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IDENTIFICATION OF CALCINEURIN REGULATED PHOSPHORYLATION SITES ON CRHSP-24

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Abstract

CRHSP-24 is a prominently regulated phosphoprotein in pancreatic acinar cells where it is the major substrate for the serine/threonine protein phosphatase, calcineurin, in response to secretagogues. We now identify the four regulated sites of CRHSP-24 phosphorylation as serines 30, 32, 41 and 52 and show that Ser^{30} and Ser^{32} are directly dephosphorylated by calcineurin. Coordinate phosphorylation/dephosphorylation of these four serines explains the multiple phosphorylated isoforms of CRHSP-24 present in acinar cells and provides a molecular framework to study CRHSP-24 regulation by secretagogues and growth factor-induced kinases and phosphatases *in vivo*.

Keywords

Calcineurin; CRHSP-24; pancreas; acinar cells; phosphoprotein; protein phosphatases

INTRODUCTION

The calcium regulated heat-stable protein of 24 kDa, CRHSP-24, was originally identified in pancreatic acinar cells by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) after ^{32}P metabolic labeling [1,6,10,11]. In acinar cells it is subject to secretagogue-induced dephosphorylation [6,10,11], an effect that is blocked by known inhibitors of calcineurin Ser/Thr phosphatase activity, cyclosporine A (CsA) and FK506 [1]. CRHSP-24 dephosphorylation can also be brought about by addition of active calcineurin to acinar lysates [1]. Thus, CRHSP-24 was purified from rat pancreas as a novel calcineurin substrate, and subsequently sequenced and characterized as a 147 amino acid proline-rich protein. It is widely distributed in various tissues, with relatively high levels of protein expression in testis, liver, pancreas and other exocrine glands, where CRHSP-24 phosphorylation occurs entirely on serine residues [2]. Although its cellular role is unclear, CRHSP-24 has been reported to immunoprecipitate

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from whole testis in a protein complex with STYX, a catalytically inactive member of the dual specificity phosphatase family [5]. CRHSP-24 is also paralogous to a phosphorylated, brain specific mRNA binding protein, termed PIPPin [3,4], which share a structural topology characterized by a central cold shock domain flanked by RNA binding motifs. In PIPPin, these motifs are essential for direct binding and regulation of translation of specific histone mRNAs. In contrast, the RNA binding capacity of CRHSP-24 has not been demonstrated and the role of regulated phosphorylation in modulating this activity in PIPPin and CRHSP-24 are still unknown.

Immuno-detection of CRHSP-24 in pancreatic acini revealed that the protein existed as multiple phosphorylated isoforms that migrated on 2-dimensional PAGE and in isoelectric focusing gels consistent with four regulated phosphorylation sites. A dephosphorylation induced shift to more alkaline bands occurred in response to agonist or second messenger treatment via a mechanism that could be blocked in acini by the immunosuppressants CsA and FK506 [2]. It has also been shown that CRHSP-24 dephosphorylation could be induced with phorbol esters or cell permeant cAMP [6] and mediated by a calyculin sensitive phosphatase [7]. These later studies support a model in which regulated dephosphorylation of CRHSP-24 involves subsets of calcineurin sensitive and PP2A sensitive sites. Although little is known about the counteracting kinases to calcineurin and PP2A in pancreas, CRHSP-24 has recently been shown to be a substrate for Akt and RSK in human embryonic kidney (HEK293) cells, and by DYRK2 phosphorylation *in vitro* [12]. To identify the physiological phosphorylation sites on CRHSP-24 we initially show that CRHSP-24 can be expressed and dephosphorylated in HEK293 cells in response to Ca^{2+}. Mutation of the fourteen serine residues present in rat CRHSP-24, individually and in combination, identified 4 phosphorylation sites (serines 30, 32, 41, 52), with Ser^{30} and Ser^{32} being the primary calcineurin sensitive residues.

METHODS

Vector subcloning and mutagenesis

For expression in cultured cells, the prevoiusly described open reading frame of rat CRHSP-24 [2] was subcloned into pcDNA3.1 using 5' *Hind*III and 3' *EcoR*I sites. The fourteen serines encoded within rat CRHSP-24 were changed to alanines using QuickChange® Site-Directed Mutagenesis Kit (Stratagene). The adjoining first two and last two serine sites ($Ser^{2,3}$ and $Ser^{146,147}$, respectively) were changed to alanines in tandem while all other serines were individually substituted to alanine. The final pcDNA3.1 expression constructs were confirmed by nucleic acid sequencing at the University of Michigan Sequencing Core. Upon identification of the four phosphorylated serines in CRHSP-24, all combinations of double, triple, and quadruple mutants were also prepared using the QuickChange® Site-Directed Mutagenesis Kit and confirmed by sequencing. Recombinant rat CRHSP-24 protein was expressed in bacteria as a GST fusion protein, purified by binding to glutathione agarose beads followed by thrombin cleavage as previously described [2].

