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**Secretagogue-dependent Phosphorylation of the Insulin Granule Membrane Protein Phogrin Is Mediated by cAMP-dependent Protein Kinase**

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Phogrin, a 60/64-kDa integral membrane protein of dense-core granules in neuroendocrine cells, is phosphorylated in a Ca2+-sensitive manner in response to secretagogue stimulation of pancreatic β-cells. Phosphorylation of the phogrin cytosolic domain by β-cell homogenates was Ca2+-independent but stimulated by cAMP. Recombinant protein kinase A (PKA) could phosphorylate phogrin directly. High performance liquid chromatography analysis of tryptic phosphopeptides, combined with site-directed mutagenesis of candidate sites, revealed the presence of two phosphorylation sites at Ser-680 and Thr-699, located in the juxtamembrane region between the transmembrane span and the protein-tyrosine phosphatase homology domain of phogrin. Full-length wild-type phogrin, as well as mutant versions where Ser-680 and Thr-699 had been replaced either by alanines or by aspartic acid residues, were targeted to secretory granules in transfected AtT20 neuroendocrine cells. Stimulation of these cells with a range of secretagogues, including K+, BaCl2, and forskolin, demonstrated that the in vivo phosphorylation sites are the same as those identified in vitro. In MIN6 β-cells, the PKA inhibitor H-89 prevented Ca2+-dependent phogrin phosphorylation in response to glucose, suggesting that Ca2+ exerts its effect on phogrin phosphorylation through regulating the activity of PKA.

Protein phosphorylation is thought to play a central role in the regulation of hormone release from dense-core secretory granules (1–3). Stimulation of pancreatic β-cells with glucose leads to depolarization of the plasma membrane, Ca2+ influx via voltage-gated channels, and an increase in free cytosolic Ca2+ (4, 5). Ca2+ regulates early as well as late events in granule exocytosis (5, 6), possibly via the activation of Ca2+-dependent protein kinases, including Ca2+/calmodulin-dependent kinase II (CaMKII) (6), myosin light chain kinase (7), and Ca2+-dependent isoforms of protein kinase C (8). However, unequivocal evidence for functional involvement of these enzymes in the secretory process is still lacking.

Other second messengers, in particular cAMP, contribute to the regulation of exocytosis (9–11). Insulinotropic hormones such as glucagon-like peptide-1 or pituitary adenylyl cyclase-activating polypeptide increase intracellular cAMP levels by stimulating adenylyl cyclase through G-protein coupled receptors on the cell surface, an effect that can be mimicked by drugs like forskolin, which activate adenylyl cyclase directly. A rise in cAMP, and the subsequent activation of protein kinase A (PKA; cAMP-dependent kinase), is generally not sufficient in itself to evoke a secretory event in β-cells, but significantly potentiates glucose-induced insulin release. A number of mechanisms appear to be stimulated by cAMP (reviewed in Ref. 10), including membrane depolarization via the ATP-dependent K+ channel (12) and non-selective cation channels (13), an increase in Ca2+ influx through voltage-gated L-type Ca2+ channels (14–17), enhanced Ca2+ mobilization from intracellular stores (18), and the recruitment of secretory granules from a reserve pool to the release sites (19, 20).

Despite numerous studies implicating secretagogue-dependent activation of protein kinases in regulated secretion, the underlying molecular mechanisms and cellular targets of phosphorylation are poorly characterized. A large number of β-cell proteins are phosphorylated in a Ca2+- or cAMP-dependent manner in cell-free extracts (reviewed in Ref. 9), but for the most part they have not been identified. In intact β-cells, the analysis of known in vitro kinase substrates as candidate proteins has demonstrated stimulus-dependent phosphorylation of synapsin I (21, 22) and microtubule-associated protein MAP-2 (23) by CaMKII, and of the glucose transporter GLUT-2 by PKA (24). It is not clear, however, if these phosphorylation events play a direct role in mediating insulin release.

