van Duijnoven, W.J.M. van de Ven, B.G. Jenks, E.W. Roubos, and G.J.M. Martens. 1990. The neuroendocrine polypeptide 7B2 is a precursor protein. J. Biol. Chem. 265:15644–15647.

Baenerke, P.A., and W.B. Huttner. 1987. Tyrosine sulfation is a trans-Golgi-spe-
cific protein modification. J. Cell Biol. 105:2655–2664.

Benjannet, S., N. Rondeau, L. Paquet, A. Boudreault, C. Lazure, M. Cretenin, and N.G. Siedah. 1993. Comparative biosynthesis, glycosylation, and effi-
 ciency of prosesgment cleavage of the prohormone convertases PC1 and PC2. Biochem. J. 294:735–743.

Benjannet, S., D. Savaria, M. Cretenin, and N.G. Siedah. 1995. 7B2 is a specific intracellular binding protein of the prohormone convertase PC2. J. Neuro-
chem. 64:2305–2311.

Bennett, D.L., E.M. Bailyes, E. Nielsen, P.C. Guest, N.G. Rutherford, S.D. Arden, and J.C. Hutton. 1992. Identification of the type 2 proenkephalin process-
ing endopeptidase as PC2, a member of the eukaryotic subtilisin family. J. Biol. Chem. 267:15229–15236.

Bowman, E.J., A. Siebers, and K. Altendorf. 1988. Bafilomycins: a class of in-
hibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl. Acad. Sci. USA. 85:7972–7976.

Braakman, I., J. Helenius, and A. Helenius. 1992: Manipulating disulfide bond
formation and protein folding in the endoplasmic reticulum. EMBO (Eur. Mol. Biol. Organ.) J. 11:1717–1722.

Braks, J.A.M., and G.J.M. Martens. 1994. 7B2 is a neuroendocrine chaperone
that transiently interacts with prohormone convertase PC2 in the secretory
pathway. Cell. 78:263–273.

Braks, J.A.M., and G.J.M. Martens. 1995. The neuroendocrine chaperone 7B2
can enhance in vitro POMC cleavage by prohormone convertase PC2. FEBS (Fed. Eur. Biochem. Soc.) Lett. 371:154–158.

Braks, J.A.M., A.M. Van Horsen, and G.J.M. Martens. 1996. Dissociation of
the complex between the neuroendocrine chaperone 7B2 and prohormone
convertase PC2 is not associated with proPC2 maturation. Eur. J. Biochem. 238:505–510.

Bresnahan, P.A., R. Leduc, L. Thomas, J. Thormer, H.L. Gibson, A.J. Brake, P.J. Barr, and G. Thomas. 1990. Human fur gene encodes a yeast KEX2-like
endoprotease that cleaves pro-beta-NFG in vivo. J. Cell Biol. 111:2851–2859.

Bu, G., and S. Kemke. 1996. Receptor-associated protein is a folding chaper-
...
Involvement of 7B2 in ProPC2 Transport and Activation

one for low density lipoprotein-receptor-related protein. J. Biol. Chem. 271: 22218–22224.

Cheney, W., S. Sundaram, N. Friedman, and P. Stanely. 1989. The Lec4A CHO glycosylation mutant arises from miscompartimentalization of a Golgi glyco-
sytransferase. J. Cell Biol. 109:2089–2096.

Creese, F.I., T.J. Strauss, F.A. Bright, J.W. Van de Loo, E. Jansen, W.J.M. Van de Ven, and J.C. Hutton. 1996. Identification of a transferable sorting domain for the regulated pathway in the prohormone convertase PC2. J. Biol. Chem. 271:25284–25291.

De Boer, D., M. Mandruzzato, D. Mulder, C. Lazure, K. Nakayama, M. Ben-
dayan, and N.G. Seidah. 1996. The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. J. Cell Biol. 135:1261–1275.

Feng, W., J.R. Huth, S.E. Norton, and R.W. Ruddon. 1995. Asparagine-linked oligosaccharides mediate human chorionic gonadotropin β-subunit folding but not assembly of preformed β with α. Endocrinology. 138:52–61.

Gething, M.J., and J. Sambrook. 1992. Protein folding in the cell. Nature (Lond.). 355:33–45.