Cell culture

Human embryonic kidney (HEK293) cells were propagated in Dulbecco Modified Eagle Medium (DMEM) with 10% fetal calf serum under 5% v/v CO_2. For expression of CRHSP-24 serine mutants, approximately 1×10^5 cells were grown overnight in 6 well plates coated with poly-L-lysine and then transfected with 1 μg of pcDNA3.1 constructs using Lipofectamine (Gibco BRL). After 48 hrs, the cells were washed and incubated in serum-free DMEM for 2 hours. When specified, 1 μM cyclosporine A (CsA) or 100 nM FK506 was present in this medium. Culture medium was then replaced with fresh DMEM containing either 5 μM ionomycin or an equivalent amount of DMSO solvent. After 15 min incubation, the wells were washed with ice cold PBS and cells solubilized in 100 μl of isoelectric focusing (IEF) buffer

containing 9 M urea, 4% IGEPAL, and 1% β-mercaptoethanol. For each sample, 5 μg of lysate protein was subjected to IEF and Western blotted onto nitrocellulose as described previously [8]. Membranes were probed using a polyclonal rabbit antibody raised against rat GST-CRHSP-24 [2], followed by HRP-conjugated secondary antibodies and detection by enhanced chemiluminescence (Amersham Pharmacia) as recorded on X-ray film.

RESULTS

In preparation for transfection studies, we initially screened untransfected cell lines for endogenous protein expression by Western blotting cell lysates and probing with antibodies to rat CRHSP-24. Small amounts of CRHSP-24 protein were found in mouse embryonic fibroblast (NIH3T3) and Chinese hamster ovary (CHO) cells, while no protein was detected in human embryonic kidney (HEK293) and transformed monkey kidney (COS) cells (not shown). When CRHSP-24 was transiently transfected into HEK293 or COS cells, several immunoreactive protein bands were identified by IEF at the acidic end of the gel. Stimulation with ionomycin, to increase cytoplasmic Ca^{2+} and activate calcineurin, shifted the CRHSP-24 banding pattern to the alkaline end of the gel. A total of five distinct immunoreactive bands were observed upon ionomycin treatment, and this effect could be totally blocked with cyclosporine A (Fig. 1A) or FK506 (not shown). Similar patterns of CRHSP-24 IEF bands were observed in COS cells and the effects in both HEK293 and COS cells paralleled what we had previously observed in rodent pancreatic acinar cells stimulated with cholecystokinin (CCK) [2,7,9].

To identify serine residues in CRHSP-24 that were phosphorylated *in vivo*, we substituted individual serines with alanines and expressed the mutant proteins in HEK293 cells. Site-directed mutagenesis of ten of the fourteen serines in rat CRHSP-24 did not alter the overall banding pattern of isoforms resolved by IEF (Fig. 1B, lanes 1-3 and 8-13). In contrast, mutation of serines at positions 30, 32, 41, or 52, caused the disappearance of the most acidic CRHSP-24 isoform and a shift of the protein pattern to more alkaline IEF bands (Fig. 1B, lanes 4-7). To determine if differential phosphorylation of serines 30, 32, 41 and 52 could account for each of the major CRHSP-24 bands resolved by IEF, we mutated all six possible doublets and three out of four possible triplets of the phosphorylatable residues for expression in HEK293 cells. As expected, the most acidic two IEF bands disappeared when any two serine residues were mutated, and three acidic bands were lost when three serines were removed (representative results from a subset of CRHSP-24 mutants are shown in Fig. 2A). Moreover, a single IEF band was observed to migrate similar to unphosphorylated, recombinant bacterial CRHSP-24 when all four serines were mutated to alanines (Figs. 2A and B). Thus, we concluded that the four phosphorylatable serines at positions 30, 32, 41 and 52, could account for the major bands resolved by IEF and that these bands represented CRHSP-24 phospho-isoforms containing 4, 3, 2, or 1 phosphate(s).

To determine the phosphoserine residues that were dephosphorylated by calcineurin, we expressed CRHSP-24 constructs containing only one of the four regulated serines (Fig. 3) in ionomycin-stimulated HEK293 cells. Ionomycin induced dephosphorylation of individual serines at positions 30 and 32, and this effect was completely blocked by addition of calcineurin inhibitor, cyclosporine A. In the case of Ser^{41}, only a small portion of the CRHSP-24 was phosphorylated prior to ionomycin-induced dephosphorylation, however, dephosphorylation of this site was not blocked by CsA. In contrast, CRHSP-24 Ser^{52} was prominently phosphorylated, but not affected by either ionomycin or CsA treatment. Thus we concluded that phosphorylation of Ser^{30} and Ser^{32} were the primary sites of calcineurin-mediated dephosphorylation and that phosphorylated Ser^{41} and Ser^{52} represent potential targets for other phosphatases, most likely PP2A or PP4 [7]. Since the presence of neighboring phosphorylation sites could alter the availability of these sites for dephosphorylation, we evaluated if

diphosphorylated CRHSP-24, at Ser^{30} and Ser^{32}, could serve as a calcineurin substrate. Indeed, CRHSP-24 with both Ser^{30} and Ser^{32} present was completely dephosphorylated in response to ionomycin in a CsA-regulated manner (data not shown).

