Purification and Characterization of a Novel Physiological Substrate for Calcineurin in Mammalian Cells*

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Although the calcium/calmodulin-regulated protein phosphatase calcineurin has been shown to play a role in a number of intracellular processes, relatively few of the downstream phosphoproteins that are dephosphorylated by this enzyme in cells have been described. Calcineurin was previously shown to play a role in amylase secretion by rat pancreatic acinar cells and to specifically dephosphorylate a 24-kDa cytosolic protein. The present study describes the purification and characterization of this novel phosphoprotein, termed CRHSP-24 (calcium-regulated heat-stable protein with a molecular mass of 24 kDa). Microgram quantities of CRHSP-24 were purified from a large-scale rat pancreas preparation in a procedure involving heat and acid precipitation, anion-exchange chromatography, preparative electrophoresis, electroelution, and two-dimensional electrophoresis. Internal amino acid sequence was obtained from two peptides following trypsin digestion and high pressure liquid chromatography. Both sequences matched with 100% identity nucleotide sequences of expressed sequence tags from human placenta and rat PC-12 cells. Two CRHSP-24 transcripts of 0.7 and 2.9 kilobases were detected in multiple rat tissues by Northern analysis, whereas a single 24-kDa protein was observed by Western blotting. The CRHSP-24 protein is 147 amino acids in length, is composed of nearly 14% proline, and is phosphorylated entirely on serine residues. Western analysis and 32P metabolic labeling of acini revealed CRHSP-24 to be maximally phosphorylated in control cells and to undergo a rapid sustained dephosphorylation on at least 3 serine residues in response to calcium-mobilizing stimuli. Dephosphorylation of CRHSP-24 was completely inhibited by pretreatment of acini with cyclosporin A or FK506. Furthermore, the inhibitory effects of FK506 were blocked by excess rapamycin. The ubiquitous expression of CRHSP-24 in rat tissues suggests that this novel calcineurin substrate plays a common role in calcium-mediated signal transduction.

Calcineurin, also known as protein phosphatase-2B, is unique among the serine/threonine protein phosphatases because its activity is highly regulated by calcium and calmodulin. This enzyme is a heterodimer composed of a 19-kDa regulatory subunit and 60-kDa catalytic subunit, the latter of which binds calmodulin (reviewed in Refs. 1–6). Both the regulatory subunit and calmodulin are calcium-binding proteins, each containing EF-hand motifs. As the holoenzyme is activated by high nanomolar/low micromolar concentrations of calcium in vitro, calcineurin is believed to be relatively inactive in cells under basal conditions of low intracellular calcium and then becomes activated following stimulation with calcium-mobilizing agonists (7).

A pivotal role for calcineurin in regulating immune cell function has emerged based on the discovery that the immunosuppressants cyclosporin A (CsA)1 and FK506 are specific inhibitors of calcineurin in T cells (reviewed in Refs. 4–6 and 8). CsA and FK506 form complexes with their respective intracellular binding proteins, cycophilin A and FKBP12, which then bind to and inhibit calcineurin phosphatase activity (9–11). Inhibition of calcineurin in T cells blocks the dephosphorylation of the transcription factor NFAT, thereby inhibiting calcium-stimulated gene transcription (12). Use of CsA and FK506 has also implicated calcineurin in a number of other cellular processes, including secretion (13–16); endocytosis, ion channel modulation, cytoskeletal organization, neurite outgrowth, and transcription (reviewed in Ref. 16); mRNA stabilization (17); Na+/K+-ATPase activity (18); and apoptosis (19, 20).