Guest, P.C., S.D. Arden, D.L. Bennett, A. Clark, N.G. Rutherford, and J.C. Hutton. 1992. The post-translational processing and intracellular sorting of PC2 in the islets of Langerhans. J. Biol. Chem. 267:22401–22406.

Hamond, C., and A. Helenius. 1994. Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling be-
tween the ER, intermediate compartment, and Golgi apparatus. J. Cell Biol. 126:41–52.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. Nature (Lond.). 381:571–580.

Hatsuzawa, K., M. Hosaka, T. Nakagawa, M. Nagase, A. Shoda, K. Murakami, P.C. Guest, P.C., S.D. Arden, D.L. Bennett, A. Clark, N.G. Rutherford, and J.C. Hutton. 1994. The neuroendocrine precursor 7B2 is a sulfated protein proteolytically processed by a ubiquitous furinlike convertase. J. Biol. Chem. 269:19279–19289.

Pelham, H.R. 1991. Recycling of proteins between the endoplasmic reticulum and Golgi complex. Curr. Opin. Cell Biol. 3:585–591.

Rothman, J.E. 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell. 59:591–601.

Rouille, Y., S.J. Dugay, K. Lund, M. Furuta, Q. Gong, G. Lipkind, A.A. Oliver, Jr., S.J. Chan, and D.F. Steiner. 1995. Proteolytic processing mechanisms in the endoplasmic reticulum of mammalian cells. Annu. Rev. Biochem. 64:323–361.

Satoh, K., H. Hirayoshi, S. Yokota, N. Hosokawa, and K. Nagata. 1996. Intracellular interaction of collagen-specific stress protein HSP47 with newly syn-
thesized precollagen. J. Cell Biol. 133:469–483.

Seidah, N.G., L. Gaspar, P. Mion, M. Mareckiwicz, M. Mbikay, and M. Chre-
tien. 1996. cDNA sequence of a novel porcine anterior pituitary polypeptide. Mol. Endocrinol. 10:2089–2096.

Seidah, N.G., M. Marcinkiewicz, S. Benjannet, L. Gaspar, M.W. Collard, and M. Chretien. 1991. Cloning and primary structure of a mouse candidate prohormone convertase PC1 homologous to PC2, furin and furin gene products: tissue-specific mRNA encoding candidates for pro-protein processing proteinsases. DNA Cell Biol. 9:415–424.

Seidah, N.G., M. Mareckiwicz, S. Benjannet, L. Gaspar, C. Lazure, M. Mbikay, and M. Chretien. 1991. Identification of a second human subtilisin-like protease gene in the mammalian endoprotease family homologous to the yeast Kex2 and furin gene products: tissue-specific mRNA encoding candidates for pro-protein processing proteinsases. DNA Cell Biol. 9:415–424.

Seidah, N.G., M. Marcinkiewicz, S. Benjannet, L. Gaspar, C. Lazure, M. Mbikay, and M. Chretien. 1991. Identification of a second human subtilisin-like protease gene in the mammalian endoprotease family homologous to the yeast Kex2 and furin gene products: tissue-specific mRNA encoding candidates for pro-protein processing proteinsases. DNA Cell Biol. 9:415–424.

Seidah, N.G., L. Gaspar, P. Mion, M. Mareckiwicz, M. Mbikay, and M. Chre-
tien. 1996. cDNA sequence of a novel porcine anterior pituitary polypeptide. Mol. Endocrinol. 10:2089–2096.

Seidah, N.G., and M. Chretien. 1994. Pro-protein convertases of subtilisin/kexin family. Methods Enzymol. 244:171–188.

Shen, F.S., I. Lindberg, and N.G. Seidah. 1993. Biosynthesis of the prohormone convertase PC2 in Chinese hamster ovary cells and in rat insulinoma cells. J. Biol. Chem. 268:24910–24915.

Shennan, K.J., N.A. Taylor, J.L. Jermann, G. Matthews, and K. Dockerty. 1995. Difference in pH optima and calcium requirements for maturation of the prohormone convertases PC2 and PC3 indicates different intracellular locations for these events. J. Biol. Chem. 270:1402–1407.