DISCUSSION

CRHSP-24 is the major calcineurin regulated phosphoprotein in pancreatic acinar cells. It was originally identified as a phosphoprotein of 24 kDa that underwent dephosphorylation in mouse and guinea pig acini in response to the secretagogues, carbachol and CCK, as well as calcium ionophore, active phorbol ester and 8Br-cyclic AMP [10,11]. Subsequent work with large format 2-D PAGE resolved CRHSP-24 as a series of heat-stable, phosphorylated isoforms among 500 phosphoprotein spots from rat acinar cells [6]. Three of these isoforms, termed Id-24/6.2, Ii-23/6.5 and Ii-23/6.8, exhibited a range of molecular weights (23 - 24 kDa) and isoelectric points (6.8 - 6.2) characteristic of a multiply phosphorylated protein. Indeed, stimulation of acinar cells with 10-100 pM CCK induced the rapid (30 sec) dephosphorylation of the most acidic, highly phosphorylated Id-24/6.2 isoform, with concomitant reciprocal accumulation of the more alkaline, dephosphorylated, Ii-23/6.5 & Ii-23/6.8 [6]. Follow-up studies demonstrated that CCK-induced dephosphorylation of CRHSP-24 isoforms could be blocked by cyclosporine A treatment, while purified calcineurin caused a Ca^{2+} and calmodulin-dependent partial dephosphorylation of the protein in ^{32}P-labeled pancreatic cytosol *in vitro* [1]. Western blots of pancreatic proteins separated by 2-D PAGE or IEF gels confirmed the existence of at least 4 phosphoisoforms recognized by polyclonal antibodies raised against CRHSP-24 [2], with each isoform of CRHSP-24 exclusively phosphorylated on multiple serine residues. Northern and Western blots also showed CRHSP-24 to exhibit a wide tissue distribution, with relatively high levels in pancreas, parotid gland and testis [2]. Subsequent purification and microsequencing of CRHSP-24 from rat pancreas allowed its molecular characterization as a novel protein of 147 amino acids [2].

The present study identified the four residues in CRHSP-24 that are phosphorylated in cells, as serines 30, 32, 41 and 52 (Fig. 5). Of these, Ser^{30} and Ser^{32} were specifically dephosphorylated in response to increased intracellular Ca^{2+} and inhibited by CsA, indicating that calcineurin is the primary phosphatase targeting these sites. In contrast, singly phosphorylated CRHSP-24 Ser^{52} was not sensitive to increased Ca^{2+}, and ionomycin-induced dephosphorylation of Ser^{41} was not inhibited by CsA, suggesting that the phosphorylation state of Ser^{41} and Ser^{52} are regulated by phosphatases other than calcineurin. Indeed, CRHSP-24 has been shown to be partially dephosphorylated in response to cAMP stimulation [6,7,10, 11], via phosphatases inhibited by calyculin A and okadaic acid, namely PP2A or PP4 [7]. Although it is currently unknown whether these phosphatases overlap in substrate preference with calcineurin, development of antibodies that specifically recognize phosphorylated serine residues 30, 32, 41 and 52 should facilitate the identification of signaling components responsible for CRHSP-24 dephosphorylation *in vivo*.

In contrast to the role of phosphatases in regulated dephosphorylation of CRHSP-24, almost nothing is known of the kinases that phosphorylate the protein under physiological conditions. Auld *et al*. have identified CRHSP-24 Ser^{52} as a phosphorylation site for protein kinase Bα (PKBα, also known as Akt) using a kinase substrate tracking and elucidation technique applied to rat liver [12]. They also showed that phosphorylation of Ser^{52} could be induced by insulin-like growth factor-1 or epidermal growth factor treatment of HEK293 cells and that inhibitors of phosphoinositide 3-kinase and/or mitogen-activated protein kinase signaling cascades could abrogate this effect. In the same study, Ser^{41} was identified as a site of phosphorylation in HEK293 cells and serines 30, 32, and 41 were shown to be phosphorylated *in vitro* by the dual-specificity tyrosine-phosphorylated and -regulated protein kinase 2 (DYRK2), although the physiological relevance of Ser^{30} and Ser^{32} phosphorylation could not be established [12].

Collectively, our results and the work of Auld *et al.* establish serines 30, 32, 41 and 52 as the primary sites of CRHSP-24 phosphorylation in cells and provide a molecular framework for studying the role of specific protein kinases and phosphatases in regulating CRHSP-24 function *in vivo*. To this end, calcineurin has been implicated in pancreatic acini as a mediator of digestive enzyme secretion [1,15], protein synthesis at the translational level [9] and growth [16,17], based on inhibition of these processes by CsA and/or FK506 or Rcan1, and direct linkage of CRHSP-24 to these functions is under study.