Phogrin (phosphatase homologue in granules of insulinoma) is a transmembrane glycoprotein localized to dense-core secretory granules in a wide range of neuronal and endocrine cell types (25, 26). It is a member of the protein-tyrosine phosphatase (PTP) family, with a single cytosolic PTP domain, but appears to lack tyrosine phosphatase activity due to the substitution of a highly conserved residue within the substrate binding pocket. The protein is initially synthesized as a 135-kDa precursor and post-translationally processed in the secretory pathway to the mature 60/64-kDa form. We have recently shown that phogrin is phosphorylated in intact MIN6 β-cells
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upon secretagogue stimulation (27). The time course of phosphorylation parallels that of insulin release, and, like secretion, phosphorylation in response to glucose or high K+ is dependent on the presence of Ca2+. Conditions that increase cAMP levels stimulate phogrin phosphorylation in a Ca2+-independent manner. The modification of phogrin by two different intracellular signaling systems, both important in regulated exocytosis, combined with its strategic location on the dense-core granule surface, make it a good candidate for a protein with a key role in the regulation of secretory granule function.

In this study, we report that phogrin is a substrate for PKA, and that phosphorylation occurs on a Ser and a Thr residue in its cytosolic domain. Our results indicate that Ca2+- and cAMP-mediated responses target the same phosphorylation sites, with the two signaling pathways converging upstream of PKA activation.

MATERIALS AND METHODS

Reagents—All chemicals were purchased from Sigma or Fisher, unless indicated otherwise. Molecular biology reagents were from New England Biolabs; cell culture supplies were obtained from Life Technologies, Inc.; forskolin, IBMX, KN-93, and H-89 were from Calbiochem.

Cell Culture—MIN6 mouse insulinoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 25 mM glucose) supplemented with 10% (v/v) fetal bovine serum, antibiotics, l-glutamine, and 50 μg β-mercaptoethanol, as described previously (27). AtT20 mouse anterior pituitary cells were grown in DMEM/F-12 (3:1 ratio) with 25 mM glucose, 10% donor calf serum with iron, 10% horse serum, and antibiotics.

In Vitro Phosphorylation of Phogrin: Analysis of Ca2+-Dependence—Glutathione S-transferase (GST) and the cytosolic domain of rat phogrin fused to GST (GST/C) (25) were purified by glutathione affinity chromatography. GST or GST/C (5 μM) was incubated at 30 °C with MIN6 extract (2 μg of protein) in 50 μl of 40 mM HEPES, pH 7.0, 10 mM MgCl2, 20 μM ATP, 2 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) in the presence of 2 mM EGTA, 0.5 mM Ca2+, or 10 μg/ml calmodulin as indicated in the figure legends. MIN6 extract was prepared by rinsing cells in phosphate-buffered saline (PBS) and homogenizing (several passes through a 28-gauge needle) in 40 mM HEPES, pH 7.4, 2 mM EGTA with protease inhibitors (10 μg pepstatin A, 10 μg E-64, 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 12,000 × g for 10 min, the pellet extracted once in 0.25 volume of the above buffer containing 0.5% Triton X-100, re-centrifuged, and the supernatant was spotted onto P-81 paper (Whatman), immersed in 5% (w/v) trichloroacetic acid to a concentration of 5% (w/v), incubated on ice for 20 min, and cleared of precipitated protein by centrifuging for 1 min at 12,000 g, pelleted by centrifugation at 14,000 × g for 10 min, the pellet extracted once in 0.25 volume of the above buffer containing 0.5% Triton X-100, re-centrifuged, and the supernatant was spotted onto P-81 paper (Whatman), immersed in 5% (w/v) trichloroacetic acid to a concentration of 5% (w/v), incubated on ice for 20 min, and cleared of precipitated protein by centrifuging for 1 min at 12,000 × g. Aliquots of each supernatant were spotted onto P-81 paper (Whatman), immersed in 5% (w/v) trichloroacetic acid to a concentration of 5% (w/v), incubated on ice for 20 min, and cleared of precipitated protein by centrifuging for 1 min at 12,000 g, pelleted by centrifugation at 14,000 × g for 10 min, the pellet extracted once in 0.25 volume of the above buffer containing 0.5% Triton X-100, re-centrifuged, and the supernatant was spotted onto P-81 paper (Whatman), immersed in 5% (w/v) trichloroacetic acid to a concentration of 5% (w/v), incubated on ice for 20 min, and cleared of precipitated protein by centrifuging for 1 min at 12,000 × g.