Siezen, R.J., J.W.M. Creemers, and W.J.M. Van der Ven. 1994. Homology mod-
eling of the catalytic domain of human furin: a model for the eukaryotic sub-
tilisin/kexin-like proprotein convertases. J. Biol. Chem. 269:255–266.

Siezen, R.J., J.A.M. Leunissen, and U. Shinde. 1995. Homology analysis of the proproteases of subtilisin-like serine proteases (subtilases). In Intramolecular Chaperones and Protein Folding. U. Shinde and N. Iinoey, editors. R.G. Landes Company, Austin, TX. 233–256.

Simons, J.F., S. Ferro-Novick, M.D. Rose, and A. Helenius. 1995. BiP/Kar2p serves as a molecular chaperone during carboxypeptidase Y folding in yeast. J. Cell Biol. 130:41–49.

Smeykens, S.P., A.S. Avruch, J. LaMendola, S.J. Chan, and D.F. Steiner. 1991. Identification of a cDNA encoding a second putative prohormone conver-
tase related to PC2 in At20 cells and islets of Langerhans. Proc. Natl. Acad. Sci. USA. 88:7756–7760.

Umayo, T., Y. Moriyaama, M. Futai, and E. Mekada. 1990. The cytoxic action of diphtheria toxin and its degradation in intact Vero cells are inhibited by bafilomycin A1, a specific inhibitor of vacuolar-type H+ (−) ATPase. J. Biol. Chem. 265:21940–21945.

Van Duinhowen, H.L., M.C. Verschuen, E.D. Timmer, P.M. Vissers, A. Groeneveld, T.A. Ayoubi, A.M. Van den Oudewaal, and W.J. Van der Ven. 1991. Application of recombinant DNA technology in epitope mapping and targeting. Development and characterization of a novel monoclonal antibi-
odies against the 7B2 neuroendocrine protein. J. Immunol. Methods. 142: 187–198.

Van Horssen, A.M., W.H. Van den Hurk, E.M. Baileys, J.C. Hutton, G.J.M. Martens, and I. Lindberg. 1995. Identification of the region within the neu-
roendocrine proprotease 7B2 responsible for the inhibition of prohormone conver-
tase PC2. J. Biol. Chem. 270:14292–14296.

Vey, M., W. Schafer, S. Bergdorfer, H.D. Klein, and W. Garten. 1994. Matura-
tion of the trans-Golgi network proprotein furin: compartmentalization of
propeptide removal, substrate cleavage, and COOH-terminal truncation. *J. Cell Biol.* 127:1829–1842.

Willnow, T.E., A. Rohlman, J. Horton, H. Otani, J.R. Braun, R.E. Hammer, and J. Herz. 1996. RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytotic receptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2632–2639.

Yoshimori, T., A. Yamamoto, Y. Moriyama, M. Futai, and Y. Tashiro. 1991. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+) ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem.* 266:17707–17712.

Zhou, Y., and I. Lindberg. 1993. Purification and characterization of the prohormone convertase PC1(PC3). *J. Biol. Chem.* 268:5615–5623.

Zhou, A., and R.E. Mains. 1994. Endoproteolytic processing of POMC and prohormone convertases 1 and 2 in neuroendocrine cells overexpressing PC1 or PC2. *J. Biol. Chem.* 269:17440–17447.

Zhu, X., Y. Ohta, F. Jordan, and M. Inouye. 1989. Prosequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. *Nature (Lond.)* 339:483–484.

Zhu, X., and I. Lindberg. 1995. 7B2 facilitates the maturation of proPC2 in neuroendocrine cells and is required for the expression of enzymatic activity. *J. Cell Biol.* 129:1641–1650.

Zhu, X., Y. Rouille, N.S. Lamango, D.F. Steiner, and I. Lindberg. 1996a. Internal cleavage of the inhibitory 7B2 carboxyl-terminal peptide by PC2: a potential mechanism for its inactivation. *Proc. Natl. Acad. Sci. USA.* 93:4919–4924.

Zhu, X., N.S. Lamango, and I. Lindberg. 1996b. Involvement of a polyproline helix-like structure in the interaction of 7B2 with prohormone convertase 2. *J. Biol. Chem.* 271:23582–23587.