In addition to our work in pancreas, identification of the sites of CRHSP-24 phosphorylation provides insight to the role of calcineurin and other phosphatases in cell culture based assays for growth, differentiation, adhesion and cell migration. Global phosphoproteomic approaches have revealed regulated phosphorylation of CRHSP-24 serines 30, 32 and 53 in growing and differentiating C2C12 muscle cells and identified a unique phosphorylation site at Ser^{2} [18] not seen in pancreas (this study) or HEK293 cells [12]. Although CRHSP-24 was not a focus of the work, calcineurin activity is known to be important for normal muscle development and in limiting the pathogenesis of dystrophic myofibers [19]. By contrast, CRHSP-24 Ser^{42} appears constitutively phosphorylated in C2C12 cells [18] and phosphorylation at this site has been observed during integrin-mediated adhesion and spreading of HeLa cells [20]. Interestingly, the Drosophila melanogaster ortholog of CRHSP-24, CG9705 (Fig. 4), was found to bind the corresponding fly proteins for calmodulin and fermitin in global yeast two-hybrid screens of protein interactions [21]. A potential interaction with calmodulin may target CRHSP-24 for subsequent Ca2+-dependent dephosphorylation by calcineurin, whereas, fermitin homologues of human and flies are found as components of integrin-containing cell adhesion structures involved in muscle assembly and maintenance [22]. Interestingly, all of the serine phosphorylation sites identified in this study are conserved in fly CG9705 (Fig. 4), thereby providing the basis to study the role of CRHSP-24 phosphorylation in regulating calmodulin and fermitin interaction in this organism.

Based on gene structure and primary amino acid sequence, CRHSP-24 is a paralog of the brain specific protein, PIPPin (Fig. 4), which has been shown to bind and inhibit the translation of specific histone mRNAs [3]. Consistent with a putative role in mRNA binding, CRHSP-24 and PIPPin contain RNA binding motifs that flank a central cold shock domain (Fig. 4), which are commonly bound to stored RNA but may also be necessary for translation. Alternatively, a subset of PIPPin found in the nucleus may play a role during early stages of mRNA synthesis within chromatin structures [23]. Of note, all of the phosphorylation sites identified in this study are located within the N-terminal RNA binding region that is shared between CRHSP-24 and PIPPin. Indeed, serine residues corresponding to Ser^{41} and Ser^{52} of CRHSP-24 are invariantly found in PIPPin and all known lower eukaryotic CRHSP-24/PIPPin orthologs (Fig. 4). Thus CRHSP-24 and PIPPin binding of mRNA, or their nucleo-cytoplasmic shuttling, could be mediated by phosphorylation and is supported by identification of specific phosphorylation of CRHSP-24 Ser^{52} in nucleus-enriched fractions of HeLa cells [24]. Moreover, retention of serines at positions analogous to Ser^{30} and/or Ser^{32} in CRHSP-24 orthologs support a role for calcineurin as an evolutionarily conserved mechanism of CRHSP-24 dephosphorylation. Finally, CRHSP-24 was also identified in a complex with STYX, an inactive dual specificity phosphatase present in spermatids [5]. Whether binding to STYX or mRNA is mediated by reversible phosphorylation remains to be determined, however, the identification and generation of CRHSP-24 mutants constrained in the phosphorylated and dephosphorylated forms should assist in determining the function of this module.

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REFERENCES

[1]. Groblewski GE, Wagner AC, Williams JA. Cyclosporin A inhibits Ca2+/calmodulin-dependent protein phosphatase and secretion in pancreatic acinar cells. J Biol Chem 1994;269:15111–15117. [PubMed: 7515049]

[2]. Groblewski GE, Yoshida M, Bragado MJ, Ernst SA, Leykam J, Williams JA. Purification and characterization of a novel physiological substrate for calcineurin in mammalian cells. J Biol Chem 1998;273:22738–22744. [PubMed: 9712905]

[3]. Castiglia D, Scaturro M, Nastasi T, Cestelli A, Di Liegro I. PIPPin, a putative RNA-binding protein specifically expressed in the rat brain. Biochem Biophys Res Commun 1996;218:390–394. [PubMed: 8573167]

[4]. Nastasi T, Scaturro M, Bellafiore M, Raimondi L, Beccari S, Cestelli A, di Liegro I. PIPPin is a brain-specific protein that contains a cold-shock domain and binds specifically to H1 degrees and H3.3 mRNAs. J Biol Chem 1999;274:24087–24093. [PubMed: 10446180]

[5]. Wishart MJ, Dixon JE. The archetype STYX/dead-phosphatase complexes with a spermatid mRNA-binding protein and is essential for normal sperm production. Proc Natl Acad Sci U S A 2002;99:2112–2117. [PubMed: 11842224]

