Neuroendocrine Protein 7B2 Can Be Inactivated by Phosphorylation within the Secretory Pathway*

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The prohormone convertases play important roles in the maturation of neuropeptides and peptide hormone precursors. Prohormone convertase-2 (PC2) is the only convertase that requires the expression of another neuroendocrine protein, 7B2, for expression of enzyme activity. In this study, we determined that 7B2 can be phosphorylated in Rin cells (a rat insulinoma cell line) and cultured chromaffin cells, but not in AtT-20 cells (derived from mouse anterior pituitary). Phosphoamino acid analysis of Rin cell 7B2 indicated the presence of phosphorylated serine and threonine. Phosphorylation of Ser115 (located within the minimally active 36-residue peptide) was confirmed by mutagenesis, although Ser115 did not represent the sole residue phosphorylated. Two independent assays were used to investigate the effect of phosphorylated 7B2 on PC2 activation: the ability of 7B2 to bind to pro-PC2 was assessed by co-immunoprecipitation, and activation of pro-PC2 was assessed in a cell-free assay. Phosphorylated 7B2 was unable to bind pro-PC2, and the phosphorylated 7B2 peptide (residues 86–121, known to be the minimally active peptide for pro-PC2 activation) was impaired in its ability to facilitate the generation of PC2 activity in membrane fractions containing pro-PC2. In vitro phosphorylation experiments using Golgi membrane fractions showed that 7B2 could be phosphorylated by endogenous Golgi kinases. Golgi kinase activity was strongly inhibited by the broad-range kinase inhibitor staurosporine and partially inhibited by the protein kinase C inhibitor bisindolylmaleimide I, but not by the other protein kinase A, Ca2+/calmodulin-dependent kinase II, myosin light chain kinase, and protein kinase G inhibitors tested. We conclude that phosphorylation of 7B2 functionally inactivates this protein and suggest that this may be analogous to the phosphorylating inactivation of BiP, which impairs its ability to bind substrate.

Prohormone convertase-2 (PC2), a member of the family of eukaryotic subtilisins, exhibits an interesting but atypical cell biology compared with other convertases. The PC2 zymogen spends an unusually long time in the endoplasmic reticulum (ER) compared with other convertases and then binds to a partner protein, 7B2, which escorts it to the Golgi (1, 2). In the Golgi, a 7B2-mediated "capacitation" process renders pro-PC2 capable of autoactivation to an active enzyme molecule (reviewed in Ref. 3). Pro-PC2 expressed in cells lacking 7B2 (such as Chinese hamster ovary (CHO) cells) may still undergo propeptide cleavage, but in this case, propeptide removal does not result in the production of enzymatically active species (4). The biochemical mechanism underlying the 7B2 capacitation effect is still enigmatic; however, it can be reproduced in a cell-free environment using Golgi membranes (5). Our current hypothesis is that 7B2 prevents pro-PC2 aggregation and/or facilitates its binding to membranes during intracellular transit. Structure-function studies have revealed that the minimal portion of 7B2 required to effect capacitation of pro-PC2 consists of a 36-residue disulfide-bonded peptide located within the 21-kDa domain (residues 1–151), which contains a putative polyproline helix and an α-helix (4–6). Proteolytic maturation of 27-kDa 7B2 to its 21-kDa form is mediated by furin or a furin-like convertase within the trans-Golgi network (7) and involves removal of a C-terminal domain (referred to as the C-terminal peptide, residues 156–186) that inhibits PC2 activity in vitro in the low nanomolar range (8–10). The inhibitory C-terminal peptide may play a role in controlling PC2 activity during transport within secretory compartments.

Protein phosphorylation is an important regulatory mechanism that affects protein synthesis, function, and subcellular localization. Many protein-protein interactions are mediated by reversible phosphorylation. Secretory proteins undergo phosphorylation within the lumen of the Golgi apparatus; these include casein (11), vitellogenin (12, 13), osteopontin (14), proline-rich protein (15), and chromogranins (16). Although the functional significance of phosphorylation of most of these proteins still remains unknown, phosphorylation of casein and vitellogenin is thought to confer calcium binding ability (17, 18). Phosphorylation of secretory proteins occurs at serine or threonine residues within the consensus sequence of a Golgi apparatus casein kinase-like enzyme, (Ser/Thr)-Xaa-(acidic residue/Ser(P)) (19). Reversible phosphorylation within the secretory pathway has thus far been described only for chaperone proteins such as BiP and GRP94 (20–23), in which phosphorylation is thought to regulate chaperone-substrate interaction in a negative manner. Phosphorylation of BiP has been shown to prevent substrate binding (21, 22, 24). 7B2 contains three potential phosphorylation sites within the minimally active 36-residue peptide responsible for binding pro-PC2; at least two of these sites are well conserved between vertebrates. In this study, we demonstrate that phosphorylation of 7B2 occurs in neuroendocrine cells at multiple sites, that it can be carried out by endogenous Golgi kinases, and that this modification results in its functional inactivation.