Transfections—Phogrin constructs in pcDNA3 were transfected into AtT20 cells with LipofectAMINE (Life Technologies, Inc.), following the manufacturer’s recommendations. Steady neomycin-resistant cell lines were selected with 1 mg/ml G418 and screened for phogrin expression by Western blotting and immunofluorescence microscopy. For each construct, several phogrin-positive cell lines were initially characterized, and representative lines were chosen for subsequent experiments. Cells were maintained in medium containing 0.25 mg/ml G418.

Gel Filtration—MIN6 cells were homogenized in 40 mM HEPES, pH 7.4, 2 mM EGTA, 200 mM sucrose, 50 mM NaCl with protease inhibitors, and centrifuged at 20,000 × g for 45 min. The supernatant ("cytosol") was subjected to gel filtration using a BioCAD 700E chromatography system (PerSeptive Biosystems). Sample (1.6 mg of protein in 0.4 ml) was fractionated on a Superdex 200 HR10/30 column (24 ml) (Amersham Pharmacia Biotech) in 40 mM HEPES, pH 7.0, 1 mM EDTA, 0.1 mM dithiothreitol, 150 mM NaCl, and collected in 0.5-ml fractions.

Protein Kinase A-Dependent In Vitro Phosphorylation—GST or GST/C (5 μM) was incubated at 30 °C in 50 μl of 50 mM Tris, pH 7.4, 10 mM MgCl2, 50 μM ATP, 2 μCi of [γ-32P]ATP with MIN6 protein in the presence of 10 mM EGTA, 10 μM cAMP. MIN6 cells were either disrupted by sonication in PBS with protease inhibitors to obtain total protein (10 μg/assay) or fractionated by gel filtration (5 μl sample/assay). (Note: this resulted in a dilution of NaCl carried over from column fractions to 15 mM and avoided inhibition of PKA activity by high NaCl.) Alternatively, recombinant PKA catalytic subunit (0.1–10 units/μl New England Biolabs) was used. Reaction products were analyzed by SDS-PAGE as described above.

HPLC Analysis of Peptides—100 μg of GST/C was phosphorylated with 5 units of PKA in the presence of [γ-32P]ATP (8 μCi) in 100 μl of 50 mM Tris, pH 7.4, 10 mM MgCl2, 50 μM ATP for 2 h at 30 °C. Samples were heated to 100 °C for 15 min, cooled, and incubated with sequencing-grade trypsin (2 μg of trypsin/100 μg of GST/C) for 5 h at 37 °C. Digestion was stopped by adding 5 μl of 10% (v/v) trifluoroacetic acid, and insoluble material was removed by centrifuging for 5 min at 9,000 × g. For HPLC fractionation (BioCAD 700E), peptides were loaded onto a C18 reverse phase column equilibrated with 0.1% trifluoroacetic acid and 10% (v/v) acetonitrile. Fractions of 1 ml or 0.2 ml (in the peak 1 and 2 regions), were collected at a flow rate of 1 ml/min, using a 60% gradient of 10% (v/v) acetonitrile for the first 60 ml, 0%–95% acetonitrile for 20 ml. Peptides were monitored by absorbance at 214 nm; 32P was measured by Cerenkov counting of fractions. Peptides of interest were sequenced by Edman degradation at the UCHSC Cancer Center core facility.

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by SDS-PAGE and phosphorimaging. B, GST/C was incubated as in A for 6 min, except that the assay mix contained EGTA (2 mM), Ca\(^{2+}\) (0.5 mM), and/or calmodulin (10 μg/ml) as indicated. C, MIN6 cytosol was fractionated by gel filtration. Fractions (25 μl/assay) were analyzed for phosphorimaging activity (15-min incubation; bottom panels) and for CaMKII activity by determining incorporation of radioactivity (cpm) into the peptide substrate autacamide-2 (10-min incubation; top panel). Plotted data are representative of two separate assays. EGTA (2 mM), Ca\(^{2+}\) (~0.5 mM free), or Ca\(^{2+}\) and calmodulin (10 μg/ml) were added as indicated. Arrowheads mark GST/C. The positions of molecular mass markers (kDa) for gel filtration are shown at the bottom, and for SDS-PAGE on the left.