[6]. Wishart MJ, Groblewski G, Goke BJ, Wagner AC, Williams JA. Secretagogue regulation of pancreatic acinar cell protein phosphorylation shown by large-scale 2D-PAGE. Am J Physiol 1994;267:G676–686. [PubMed: 7943332]

[7]. Schafer C, Steffen H, Krzykowski KJ, Goke B, Groblewski GE. CRHSP-24 phosphorylation is regulated by multiple signaling pathways in pancreatic acinar cells. Am J Physiol Gastrointest Liver Physiol 2003;285:G726–G734. [PubMed: 12801884]

[8]. Groblewski GE, Yoshida M, Yao H, Williams JA, Ernst SA. Immunolocalization of CRHSP28 in exocrine digestive glands and gastrointestinal tissues of the rat. Am J Physiol 1999;276:G219–G226. [PubMed: 9886999]

[9]. Sans MD, Williams JA. Calcineurin is required for translational control of protein synthesis in rat pancreatic acini. Am J Physiol 2004;287:C310–C319.

[10]. Burnham DB, Munowitz P, Hootman SR, Williams JA. Regulation of protein phosphorylation in pancreatic acini. Distinct effects of Ca2+ ionophore A23187 and 12-O-tetradecanoylphorbol 13-acetate. Biochem J 1986;235:125–131. [PubMed: 2427068]

[11]. Burnham DB, Sung CK, Munowitz P, Williams JA. Regulation of protein phosphorylation in pancreatic acini by cyclic AMP-mediated secretagogues: interaction with carbamylcholine. Biochim Biophys Acta 1988;969:33–39. [PubMed: 2450590]

[12]. Auld GC, Campbell DG, Morrice N, Cohen P. Identification of calcium-regulated heat-stable protein of 24 kDa (CRHSP24) as a physiological substrate for PKB and RSK using KESTREL. Biochem J 2005;389:775–783. [PubMed: 15910284]

[13]. Campbell LE, Proud CG. Differing substrate specificities of members of the DYRK family of arginine-directed protein kinases. FEBS Lett 2002;510:31–36. [PubMed: 11755526]

[14]. Woods YL, Cohen P, Becker W, Jakes R, Goedert M, Wang X, Proud CG. The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bepsilon at Ser539 and the microtubule-associated protein tau at Thr212: potential role for DYRK as a glycogen synthase kinase 3-priming kinase. Biochem J 2001;355:609–615. [PubMed: 11311121]

[15]. Waschulewski IH, Hall DV, Kern HF, Edwardson JM. Effects of the immunosuppressants cyclosporin A and FK 506 on exocytosis in the rat exocrine pancreas in vitro. Br J Pharmacol 1993;108:892–900. [PubMed: 7683567]

[16]. Tashiro M, Samuelson LC, Liddle RA, Williams JA. Calcineurin mediates pancreatic growth in protease inhibitor-treated mice. Am J Physiol Gastrointest Liver Physiol 2004;286:G784–G790. [PubMed: 14684381]

[17]. Gurda GT, Williams JA. Calcineurin NFAT-pathway is a critical determinant of adult pancreatic growth plasticity. Gastroenterology 2008;134:T1845.

[18]. Puente LG, Voisin S, Lee RE, Megeney LA. Reconstructing the regulatory kinase pathways of myogenesis from phosphopeptide data. Mol Cell Proteomics 2006;5:2244–2251. [PubMed: 16971385]

[19]. St-Pierre SJ, Chakkalakal JV, Kolodziejczyk SM, Knudson JC, Jasmin BJ, Megeney LA. Glucocorticoid treatment alleviates dystrophic myofiber pathology by activation of the calcineurin/NF-AT pathway. FASEB J 2004;18:1937–1939. [PubMed: 15456738]

[20]. Chen Y, Lu B, Yang Q, Fearns C, Yates JR, Lee JD. Combined integrin phosphoproteomic analyses and small interfering RNA-based functional screening identifying key regulators for cancer cell adhesion and migration. Cancer Res 2009;69:3713–3720. [PubMed: 19351860]

[21]. Giot L, et al. A protein interaction map of Drosophila melanogaster. Science 2003;302:1727–1736. [PubMed: 14605208]

[22]. Bai J, Binari R, Ni JQ, Vijayakanthan M, Li HS, Perrimon N. RNA interference screening in Drosophila primary cells for genes involved in muscle assembly and maintenance. Development 2008;135:1439–1449. [PubMed: 18359903]

[23]. Bono E, Compagno V, Proia P, Raimondi L, Schiera G, Favaloro V, Campo V, Donatelli M, Di Liegro I. Thyroid hormones induce sumoylation of the cold shock domain-containing protein PIPPin in developing rat brain and in cultured neurons. Endocrinology 2007;148:252–257. [PubMed: 17053029]