**MATERIALS AND METHODS**

Antisera — The antisera against PC2 (LSU13BF) was directed against a C-terminal peptide of mature PC2 (25). The antisera against 7B2 (LSU13BF) was raised against residues 23–39 of 7B2 (26). The antisera against PC1 (LSU2B6) was directed against an N-terminal peptide of mature PC1 (27). Peptides were synthesized as described previously (28).
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In Vivo Phosphorylation—RinPE/7B2 and AtT-20/PC2/7B2 cells (rat insuloma and mouse pituitary cells, respectively) (26) were split into 6-well plates at 3 \times 10^6 and 1 \times 10^6 cells/well, respectively, 1 day before labeling. Cells were washed twice with 3 ml of phosphate-free Dulbecco’s modified Eagle’s medium (ICN Pharmaceuticals, Inc., Costa Mesa, CA) containing 10 mM Hepes (pH 7.4), and 1 ml of phosphate-free Hepes-buffered Dulbecco’s modified Eagle’s medium was added. Cells were then incubated in 6% CO_2 at 37 °C for 1 h. Cells were labeled with 1 ml of phosphate-free Hepes-buffered Dulbecco’s modified Eagle’s medium containing 0.5 mM phenylmethanesulfonyl fluoride and 10 \mu M NaCl, and 0.9% NaCl containing 10 \mu M Hepes (pH 7.4), and 1 ml of phosphate-free Hepes-buffered Dulbecco’s modified Eagle’s medium was added. Cells were then incubated in 6% CO_2 at 37 °C for 1 h. Cells were labeled with 1 ml of phosphate-free Hepes-buffered Dulbecco’s modified Eagle’s medium containing 0.5 \mu Ci/ml [32P]H_3PO_4 (ICN Pharmaceuticals, Inc.) and 50 \mu g/ml gentamicin (Invitrogen) at 37 °C for 16 h. After labeling, the medium was discarded, and cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with 1 ml of AG buffer (0.1 M sodium phosphate (pH 7.4), 1 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40, and 0.9% NaCl) containing 10 \mu M of 100 mM phenylmethanesulfonyl fluoride (Roche Applied Science) and 10 \mu M of 10 mM p-chloromercuriphenylsulfonic acid (Sigma). Cell extracts were centrifuged at 15,000 \times g for 5 min, and immunoprecipitation of the supernatant was performed using antiserum against 7B2 and PC2.

Two-day-old primary cultures of bovine adrenal chromaffin cells (6 \times 10^6 cells) (29) were labeled for 20 h, extracted with 1 ml of cold 0.1 N HCl containing 50 mM 2-mercaptoethanol, frozen, thawed, and centrifuged. The supernatant was lyophilized and resuspended in 1 ml of AG buffer. Immunoprecipitations of [32P]-labeled chromaffin cell extracts were performed using anti-7B2 antiserum. Twenty micrograms of purified recombinant 7B2 were added to one set of samples during immunoprecipitation as a blocking control.