**RESULTS**

**Phosphorylation of Recombinant Phogrin in Vitro**—We have previously shown that glucose-stimulated phogrin phosphorylation in intact β-cells is dependent on the presence of extracellular Ca\(^{2+}\). Inhibitor studies targeting different protein kinases were consistent with CaMKII activation being involved (27). To examine this phosphorylation event further, the phogrin cytoplasmic domain fused to GST (GST/C) was incubated in vitro with MIN6 β-cell protein extract in the presence of \[γ^{32}\]P]ATP. Analysis on SDS-PAGE gels showed phosphate incorporation into the fusion protein (Fig. 1A). However, this was not stimulated by the addition of Ca\(^{2+}\) and/or calmodulin and still occurred in the presence of a Ca\(^{2+}\) chelator (Fig. 1B). Fractionation of MIN6 cytosol by gel filtration revealed that CaMKII activity eluted as a high molecular weight peak (Fig. 1C, top panel), consistent with the existence of the enzyme in vivo as a multimeric complex of 8–12 subunits (6). Ca\(^{2+}\)/calmodulin also stimulated the incorporation of phosphate into several endogenous proteins in the same fractions (the bands of about 55 kDa on SDS-PAGE gels may represent autophosphorylated CaMKII monomers); however, no phosphorylation of phogrin was seen under these conditions (bottom panels). Similarly, phogrin was a poor substrate for recombinant CaMKII (data not shown).

**In vitro phosphorylation of phogrin GST/C by MIN6 extract** was markedly stimulated by cAMP or its non-hydrolyzable analogue 8-Br-cAMP (Fig. 2A). In MIN6 cytosol fractionated by gel filtration, cAMP-stimulated phosphorylating activity eluted in two broad peaks (Fig. 2B, upper panels). Western blot analysis of PKA distribution with antibodies to the catalytic subunit of the kinase showed a similar profile, both for the α- and the β-isoforms (lower panels). The enzyme’s presence in high molecular weight fractions may reflect the formation of a complex with regulatory subunits, and presumably interactions with anchoring proteins (28). Incubation of GST/C with the recombinant catalytic subunit of PKA alone phosphorylated the fusion protein directly, in a concentration-dependent manner (Fig. 2C).

Phosphorylation of mature phogrin in pancreatic β-cells induces a characteristic mobility shift on SDS-PAGE gels, giving rise to a 60-kDa basal form and a phosphorylated protein migrating with an apparent molecular mass of 64 kDa. When in vitro phosphorylated GST/C was examined on SDS-PAGE gels, three distinct molecular forms were observed after Coomassie staining. Phosphorimaging revealed that two of these...
had incorporated phosphate (Fig. 2D). Phosphorylated form 1 (P1) migrated with an apparent molecular mass of around 2 kDa higher than non-phosphorylated GST/C; P2 showed a shift of about 4 kDa. In a time-course experiment, P1 was observed initially, and the P2 form at later time points (data not shown), indicating the existence of more than one phosphorylation site.

**Identification of PKA Phosphorylation Sites**—The cytosolic domain of phogrin contains 56 serine or threonine residues, 7 of these in classic PKA recognition motifs (29). To narrow down the location of the target sites, phogrin GST/C phosphorylated in vitro by recombinant PKA in the presence of [γ-32P]ATP was subjected to proteolytic digestion with trypsin and fractionated by HPLC. Two peaks of 32P incorporation were detected (peaks 1 and 2) (Fig. 3A), in both cases corresponding to well resolved peptide peaks monitored as absorbance at 214 nm (Fig. 3B). Comparison of profiles of tryptic peptides derived from phosphorylated and non-phosphorylated GST/C demonstrated a shift of the two A214 peaks toward elution at lower solvent