Despite the numerous studies addressing the function of calcineurin in cells, little is known of the specific biochemical events associated with its activation. The most well-characterized calcineurin substrates are the protein phosphatase-1 inhibitors DARPP-32 (18, 19) and inhibitor-1, which are dephosphorylated on a specific threonine residue (3–6), and NFAT, which is dephosphorylated on multiple serine residues (12). Other less well characterized phosphoproteins reported to be dephosphorylated by calcineurin include the inositol trisphosphate receptor (22), the microtubule-associated protein MAP2 (23), the synaptic nerve terminal protein dynamin (24), the dual leucine zipper-bearing kinase (25), and the transcription factor ELK-1 (26). Thus, in contrast to the apparent multitude of cellular substrates for the type 1 and 2A serine/threonine phosphatases in cells (3–6), relatively few in vivo cellular targets of calcineurin have been described, especially outside of the nervous system.

Pancreatic acinar cells are among the most well character-

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1 The abbreviations used are: CsA, cyclosporin A; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; GST, glutathione S-transferase; PBS, phosphate-buffered saline; CCK, cholecystokinin.
CRHSP-24, a Novel Calcineurin Substrate

CRHSP-24 was phosphorylated on serine residues—Based on its isoelectric focusing pattern, CRHSP-24 was determined to be phosphorylated on multiple residues in control cells and to undergo a pronounced alkaline shift in response to the calcium-mobilizing secretagogue cholecystokinin (CCK) (Fig. 1) or treatment with calcium ionophore (data not shown). In the basal state, CRHSP-24 was maximally phosphorylated and

This method and its application to acini of different ages and with different secretagogue exposures have been described previously (27). Rat acini were cultured for 3 days in the absence of secretagogue to obtain basal conditions. Acini were then washed twice with Hanks’ balanced salt solution (HBSS) and placed in a medium containing 5 mM glucose, 25 mM HEPES, pH 7.4, and 0.1% bovine serum albumin for 30 min. The acini were then rinsed three times with HBSS, and calcium-stimulated secretion in acutely isolated rat acinar cells was measured as described previously (27). CaCl2 was added to the medium at a final concentration of 1.0 mM, and 100 nM CCK was added in parallel to assess the secretagogue-induced response (27). The secretagogue-induced response was calculated as the percentage increase above basal (no secretagogue) activity.

The isolated acini were washed three times with ice-cold HBSS, and the final wash was used to establish the basal secretion (no secretagogue). The acini were then incubated for 30 min at 37°C in the absence of secretagogue in HBSS containing 0.1% bovine serum albumin and 25 mM HEPES, pH 7.4. The final wash was aspirated, and the acini were incubated for 5 min in the presence of 5 mM glucose, 25 mM HEPES, pH 7.4, and 0.1% bovine serum albumin. The acini were then rinsed three times with HBSS, and calcium-stimulated secretion in acutely isolated rat acinar cells was measured as described previously (27). CaCl2 was added to the medium at a final concentration of 1.0 mM, and 100 nM CCK was added in parallel to assess the secretagogue-induced response (27). The secretagogue-induced response was calculated as the percentage increase above basal (no secretagogue) activity.

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FIG. 1. CRHSP-24 is phosphorylated on multiple serine residues. Heat-stable proteins from control and cholecystokinin-treated (1 nM, 5 min) 32P-labeled pancreatic acinar cells were separated by two-dimensional electrophoresis and transferred to polyvinylidene difluoride membrane. Upper panels, phosphorylated CRHSP-24 was detected by autoradiography. Lower panel, CRHSP-24 phospho isoforms were excised from the membrane and subjected to phosphoamino acid analysis. Positions of phosphoserine, phosphothreonine, and phosphotyrosine standards are indicated on the right.

predominantly present as its most acidic isoform (Fig. 1, spot 1, pI 6.2). Following stimulation of cells with CCK, there was a large decrease in the phosphate content of spot 1, with significant increases in the intensities of three more alkaline isoforms of CRHSP-24 (spots 2–4, pI 6.3, 6.5, and 6.8, respectively). Phosphoamino acid analysis of each of the CRHSP-24 isoforms before and after CCK treatment revealed the protein to be phosphorylated entirely on serine residues (Fig. 1). The absence of tyrosine phosphorylation was confirmed by immunoblotting with anti-phosphotyrosine antibodies (data not shown). Collectively, the presence of the four phospho isoforms of CRHSP-24 as seen by isoelectric focusing indicates that the protein is phosphorylated on a minimum of 4 separate serine residues in acinar cells.