Metabolic Labeling and Immunoprecipitation—RinPE and AtT-20/PC2 cells were plated into 6-well plates at 3 \times 10^6 and 1 \times 10^6 cells/well, respectively, 1 day before labeling. Cells were labeled with 0.5 \mu Ci/ml [35S]methionine/cysteine Pro-mix (Amersham Biosciences) for 20 min and either chased for 30 min in Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum or lysed without chase. The cells were boiled for 5 min in 100 \mu l of 50 mM sodium phosphate (pH 7.4), 1% SDS, 50 mM 2-mercaptoethanol, and 2 mM EDTA and then diluted with 0.9 ml of AG buffer containing 10 \mu l of 100 mM phenylmethanesulfonyl fluoride and 10 \mu l of 10 mM p-chloromercuriphenylsulfonic acid. Cell extracts were preincubated with 0.1 ml of 20% protein A-Sepharose CL-4B (Amersham Biosciences AB, Uppsala, Sweden), hydrated, and washed with AG buffer at 4 °C for 1 h, and then centrifuged. Five microliters of antiserum against PC2, 7B2, or PC1 were then added to the supernatant, along with 0.5 mM phenylmethanesulfonyl fluoride and 0.5 mM p-chloromercuriphenylsulfonic acid. Samples were incubated overnight at 4 °C with agitation. One-hundred microliters of 20% protein A-Sepharose, hydrated and washed three times with AG buffer, were then added, and the samples were rocked at 4 °C for 1 h. The beads were washed twice with AG buffer, once with 0.5 M NaCl in PBS, and twice with PBS. Immunoprecipitates were resuspended in Laemmli sample buffer containing 6 M urea and analyzed using either 8.8% (for PC2 and PC1) or 15% (for 7B2) SDS-polyacrylamide gel. The dried gels were exposed to PhosphorImager screens and analyzed using a Typhoon 9410 variable mode imager and ImageQuant software (Amerham Biosciences).

Phosphoamino Acid Analysis—For phosphoamino acid analysis of [32P]-labeled 7B2, 7B2 was immunoprecipitated from [32P]-labeled RinPE/7B2 cell extracts using antiserum against 7B2. The immunoprecipitated 7B2 was subjected to electrophoresis on a 15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) for 1.5 h at 200 mA. The polyvinylidene difluoride membrane was rinsed with PBS, air-dried, and exposed to a Phospho-Imager screen. The slice containing phosphorylated 7B2 was excised from the polyvinylidene difluoride membrane, and the protein was hydrolyzed by incubation in 200 \mu l of 6 N HCl for 30 min (or in some cases, for 1 h at 110 °C). Amino acids were eluted from the membrane with 30% methanol and 0.1 N HCl (three changes, 200 \mu l each). The 6 N HCl hydrolysate and the three eluates were pooled and dried by lyophilization. The sample was resuspended in 200 \mu l of distilled water, lyophilized, and resuspended again in 20 \mu l of distilled water, and 3 \mu l were applied to an Eastman Kodak Chromagram sheet (6064 cellulose, 20 \times 20 cm^2). Phosphoamino acids (Sigma) were applied to a separate lane as well as to the same spot with the sample. The samples were subjected to TLC in a solvent composed of 5:3 isobutyric acid and 0.5 M NH_4OH (30) for 4 h. The TLC plate was removed and dried under hot air. The plate was then sprayed with ninhydrin solution (0.2% ninhydrin in ethanol; Sigma) and dried with hot air to visualize the phosphoamino acid standards. The TLC plate was exposed to a PhosphorImager screen to determine the positions of [32P]-labeled phosphoamino acids. To align the autoradiogram with the TLC plate, a radioactive marker was spotted on the dried TLC plate.

Mutagenesis of 7B2 at the Putative Phosphorylation Sites and Location of Conformations—Mutations were generated in rat 7B2 cDNA by PCR-mediated methods as described previously (26, 31). For mutation at the putative phosphorylation site within peptide 86–121, the minimally active 7B2 peptide (28), the N-terminal primer containing the mutations was 5’-ACTGCAAGTTGCGCCGAGAAATTCGAG3’ for S7B2A, and the C-terminal primer was 5’-CGGCGGGGATCCTTATCTTGAGCTCCTTCTC3’—The product from the first round of PCR was used as a C-terminal primer with a new N-terminal primer (5’-GGCGCAAGCTTACCATGAGATTCAGGATGGG3’) in the second round of PCR. The PCR fragments were cloned into the pCEP4 vector (Invitrogen) at the HindIII and BamHI restriction sites. All cDNA generated by PCR was verified by DNA sequencing. Mutant 7B2 cDNAs were transfected into RinPE cells (32) using 30 \mu g of plasmid and 30 \mu l of Lipofectin (Invitrogen) in a 10-cm dish. For each construct, high-expressing stable clones were selected in hygromycin and used in the labeling experiments.

Co-immunoprecipitation of 7B2 and PC2—For co-immunoprecipitations, cells were treated with 2 ml of 0.5 M iodoacetamide (Sigma) in PBS for 10 min on ice after metabolic labeling to block free sulphydryl groups and to prevent the formation of biologically irrelevant disulfide bridges during and after cell lysis. All of the following steps of extraction and immunoprecipitation were performed at 4 °C. The iodoacetamide solution was discarded, and proteins were extracted with 0.5 ml of 25 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100, 0.1 M NaCl, 10 mM iodoacetamide, 5 mM EDTA, 0.5 mM p-chloromercuriphenylsulfonic acid, and 1 mM phenylmethanesulfonyl fluoride. The extracts were centrifuged at 15,000 \times g for 10 min. Supernatants were diluted to 0.5% Triton X-100 with AG buffer lacking Triton, and immunoprecipitation was performed as described above.