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**Fig. 2.** Cyclic AMP and PKA-dependent phosphorylation of phogrin in vitro. A, GST or GST/C was incubated with MIN6 protein (10 μg) and [γ-32P]ATP for the indicated periods of time in the presence (+cAMP) or absence (−cAMP) of 8-Br-cAMP (10 μM). Samples were analyzed by SDS-PAGE and phosphorimaging, and the phosphorylated bands quantified. The upper panel shows the 30-min time point. A representative experiment is shown; addition of 8-Br-cAMP typically stimulated phosphorylation 4.1 ± 0.7-fold (n = 5). B, upper panels, cAMP-stimulated phogrin phosphorylating activity was determined in cytosol fractionated by gel filtration. GST/C was incubated with fractions (5 μl/assay) for 20 min as in A. Lower panels, fractions (40 μl) were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and Western-blotted for PKA using antibodies recognizing either the α-isoform or the α- and β-isoforms of the catalytic subunit. C, GST/C was incubated with recombinant PKA catalytic subunit and [γ-32P]ATP. Left panel, incubation with 1 unit of PKA for 1–30 min; right panel, incubation for 30 min with 0.1–10 units of enzyme. (Note: left panel shows a longer exposure than right panel.) D, GST/C was incubated as above with or without PKA. Gels were run for an extended period of time to separate closely spaced bands. Proteins were stained with Coomassie Blue, followed by phosphorimaging to determine incorporation of radioactivity.
concentrations upon incorporation of negatively charged phosphate groups into the peptides.

N-terminal sequencing of peptide 1 yielded Ile-Asn-Ser-Val-Ser, identifying it as a 19-residue tryptic peptide spanning amino acids 678–696. For peptide 2, Ser-Ser-Thr-Ser-Ser was obtained, corresponding to a 32-residue peptide from amino acids 697 to 728 (Fig. 3C). Both peptides form part of a region highly enriched in serines that is located between the transmembrane span and the PTP homology domain of phogrin.

The majority of PKA sites conform to the consensus targets Arg-X-(Ser/Thr) or Arg-X-X-Ser/Thr (29). Based on this, site-directed mutagenesis was performed on candidate residues within peptides 1 and 2. Mutants were expressed as cytosolic domain GST fusion proteins and tested for in vitro phosphorylation by recombinant PKA. Substitution of Ser-680 with alanine resulted in a significant decrease in phosphate incorporation, and in a reduced mobility shift on SDS-PAGE gels (Fig. 4A). Even after prolonged incubation times, S680A did not reach the P2 position of fully phosphorylated GST/C. Additional point mutations introduced at either Ser-698 or Ser-700 did not significantly alter the phosphorylation pattern. However, a S680A/T699A double mutant neither incorporated phosphate nor showed the corresponding bandshift (Fig. 4B), suggesting that Ser-680 and Thr-699 were the PKA target sites in rat phogrin.

Subcellular Localization and Phosphorylation of Recombinant Phogrin in AtT20 Cells—AtT20 pituitary corticotroph tumor cells were chosen to examine the behavior of mutant forms...
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of phogrin in a cellular context because these cells express very low levels of the endogenous molecule (Fig. 5, A (lane 1) and B (first panel)). Cells stably transfected with full-length wild-type phogrin analyzed by immunoblotting with an antibody directed against the lumenal domain of the protein showed a doublet band at 60/64 kDa, the expected size for mature, post-translationally processed phogrin (Fig. 5A). In addition, significant levels of the 135-kDa precursor and a 71/75-kDa doublet, presumably a processing intermediate, were seen.

Immunofluorescence microscopy did not detect significant phogrin expression in the parental cell line or in cells transfected with vector alone. Cells transfected with wild-type phogrin showed strong labeling at the tips of cell extensions, as well as in the perinuclear region and in punctate structures throughout the cell body (Fig. 5B). The distribution of phogrin overlapped extensively with the labeling for the granule marker ACTH, both in the cell processes and in phogrin-positive cytoplasmic vesicles, suggesting that AtT20 cells, like pancreatic β-cells, targeted the recombinant molecule to secretory granules. When protein synthesis was inhibited with cycloheximide for 2.5 h, the perinuclear pool largely disappeared while labeling was also seen for phogrin, and a small number of phogrin-positive, ACTH-negative vesicles were evident, especially in the cytoplasm. This is consistent with differences in trafficking between granule membrane components, which are retained and are thought to recycle after exocytosis, and content proteins, which are released.