Purification and Identification of CRHSP-24—Efforts to identify CRHSP-24 using specific antibodies to candidate phosphoproteins were unsuccessful, suggesting that CRHSP-24 was novel. Therefore, the amino acid sequence of CRHSP-24 was elucidated by purifying the protein from a large-scale preparation of over 600 rat pancreases. Purification was facilitated by the thermal stability of CRHSP-24 as well as its high solubility in aqueous solutions. As a final step in the procedure, CRHSP-24 was immobilized to polyvinylidene difluoride membrane and detected by staining with Coomasie Brilliant Blue dye and autoradiography (data not shown). Microgram quantities of CRHSP-24 were obtained from each of the three most acidic phospho isoforms of the protein (Fig. 1, spots 1–3). These pieces of membrane were combined and treated with trypsin; the resulting peptides were separated by reversed-phase HPLC (Fig. 2A). Eluant fractions containing CRHSP-24 peptides were submitted for microsequence analysis. High quality sequence was obtained from two peptides of 19 and 20 amino acids in length.

A search of the GenBank™ Data Bank revealed that both of the purified CRHSP-24 amino acid sequences were positioned in the same reading frame of an expressed sequence tag from a human placenta cDNA library. Upon sequencing, this 722-base pair cDNA was found to contain a Kozak consensus sequence (34) positioned 12 nucleotides from the 5’-end, which aligns it with a TAG stop codon beginning at nucleotide 552. This defined coding sequence predicts a 147-amino acid protein containing both of the purified rat CRHSP-24 peptides positioned contiguously within the molecule. An expressed sequence tag from rat PC-12 cells showing high homology to the human clone was subsequently identified. This 2.9-kilobase cDNA was found to code for a protein having >96% homology to the human protein and, likewise, contained both of the purified peptide sequences (Fig. 2C). In addition to an expanded 3’-untranslated region, the larger rat cDNA has a 30-nucleotide 5’-untranslated region that contains no other translational start sites.

In the nonphosphorylated state, rat CRHSP-24 has a calculated molecular mass of 15.8 kDa and an isoelectric point of 7.8. CRHSP-24 is predicted to be abundantly hydrophilic, consistent with its presence in the soluble fraction following high speed centrifugation. It is rich in proline, containing 20 residues that account for nearly 14% of the protein by frequency. This high proline content is especially noted in the amino terminus, where a stretch of 7 prolines is part of a single glutamine residue. Finally, rat CRHSP-24 also contains 14 serine, 13 glycine, and 13 valine residues, which collectively constitute >27% of the protein.

The CRHSP-24 protein has an ~62% identity to the predicted coding region of a recently identified cDNA that encodes a putative double-stranded RNA-binding protein termed PIPPIN (35). Similar to CRHSP-24, PIPPIN is a small (154 amino acids) hydrophilic protein that, when expressed in vitro, undergoes anomalous migration on SDS-PAGE. The highest identity between the two proteins is located carboxyl-terminal to the first 40 amino acids of CRHSP-24; conversely, a much lower identity is noted in the amino termini of the two molecules. Unlike the broad tissue distribution of CRHSP-24 (see below), the PIPPIN mRNA was found exclusively in rat brain. In addition, PIPPIN is predicted to contain two double-stranded RNA-binding domains that are partially conserved in CRHSP-24. Within one of these domains, CRHSP-24 contains a 19-amino acid region (residues 73–92) with 73% identity to the E. coli cold-shock proteins. Bacterial cold-shock proteins are a family of low molecular mass (~70 amino acids) DNA- and RNA-binding proteins that are believed to function as transcriptional and/or translational regulatory proteins (36).