Alkaline Phosphatase Treatment—Protein A beads containing immunoprecipitated 7B2 obtained from RinPE/7B2 and AtT-20/PC2/7B2 cells were washed twice with PBS and resuspended in 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl_2, and 0.025% Triton X-100. To half of the beads were added 50 units of alkaline phosphatase (from calf intestinal mucosa; New England Biolabs Inc., Beverly, MA), and the reaction was incubated for 3 h at 37 °C. The other half of the beads was incubated for 3 h at 70 °C in the absence of alkaline phosphatase. The reaction was stopped by addition of 5X Laemmli sample buffer containing 6 M urea and analyzed following separation via 15% SDS-PAGE.
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PC2 Enzyme Assay of Synthetic 7B2 Peptides in Golgi-enriched Membranes—Golgi-enriched subcellular fractions were prepared from CHO/PC2 cells as described previously (5). Briefly, Golgi-enriched fractions (1.5 μg of cells) were incubated in 100 mM sodium acetate (pH 5.0) containing 5 mM CaCl₂, 0.1 mg/ml bovine serum albumin, 1% Triton X-100, and a proteinase inhibitor mixture (1 μM pepstatin, 1 μM trans-epoxysuccinic acid, 280 μM tosylphenylalanyl chloromethyl ketone, and 140 μM tosyllysyl chloromethyl ketone (final concentrations)) in the presence of either 1 μM synthetic peptide 86–121 (28) or 1 μM synthetic peptide 86–121 containing phosphoserine at position 115 (termed phosphopeptide 86–121). To inhibit phosphorylation activity that might be present in Golgi-enriched membrane fractions, 2 μl of a stock solution of a phosphatase inhibitor mixture (2.5 mM (-)-p-bromotetramisole, 0.5 mM cantharidin, and 0.5 μM microcystin LR; Calbiochem) were added to each 50 μl of reaction mixture. To determine the effect on Golgi kinase activation of several protein kinase inhibitors, highly selective Ser/Thr kinase inhibitors (1 μM staurosporine, 1 μM bisindolylmaleimide I, 1 μM H-89, dihydrochloride, 2 μM KN-93, and 2 μM ML-7; final concentrations), as well as a protein kinase G inhibitor (200 μM final concentration; Calbiochem), were individually added to 50 μl of reaction mixture. The incubation was conducted at 37 °C for 4 h. The PC2 enzyme assay was performed as described previously (8) using 200 μM <Glu-Arg-Thr-Lys-methylcoumarin amide (where <Glu is pyroglutamic acid) as a substrate. Enzyme activity was measured in triplicate and is given in fluorescence units/min; 1 fluorescence unit corresponds to 5.33 pmol of aminomethyl coumarin product.

**Phosphorylation of 7B2 in Golgi-enriched Membranes**—Recombinant 7B2 proteins were prepared by prokaryotic expression as described previously (9). Kinase assays were performed in 50 mM MOPS (pH 7.0) containing 10 mM MgCl₂, 2 mM EGTA, 1 mM dithiothreitol, 0.4% Triton X-100, 0.1 mg/ml bovine serum albumin, a proteinase inhibitor mixture (see above), a phosphatase inhibitor mixture (see above), and the respective protein kinase inhibitors (see above) in a 100-μl reaction mixture. Purified His-tagged 27-kDa 7B2 (3.6 mg) and 21-kDa 7B2 (5.8 μg) were incubated with either Golgi-enriched membranes (0.3 mg/ml) or casein kinase II (a gift from Dr. Melanie Cobb, University of Texas Southwestern Medical Center) in the presence of 0.1 mM [γ-³²P]ATP (3 Ci/mmol; Amersham Biosciences) at 37 °C for 6 h. Immunoprecipitation was performed as described above. The immunoprecipitates were resuspended in 50 μl of Laemmli sample buffer and analyzed on NuPAGE™ 4–12% BisTris gels (Invitrogen). The dried gels were exposed to PhosphorImager screens and analyzed using a Typhoon 9410 variable mode imager and ImageQuant software.