To evaluate the AtT20 cells’ ability to phosphorylate phogrin in response to secretagogue stimulation, cells transfected with wild-type phogrin were pre-equilibrated with [32P]Pi, followed by incubation either in basal or in stimulation medium containing 10 μM forskolin and 0.5 mM IBMX. Immunoprecipitation of phogrin showed little background in cells transfected with vector alone. In phogrin-expressing cells, some 32P labeling was observed under basal conditions, which was markedly increased in response to forskolin stimulation (Fig. 5C). Phosphate was incorporated both into the fully processed 60/64-kDa phogrin and the 71/75-kDa intermediate form.

Expression of Phosphorylation Mutants in AtT20 Cells—To study phogrin phosphorylation in a cellular context, point mutations were introduced into the full-length molecule. The PKA target residues were replaced with either alanine to prevent their phosphorylation, or aspartic acid to mimic constitutively phosphorylated residues. Phogrin S680A/T699A (AA) or S680D/T699D (DD) appeared to be post-translationally processed as efficiently and reached steady-state levels similar to those for the wild-type molecule (Fig. 6A). The subcellular localization of phogrin/AA and phogrin/DD also resembled that of the wild-type protein, with prominent immunolabeling at the tips of cell processes and in the perinuclear region, as well as in punctate structures throughout the cell (Fig. 6B). For all three phogrin variants, the steady-state distribution showed extensive co-localization with the secretory granule marker ACTH. As observed for the wild-type molecule, inhibition of protein synthesis resulted in reduction of the perinuclear pool of phogrin/AA or DD, but did not affect staining in the tips of processes where the majority of granules are located (Fig. 6C).

To determine if phogrin phosphorylation in intact cells occurred at Ser-680 and Thr-699, and to examine if further residues in addition to the target sites identified in vitro were involved, cells expressing equivalent amounts (see Fig. 6A) of wild-type, AA or DD phogrin were analyzed by [32P]Pi labeling. Different types of secretagogues were used, including those acting via cAMP-dependent pathways (the adenylyl cyclase activator forskolin and the phosphodiesterase inhibitor IBMX),
and those relying on Ca\(^{2+}\)-dependent pathways (depolarizing concentrations of K\(^+\), or Ba\(^{2+}\), which mimics the effect of Ca\(^{2+}\) on exocytosis). Stimulation of cells with either 55 mM KCl, 1 mM BaCl\(_2\) or 10 mM forskolin/0.5 mM IBMX, or with a combination of the above, resulted in the incorporation of phosphate into wild-type phogrin, but neither into the AA nor the DD mutants (Fig. 7). These findings suggested that Ser-680 and Thr-699 represent the physiologically relevant target sites for secretagogue-dependent phosphorylation.

PKA-dependent Versus Ca\(^{2+}\)-dependent Phosphorylation—

The highest levels of phogrin phosphorylation were achieved by treating cells with 10 mM forskolin in the presence of 0.5 mM IBMX, a combination of compounds aimed at maximally stimulating PKA by increasing the rate of cAMP synthesis while inhibiting its breakdown by phosphodiesterases. Depolarization with K\(^+\) or stimulation with BaCl\(_2\) resulted in lower but reproducible levels of phosphorylation. When the cells received a high K\(^+\) or a BaCl\(_2\) stimulus in addition to forskolin/IBMX, phosphate incorporation into phogrin did not increase beyond that induced by forskolin/IBMX alone (Fig. 7). This is suggestive of the convergence of the Ca\(^{2+}\)-dependent and the cAMP-mediated signaling pathways upstream of PKA. To investigate if PKA was required for Ca\(^{2+}\)-dependent phogrin phosphorylation, the effect of the PKA inhibitor H-89 was examined in glucose-stimulated MIN6 \(\alpha\)-cells. H-89 (20 mM) suppressed phosphorylation induced by 10 mM forskolin, either by \(^{32}\)P\(_2\) labeling of phogrin or by Western blotting to assay for the bandshift characteristic of phosphate incorporation (Fig. 6).
Phosphorylation of phogrin/AA and phogrin/DD in AtT20 cells. Cells stably expressing wild-type phogrin, or either AA or DD mutant phogrin, were pre-equilibrated with \[^{32}P\]Pi, for 2 h, followed by a 30-min incubation in basal or stimulating buffer. Stimulation buffers contained 55 mM KCl (K\(^+\)), 1 mM BaCl\(_2\) (Ba\(^{2+}\)), 10 \(\mu\)M forskolin + 0.5 mM IBMX (F/I), or either KCl or BaCl\(_2\) in combination with forskolin + IBMX. As a control, cells transfected with vector alone were stimulated with forskolin + IBMX. Phogrin was immunoprecipitated using an antiserum to the lumenal domain. The bottom panel represents quantitation of phosphate incorporation into the 60/64-kDa form of phogrin. Results shown are representative of two independent experiments. i., intermediate; m., mature protein; wt, wild-type.