[24]. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villén J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci USA 2004;101:12130–12135. [PubMed: 15302935]

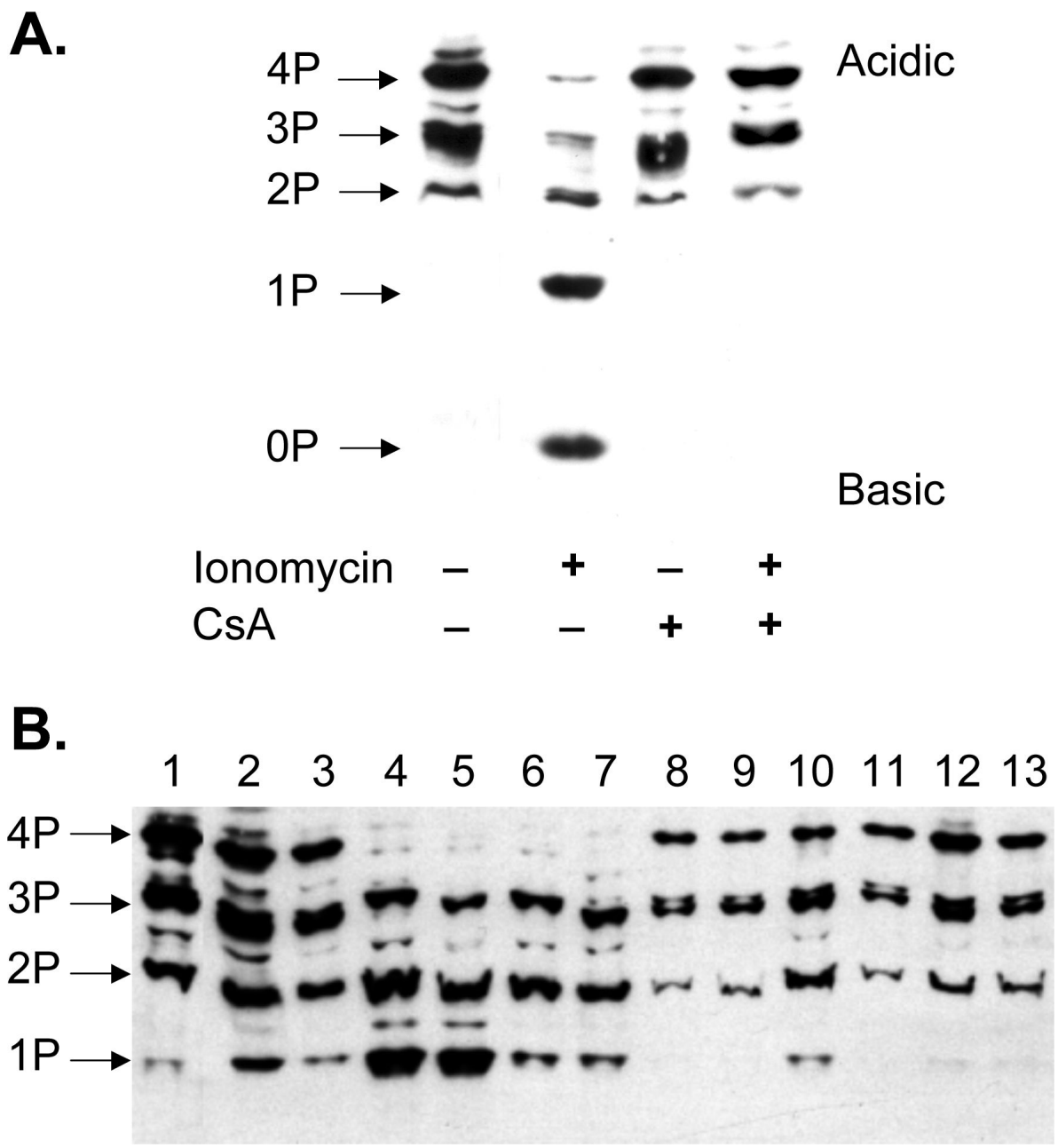

Figure 1.

A. Calcium induced dephosphorylation of CRHSP-24 is mediated by calcineurin in HEK293 cells. Cells were transfected with rat CRHSP-24 and incubated for 48 h before being stimulated for 15 min with 5 μM ionomycin to increase intracellular calcium. When indicated, cells were exposed to 1 μM cyclosporine A (CsA) prior to ionomycin treatment. After incubation, protein lysates were separated by IEF, Western blotted, and CRHSP-24 visualized by immunoprobing and ECL detection. Arrows indicate bands containing predicted number of phosphorylated residues as described in text. **B**. Identification of phosphorylated serine residues in CRHSP-24. Constructs containing serine to alanine substitutions of CRHSP-24 were expressed in HEK293 cells for 48 h followed by IEF and Western blotting of unstimulated cells. Ser was replaced with Ala at the following residues: Lane 1, none; Lane 2, $Ser^{2,3}$; Lane 3, Ser^{17}; Lane 4, Ser^{30}; Lane 5, Ser^{32}; Lane 6, Ser^{41}; Lane 7, Ser^{52}; Lane 8, Ser^{58}; Lane 9, Ser^{73}; Lane 10,

Ser^{93}; Lane 11, Ser^{113}; Lane 12, Ser^{141}; Lane 13, Ser^{146,147}. Arrows indicate bands containing predicted number of phosphorylated residues as described in text.