**RESULTS**

7B2 Is Phosphorylated in Rin Cells and Primary Bovine Adrenal Chromaffin Cells, but Not in AtT-20 Cells—To determine whether 7B2 can be phosphorylated in endocrine cells, we labeled RinPE/7B2, AtT-20/PC2/7B2, and primary cultures of bovine adrenal chromaffin cells with [³²P]orthophosphate and immunoprecipitated labeled cell extracts using antiserum against 7B2. As shown in Fig. 1, antiserum directed against 7B2 immunoprecipitated both non-phosphorylated and phosphorylated 7B2 proteins; only 7B2 obtained from RinPE/7B2 cells contained radioactive phosphate. [³⁵S]labeling of these cells showed that 7B2 was actually synthesized in both RinPE/7B2 and AtT-20/PC2/7B2 cells. [³⁵S]-Labeled 7B2 obtained from Rin cells was microheterogeneous, i.e. appeared as several bands, indicating the presence of different post-translational modifications; only the uppermost band disappeared after treatment with alkaline phosphatase (Fig. 2A). 7B2 obtained from AtT-20 cells consisted of only two bands. Because this upper band was not phosphorylated (Fig. 1), it likely corresponds to sulfated 7B2, a known post-translational modification of this protein (7). Interestingly, after alkaline phosphatase treatment, the uppermost bands of 7B2 obtained from Rin cells shifted to the same positions as the two 7B2 bands obtained from AtT-20 cells (data not shown), supporting the idea that these AtT-20 bands are not phosphorylated and indicating that phosphorylation of 7B2 does not require sulfation. It has been previously shown that sulfated 7B2 can coprecipitate with pro-PC2 (1). We also determined whether endogenous 7B2 can be phosphorylated using primary cultures of chromaffin cells. Fig. 2C (lanes 2 and 3) confirms the presence of phosphopeptide-labeled 21-kDa 7B2. [³⁵P]-Labeled 7B2 was not observed in samples incubated with an excess of antigen during immunoprecipitation (Fig. 2C, lane 1). In addition, another band was observed in the position of a dimeric form of 7B2 (Fig. 2C, lane 2, asterisk).

Phosphorylated 7B2 Cannot Interact with Pro-PC2—To study the functional role of phosphorylated 7B2, we tested whether phosphorylated 7B2 can interact with PC2 by co-immunoprecipitation. Fig. 2 shows immunoprecipitated [³⁵S]- or [³²P]-labeled 7B2 obtained from RinPE/7B2 and AtT-20/PC2/7B2 cells. When we used antiserum directed against PC2 for coprecipitation of 7B2, smaller amounts of coprecipitated 7B2 were detected than when we used 7B2 antiserum, possibly due to dissociation caused by the decreased affinity between pro-PC2 and 21-kDa 7B2, which occurs as a gradual process during cellular transport (1, 6). In our experiments, after chasing cells in methionine-containing medium for 30 min, pro-PC2 was the major form detected by 12% SDS-PAGE (data not shown). A portion of [³⁵S]-labeled 7B2 obtained from RinPE/7B2 cells was sensitive to alkaline phosphatase, whereas 7B2 obtained from AtT-20/PC2/7B2 cells was unaffected by phosphatase (Fig. 2A). We confirmed these results using [³²P]-labeled 7B2 obtained from RinPE/7B2 cells; the mobility of the [³²P]-labeled band was identical to that of the uppermost band of [³⁵S]-labeled 7B2, and the band completely disappeared following alkaline phosphatase treatment (Fig. 2B). Treatment of immunoprecipitated 7B2 proteins with alkaline phosphatase revealed that only non-phosphorylated 7B2 coprecipitated with PC2 (Fig. 2A). To determine the specificity of the co-immunoprecipitation reaction, co-immunoprecipitation with antiserum against PC1 was performed. These data confirm that no form of 7B2 can interact with PC1. The identity of the PC2 and 7B2 bands was further confirmed by Western blotting using anti-PC2 and 7B2 antisera, respectively (data not shown).