Phogrin phosphorylation in intact MIN6 \(\beta\)-cells in response to glucose or K\(^+\) only occurs in the presence of extracellular Ca\(^{2+}\), and is blocked by inhibitors of CaMKII. Ca\(^{2+}\)-independent phosphorylation, however, can be evoked by cAMP, presumably via the activation of PKA (27). Attempts to reconstitute Ca\(^{2+}\)-dependent phosphorylation in vitro using MIN6 homogenate, fractionated cytosol, or recombinant CaMKII as a source of enzyme were unsuccessful, although the kinase was clearly active under the assay conditions used. On the other hand, MIN6 preparations phosphorylated phogrin in vitro in a CaM-dependent manner, an activity that co-fractionated with endogenous PKA and could be mimicked by recombinant PKA catalytic subunit alone.

Recombinant phogrin cytosolic domain, upon phosphorylation with PKA, underwent a mobility shift similar to that characteristic of phosphorylation of the native protein, supporting the hypothesis that the in vitro modifications reflect events in a stimulated \(\beta\)-cell. We showed that phosphorylation occurred at Ser-680 and Thr-699 on two adjacent tryptic peptides, both located within a stretch of around 50 amino acids containing a total of 18 serines and threonines. This cluster of serine residues is positioned between the transmembrane span and the PTP domain, a part of the molecule that is highly susceptible to proteases in native phogrin (25). Thus, this region may represent a flexible hinge linking the transmembrane segment to the more tightly folded globular PTP domain. In such a model, phosphorylation of residues in the hinge region could result in a conformational change within the cytoplasmic domain to permit or exclude interactions with binding partners.

To study the behavior of mutant phogrin molecules in a neuroendocrine cell type, AtT20 cells were chosen as a model system because of their low levels of endogenous protein. Exogenous phogrin was efficiently targeted to secretory granules when expressed at levels comparable to those found in MIN6 cells. Under steady-state conditions, the protein is present both in granules, which in AtT20 cells are concentrated in the tips of...
processes, and in the perinuclear region of the cell, presumably the Golgi. The latter pool was found to represent newly synthesized protein which completes its transit through the Golgi and trans-Golgi network over a period of 2 h. Phogrin localizing to the cell extensions, or to ACTH-positive cytoplasmic vesicles, was much more stable, consistent with secretory granules being the main site of residence in the cell. AtT20 cells transfected with wild-type phogrin were capable of phosphorylating the molecule in response to stimulation with a range of secretagogues. Phogrin mutants lacking both phosphorylation sites identified in vitro no longer incorporated phosphate upon stimulation, suggesting that the same residues are phosphorylated in vivo. No additional residues appear to be targeted in intact cells, neither when Ser-680 and Thr-699 were replaced by alanines, nor when PKA phosphorylation was mimicked by aspartic acid residues.