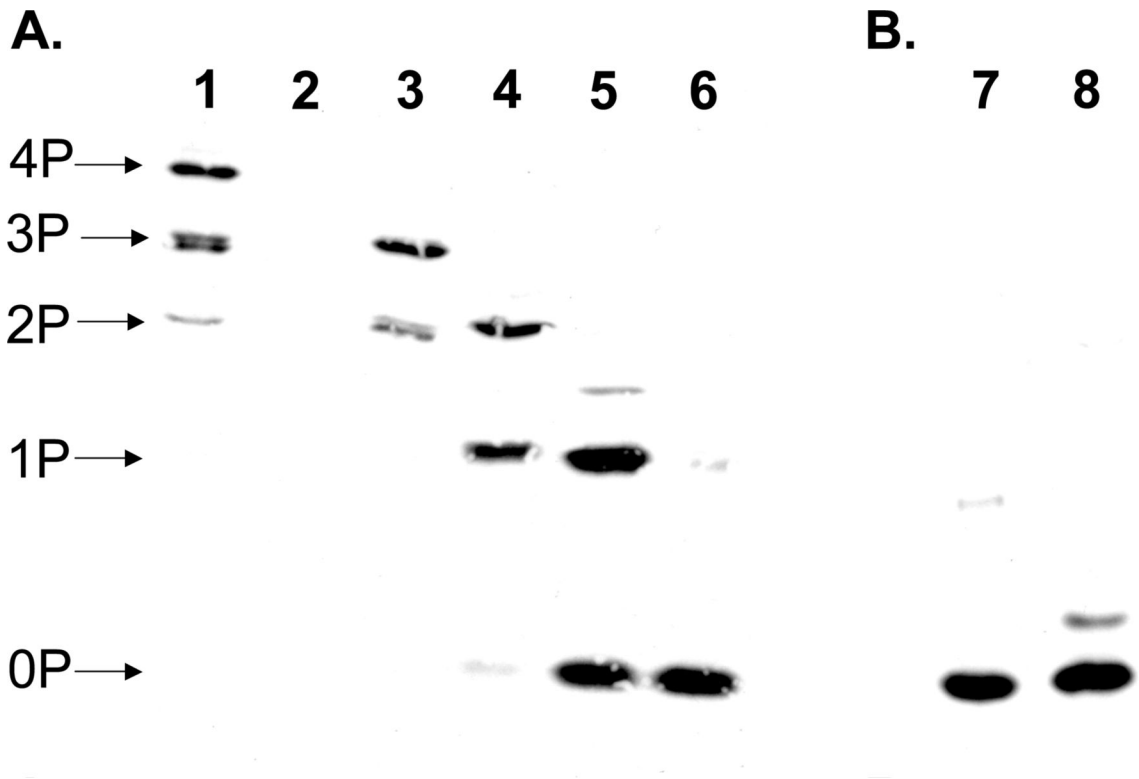

Figure 2.

Multiple serine deletion defines the four phosphorylation sites of CRHSP-24. HEK293 cells were transfected with various constructs coding for wild-type or Ser to Ala mutations of CRHSP-24 for 48 h followed by IEF and Western blotting of CRHSP-24. **A**. Lane 1, wild-type CRHSP-24; Lane 2, pcDNA vector; Lane 3, Ser^{41} single mutation; Lane 4, Ser32,41 double mutation; Lane 5, $Ser^{30,32,41}$ triple mutation; Lane 6, $Ser^{30,32,41,52}$ quadruple mutation **B**. Comparison of Ser to Ala at position 30, 32, 41, 52 (Lane 7) to bacterially expressed wild-type CRHSP-24 (Lane 8).