Phosphorylation of 27-kDa 7B2 Occurs in a Golgi Compartment prior to Furin-mediated Cleavage—To determine in which cellular compartment 7B2 becomes phosphorylated, we immunoprecipitated 7B2 from RinPE/7B2 cells labeled with either [³⁵S]Met/Cys or [³²P]orthophosphate in the presence and absence of brefeldin A, a drug that blocks...
FIGURE 2. Phosphorylated 7B2 fails to interact with pro-PC2. RinPE/7B2 and AtT-20/PC2/7B2 cells were labeled with [35S]Met/Cys (A) and [32P]orthophosphate (B). Cells were extracted in the presence of iodoacetamide for co-immunoprecipitation using antiserum against either 7B2 or PC2. Immunoprecipitated 7B2 was treated with alkaline phosphatase (AP) for 3 h at 37 °C.

Co-immunoprecipitation using antiserum against PC1 was performed as a negative control. The inset in A shows 7B2 modified using ImageQuant software to more clearly show the 7B2 band shifting after alkaline phosphatase treatment. Immunoprecipitated 7B2 proteins from 32P-labeled chromaffin cell cultures were subjected to SDS-PAGE (C). Lane 1, control immunoprecipitate formed in the presence of excess antigen; lanes 2 and 3, specific immunoprecipitate (two independent experiments). The expected positions of 7B2, pro-PC2, and PC1 are indicated. The identities of the PC2 and 7B2 bands were confirmed by Western blotting (data not shown). The experiment was repeated four times with similar results. Asterisk, potential dimer.
movement of newly synthesized proteins to the trans-Golgi network. Fig. 3 shows the presence of phosphate-labeled 27-kDa 7B2, indicating that phosphorylation takes place prior to furin cleavage of the 27-kDa protein to the 21-kDa form. In the presence of brefeldin A, cleavage to the 21-kDa form was completely blocked, suggesting the idea that phosphorylation occurs prior to cleavage and to sulfation (sulfation is known to occur in the trans-Golgi network prior to cleavage) (7) either in the ER or in an early Golgi compartment.

Serine and Threonine Residues Are Phosphorylated in 7B2—Phosphorylated amino acids within 7B2 were identified in radiolabeling experiments. RinPE/7B2 cells were labeled with [32P]orthophosphate, and cell extracts were immunoprecipitated with anti-7B2 antiserum under denaturing conditions. Immunoprecipitates were analyzed by 15% SDS-PAGE. The positions of the 27- and 21-kDa 7B2 proteins are indicated. The experiment was repeated three times with similar results.

whether this site is phosphorylated. RinPE cells stably expressing S7B2A or wild-type 7B2 were labeled with [32P]orthophosphate, and the phosphorylating acids were analyzed by SDS-PAGE (Fig. 6A) and TLC (Fig. 6B). The data show a reduction in the amount of phosphorylated protein as well as phosphoserine, indicating that Ser115 is indeed phosphorylated in Rin cells. Note that serine phosphorylation was not abolished; this may be due both to the continued expression of endogenous wild-type 7B2 and to the phosphorylation of serines other than Ser115. Unfortunately, there are no known neuroendocrine cell lines that do not express 7B2. (Although a neuroepithelioma line exists that expresses PC2 but not 7B2 (35), this cell line does not store peptide products (36) and is thus more constitutive than neuroendocrine in character.)

Phosphopeptide 86–121 Fails to Facilitate PC2 Activity—To study whether phosphorylation of 7B2 affects its ability to facilitate the maturation of pro-PC2 to an active enzyme species, we synthesized peptide 86–121 containing phosphoserine at position 115 (phosphopeptide 86–121) and peptide 86–121. The peptides were tested in an in vitro system using Golgi-enriched membrane fractions obtained from CHO/PC2 cells (5). Phosphopeptide 86–121 and peptide 86–121 (1 µM final concentration) were incubated with Golgi-enriched fractions obtained from CHO/PC2 cells in the presence of a phosphatase inhibitor mixture. Fig. 7 shows that inclusion of phosphopeptide 86–121 resulted in much less PC2 activity compared with peptide 86–121, indicating that phosphopeptide 86–121 is not able to assist in the production of active PC2 in vitro.

To characterize the kinase reaction, we added the broad-range Ser/Thr kinase inhibitor staurosporine (37) to the reaction mixture. Fig. 7 data shows that 1 µM staurosporine partially increased PC2 activity. This result suggests 1) that Golgi-enriched membrane fractions contain kinase(s) that may be responsible for the phosphorylation of peptide 86–121 and 2) that the activity of the Golgi kinase(s) is partially inhibited by staurosporine under our PC2 enzyme assay conditions (pH 5.0). However, possibly because our assay conditions are not optimal for kinase action, the effect was not large.