These experiments also provided insight into the interplay between the Ca\(^{2+}\)- and the cAMP-dependent signaling pathways involved in regulating phogrin phosphorylation (27). The two sites phosphorylated by PKA appeared to be the same ones targeted in response to stimuli relying on Ca\(^{2+}\)-dependent pathways (K\(^+\) and BaCl\(_2\)), consistent with the Ca\(^{2+}\)-activated regulatory step being located upstream of PKA. In MIN6 \(\beta\)-cells, the PKA inhibitor H-89 completely blocked phosphorylation of endogenous phogrin induced by glucose or K\(^+\), stimuli whose mode of action relies on elevating cytosolic Ca\(^{2+}\). On the other hand, the effects of W-7 or KN-93, while suppressing glucose and K\(^+\)-induced phogrin phosphorylation, could be bypassed by stimulating PKA directly with forskolin. This argues for PKA playing a key role downstream of Ca\(^{2+}\) signaling.

From our initial inhibitor studies (27), CaMKII emerged as a key kinase for the enzyme mediating the Ca\(^{2+}\)-dependent step since phosphorylation was inhibited by KN-93, but not by the same concentration of its inactive structural analogue KN-92. Moreover, glucose or K\(^+\) stimulation is known to activate \(\beta\)-cell CaMKII (30) and results in the phosphorylation of several endogenous substrates (21, 23). However, a recent study found that KN-93 suppressed Ca\(^{2+}\) influx into intact stimulated \(\beta\)-cells much more potently than KN-92 (31); hence, the effects of these compounds on Ca\(^{2+}\)-dependent phogrin phosphorylation could result from an inhibition of Ca\(^{2+}\) influx rather than CaMKII activity. This is consistent with our in vitro experiments, which led us to conclude that phogrin is not phosphorylated by CaMKII. It is possible that the link between Ca\(^{2+}\) and PKA is at the level of adenylyl cyclase. At least two members of this enzyme family, adenylyl cyclases 1 and 8, are activated by Ca\(^{2+}\) and calmodulin (32). The expression of these isoforms has recently been reported in pancreatic \(\beta\)-cells (33), and it will be of interest to investigate the effects of secertagogue stimulation upon their activity.

How might phosphorylation regulate phogrin function? Studies in pancreatic \(\beta\)-cells have shown that only a small fraction of granules are in a docked, readily releasable state (20). It has been suggested that cAMP and PKA play an important role in recruiting intracellular granules to the sites of exocytosis to replenish this pool under conditions of prolonged stimulation (19, 34). Since this step is likely to be subject to regulation by secretagogues, we hypothesize that phogrin may be involved in this aspect of granule function, possibly by promoting the interaction of granules from a reserve pool with components of the microtubule- or actin-based cytoskeleton in a phosphorylation-dependent manner. Alternatively, phosphorylation may play a role in phogrin recycling subsequent to granule exocytosis by affecting its trafficking in the endocytic pathway, similar to what has been proposed for the protein kinase C-mediated phosphorylation of integral membrane peptideglycine \(\alpha\)-amidating monoxygenase (35, 36).

The expression of phogrin phosphorylation site mutants in AtT20 cells did not significantly affect hormone release (data not shown). However, if phogrin was involved in mobilizing cytoplasmic granules to refill a pool of vesicles released during an initial burst of exocytosis, its effect on cumulative release of hormone over a comparatively short period of time would be small. The number of granules docked and primed for rapid exocytosis is not known for AtT20 cells, but appears to vary greatly between cell types, from an estimated 40 granules in the pancreatic \(\beta\)-cell (20) to approximately 800 or 3000 in adrenal chromaffin cells or pituitary melanotrophs, respectively (37). Examination of the effects of phogrin phosphorylation mutants on the secretory response in pancreatic \(\beta\)-cells will therefore be of interest. However, such studies are complicated by the high levels of wild-type phogrin present in these cells. Previous attempts to generate MIN6 lines stably expressing various mutant phogrin molecules resulted in expression levels significantly lower than those of the endogenous protein, and strategies such as antisense suppression of endogenous phogrin or a phogrin knockout may be required to allow functional expression of phosphorylation mutants in \(\beta\)-cells. Such studies are warranted since the substantially higher levels of endogenous phogrin found in \(\beta\)-cells may reflect a greater functional dependence of their secretory mechanisms on this molecule.

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