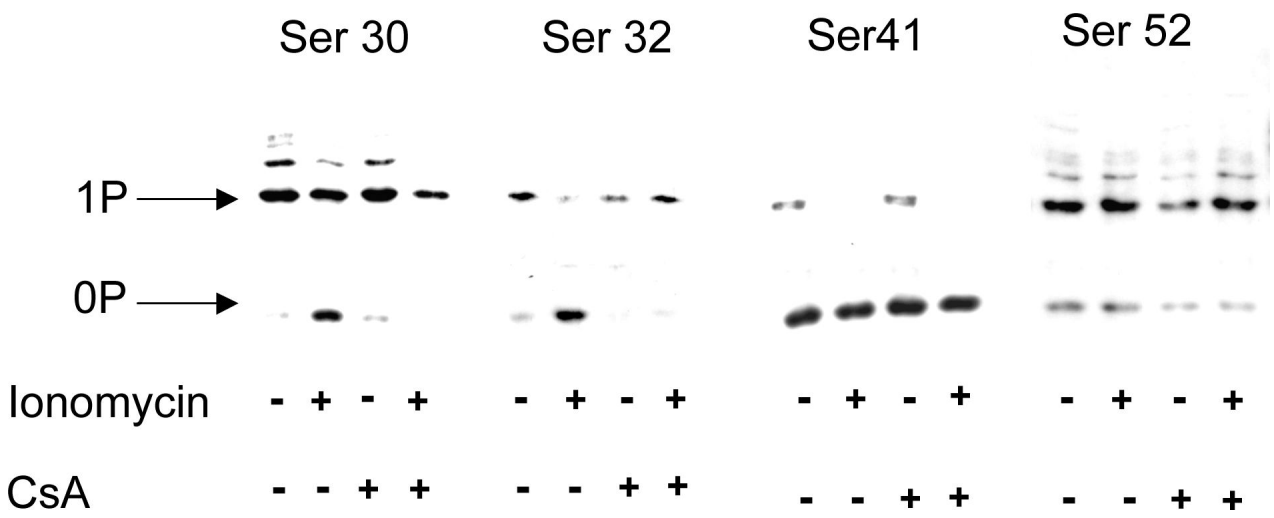

Figure 3.

Cyclosporine A differentially inhibits dephosphorylation of CRHSP-24 on individual phosphoserine residues. CRHSP-24 was mutated to alanine at three of the four phosphorylatable Ser residues at positions 30, 32, 41 and 52, leaving only a single phosphorylatable site as indicated. Cells were transfected and after 48 h stimulated with ionomycin followed by IEF and Western blotting of lysates as indicated in Fig. 1. Each construct was studied in three 3 replicate experiments.

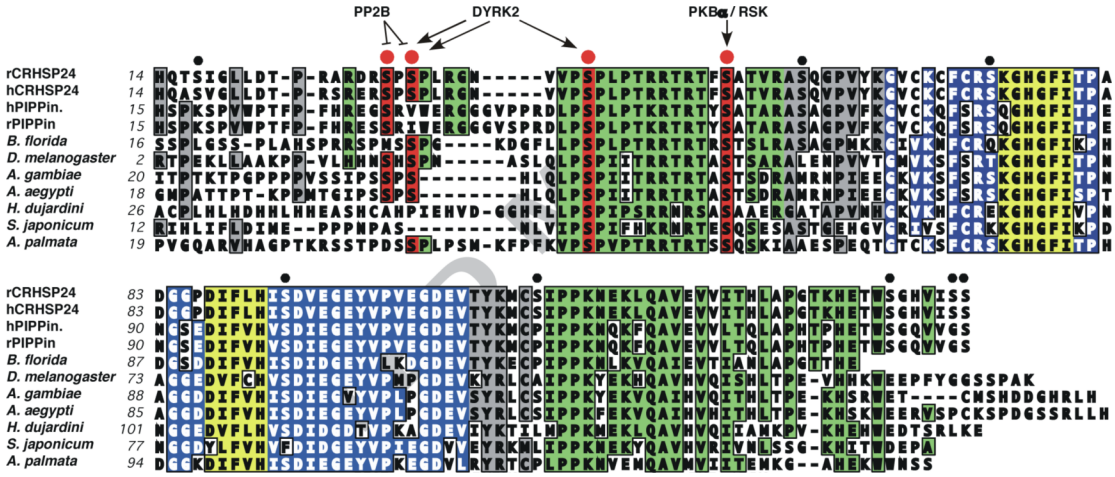

Figure 4.

CRHSP-24, PIPPin and lower eukaryotic orthologs contain conserved protein domain structures and sites of regulated phosphorylation. Regions of amino acid similarity are boxed and shaded by content: cold-shock domain (*blue*), ribonucleoprotein motifs (*yellow*), double-stranded RNA binding domains (*green*) as previously described [4]. Sites of serine phosphorylation in rat CRHSP-24 are indicated (*red dots*), along with positions of other serines investigated in this study (asterisks). Sites of regulated phosphorylation by protein kinases (DYRK2, PKBα, RSK) and phosphatases (PP2B) are also provided. Multiple sequence alignments were generated using ClustalW algorithms (MacVector) from GenBank sequences: rat CRHSP24, NP_690003; human CRHSP24, NP_055131; human PIPPin, NP_055275; rat PIPPin, Q63430; *Bracnchiostoma florida*, XP_002206249; *Drosophila melanogaster* CG9705, NP_730197; *Anopheles gambiae*, XP_315660; *Aedes aegypti*, XP_001659860; *Hypsibius dujardini*, CK326662; *Schistosoma japonicum*, AAW27166; *Acropora palmata*, DR984419.