Recombinant 7B2 Can Be Phosphorylated by Kinasen Present in Golgi-Enriched Membrane Fractions—To investigate whether the putative Golgi kinases are able to phosphorylate purified recombinant 7B2, we attempted to label the recombinant 21- and 27-kDa forms with [γ-32P]ATP using Golgi-enriched membrane fractions (see “Materials and Methods”). Fig. 8 shows the presence of both phosphate-labeled 27-kDa forms, indicating that 7B2 can be phosphorylated by endogenous Golgi kinases. Because 7B2 also has potential phosphorylation sites for casein kinase II ((Ser/Thr)-Xaa-Xaa-(Glu/Asp/Ser/P/Tyr) (Fig. 4) (38), purified casein kinase II, which is not normally present in Golgi fractions (39), was used in a parallel incubation as a positive control. Two putative phosphorylation sites exist for casein kinase II in the N-terminal domain (19) and at position 115 (39), and one site is present in the C-terminal 31-residue peptide (179SEEE182) (Fig. 4). Interestingly, although most of the putative phosphorylation sites are in the N-terminal domain of 7B2 (Fig. 4), both kinases (especially casein kinase II) preferentially phosphorylated the 27-kDa form, with much less incorporation of 32P into the 21-kDa protein (Fig. 8, inset). This result may indicate that the conformation of the two 7B2 proteins differs, resulting in differential exposure of phosphorylation sites. Alternatively, Golgi kinases may simply preferentially phosphorylate the C-terminal domain of 7B2 in vitro.

To characterize the putative Golgi kinase(s) further, we investigated the ability of several inhibitors of Ser/Thr kinase inhibitors to inhibit Golgi kinase activity against recombinant 7B2. We used 1 µM staurosporine (a broad-range Ser/Thr kinase inhibitor) (37), 1 µM bisindolyl-
maleimide I (a protein kinase C inhibitor) (40), 1 μM H-89 dihydrochloride (a protein kinase A inhibitor) (41), 2 μM KN-93 (a Ca2+/calmodulin-dependent kinase II inhibitor) (42), 2 μM ML-7 (a myosin light chain kinase inhibitor) (43), and 200 μM protein kinase G inhibitor (44). Fig. 9 shows that staurosporine strongly reduced and bisindolylmaleimide I partially reduced the in vitro phosphorylation of 7B2 by the Golgi kinase(s). The data support the idea that a particular kinase present in Golgi-enriched membrane fractions is responsible for the phosphorylation of 7B2.

**DISCUSSION**

Cell Line-specific Phosphorylation of 7B2—The full-length 27-kDa 7B2 molecule has been shown to undergo numerous post-translational modifications, including early furin cleavage (7, 45), tyrosine sulfation (7), and phosphorylation within the C-terminal peptide (46). The data presented in this work indicate that the N-terminal domain of 7B2 can...
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also be phosphorylated. Curiously, only cells of pancreatic origin, such as Rin and βTC3, were able to phosphorylate 7B2; AtT-20 cells were unable to perform this modification. Primary anterior pituitary cells are capable of phosphorylating adrenocorticotropic hormone (ACTH); this capability increases with time in culture, whereas intermediate lobe cells gradually lose the ability to phosphorylate this peptide over time in culture (47). These data support the notion of cell type-specific control of phosphorylation events. To our knowledge, no phosphorylation of secretory proteins has been demonstrated in AtT-20 cells. Why AtT-20 cells are unable to phosphorylate 7B2 remains unclear; potential reasons include high levels of phosphatase activity within the secretory pathway, lack of expression of a specific kinase, or both. We attempted to determine whether tissues derived from the pituitary, pancreas, and adrenal gland would also show tissue-specific phosphorylation demonstrable by microheterogeneity. Although the levels and/or turnover of endogenous 7B2 in the pituitary and pancreas, including freshly dissected rat islets, were unfortunately too low to detect either by Western blotting or by metabolic labeling primary bovine adrenal chromaffin cells did phosphorylate endogenous 7B2. This result, together with the fact that a considerable proportion of 7B2 was phosphorylated in Rin cells (as assessed by methionine labeling with and without phosphatase treatment) (Fig. 2A), supports the idea that phosphorylation may represent a physiologically important cellular event.

We have previously shown that 7B2 interacts with fully folded pro-PC2 in the ER and that the complex is then transported to the Golgi; free 7B2 also travels uncomplexed to the Golgi apparatus (5). The present data showing that brefeldin A can block furin cleavage of phosphate-labeled 7B2 clearly demonstrate that phosphorylation precedes furin processing and sulfation, events occurring in the trans-Golgi network (7); phosphorylation must thus occur either in the ER or in early Golgi compartments. It is unlikely that 7B2 molecules already bound to pro-PC2 can become phosphorylated because we would have observed co-immunoprecipitation of phosphate-labeled 7B2 with pro-PC2. We speculate that phosphorylation of 7B2 serves as a mechanism to regulate binding of 7B2 to pro-PC2/PC2 forms, much like phosphorylation of Bip prevents its interaction with substrate (21, 22, 24). In vitro phosphorylation experiments provided further evidence that 7B2 can be phosphorylated within the secretory pathway.

Functional Significance of Phosphorylation within the Secretory Pathway—Peptide precursors such as proopiomelanocortin (48, 49), gastrin (50), proatrial natriuretic factor (51), and proenkephalin (52, 53) also naturally undergo phosphorylation. Recently, Giorgianni et al. (54) identified six phosphorylated proteins from a whole human pituitary proteome by liquid chromatography-tandem mass spectrometry analysis: human growth hormone, chromogranin A, secretogranin I/chromogranin B, 60 S ribosomal protein P1 and/or P2, DNAJC5 (DNA homolog subfamily C member 5), and galanin. However, the functional role for phosphorylation of peptide precursors still remains unknown. For example, ~30% of human ACTH is phosphorylated, but phosphorylated and non-phosphorylated forms of ACTH are biologically equipotent (55). Phosphorylation of ACTH in primary anterior pituitary lobe cultures is unaffected by cell stimulation (47). Phosphorylation of a gastrin precursor has been shown to modulate its rate of cleavage (56); in this peptide, the phosphoserine is present in close proximity to a known convertase cleavage site. A similar situation may apply to the 7B2 C-terminal peptide, although, in this instance, the phosphoserine is located six residues C-terminal to the known furin cleavage site (46).

In the case of the 21-kDa domain of 7B2, we noted the presence of several potential phosphorylation sites within the 36-residue peptide previously shown to represent the minimal portion of 7B2 that can effect capacitation of pro-PC2 (28). We have previously proposed that a portion of this 36-residue peptide may exist in an α-helical conformation (28); the introduction of a phosphate group into this sequence at Ser115 would be expected to disrupt the secondary structure of this sequence. Our results demonstrating no co-immunoprecipitation of phosphorylated forms of 7B2 with pro-PC2 clearly indicate that phosphorylation severely reduces, if not totally eliminates, the ability of 7B2 to bind to pro-PC2. A functional role for phosphorylation of 7B2 is further supported by studies showing that the facilitatory effect of a Ser115 phosphopeptide (corresponding to the minimally active 36-residue sequence within 7B2) is small. We were not able to compete binding of purified recombinant 7B2 to recombinant PC2 with either the phosphorylated or non-phosphorylated 36-residue peptide, possibly due to the much stronger binding affinity of full-length 7B2 for PC2. Although we believe that the conserved residue Ser115 represents a likely site of phosphorylation within the 36-residue peptide, other sites within 7B2 are also apparently phosphorylated; for example, we obtained clear evidence for major phosphorylation of threonine. Three putative serine and three threonine “Golgi apparatus casein kinase” consensus sequence sites (19) are contained within the 21-kDa domain (shown in Fig. 4). Of these, one site is conserved in the 36-residue peptide in all vertebrate 7B2 proteins (Fig. 4, underlined) (34), suggesting that they may be functionally important.

The kinases responsible for phosphorylation within the secretory pathway have not yet been isolated. Because secretory proteins are often phosphorylated at physiological casein kinase consensus sequences (Ser-Xaa-acidic residue), casein kinase-like enzymes have been proposed to represent the physiological enzymes accomplishing secretory pathway phosphorylation (Ref. 57 and references therein). A promising candidate for phosphorylation occurring within the Golgi may be an enzyme termed Golgi apparatus casein kinase (19, 58–61); the Golgi apparatus casein kinase consensus sequence is (Ser/T)-Xaa-(acidic residue/Ser(P)). Several substrates of Golgi apparatus casein kinase have been reported thus far, including GRP94 (glucose-regulated protein of 94 kDa) (62), osteopontin (39), and proline-rich protein (15, 33). Osteopontin isolated from bovine milk contains 60 potential phospho-

2 J. R. Hwang and I. Lindberg, unpublished data.
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