Tissue Distribution of CRHSP-24 in Rat—The rat CRHSP-24 cDNA was used to probe a Northern blot of rat pancreas poly(A) mRNA (Fig. 3A). Two CRHSP-24 transcripts of ~2.9 and 0.7 kilobases were identified. The larger transcript was similar in size to the rat cDNA, suggesting that this clone potentially represents a full-length CRHSP-24 mRNA. Similarly, the smaller 0.7-kilobase message was consistent with the size of the human cDNA. An analysis of mRNA from various rat tissues indicated that both of the CRHSP-24 transcripts were present in all organs tested. CRHSP-24 mRNA was particularly abundant in pancreas, testis, liver, and lung, whereas lower levels were found in spleen, brain, and heart. Much lower amounts were seen in kidney and skeletal muscle. Similar results were obtained when analyzing the relative expression of the CRHSP-24 protein (Fig. 3B). Immunoblotting using specific anti-rat CRHSP-24 antiserum (see below) indicated a ubiquitous expression of a single 24-kDa protein in rat. The presence of a single protein in pancreas and other tissues using a polyclonal antiserum raised against the full-length CRHSP-24 protein suggests that both of the mRNA transcripts detected by Northern blotting potentially code for the same protein and may differ in their untranslated regions. Alternatively, it is also possible that the smaller CRHSP-24 transcript codes for a homologous or alternatively spliced protein that is not recognized by the antibodies. The highest levels of
CRHSP-24 protein expression occurred in pancreas, parotid gland, liver, testis, and lung, whereas the lowest levels were found in stomach, small intestinal mucosa, and kidney.

**Bacterial Expression of CRHSP-24 and Production of Specific Antiserum—**

The predicted coding sequence from the rat CRHSP-24 cDNA was subcloned into a pGEX-Kt expression vector and expressed in bacteria as a GST fusion protein. GST-CRHSP-24 was purified from bacterial lysates, and the CRHSP-24 portion was recovered by thrombin cleavage (Fig. 4A, lane 1). Despite its predicted molecular mass of 15.8 kDa, recombinant CRHSP-24 migrated at 24 kDa following 12.5% SDS-PAGE, in close agreement with the size of the purified rat protein.

Reduced mobility on SDS-PAGE is a property shared by a number of other heat-stable molecules, including inhibitor-1 and -2, DARPP-32 (3), stathmin (37), myristoylated alanine-rich C kinase substrate protein (38), PHAS-I (39), and CRHSP-28 (30).

Polyclonal rabbit antisera raised against recombinant GST-CRHSP-24 recognized a 24-kDa protein in acinar cell lysates (Fig. 4A, lane 2). The predicted molecular mass of 15.8 kDa, recombinant CRHSP-24 migrated at ~24 kDa following 12.5% SDS-PAGE, in close agreement with the size of the purified rat protein. Reduced mobility on SDS-PAGE is a property shared by a number of other heat-stable molecules, including inhibitor-1 and -2, DARPP-32 (3), stathmin (37), myristoylated alanine-rich C kinase substrate protein (38), PHAS-I (39), and CRHSP-28 (30).

**Fig. 3. Expression of CRHSP-24 in rat tissues. A.** Northern blot of CRHSP-24 mRNA. Pancreatic poly(A) mRNA (2 μg) was separated by electrophoresis and immobilized on a nylon membrane. Poly(A) mRNA (2 μg/lane) from the indicated rat tissues was also obtained from a multi-tissue blot. The CRHSP-24 message was detected using 32P-labeled rat CRHSP-24 cDNA as a probe. B, immunoblot of the CRHSP-24 protein. Equal amounts of whole tissue homogenates (50 μg/lane) were separated by 12.5% SDS-PAGE and immunoblotted using anti-rat-CRHSP-24 serum (1:1000). kb, kilobases.

CRHSP-24 protein expression occurred in pancreas, parotid gland, liver, testis, and lung, whereas the lowest levels were found in stomach, small intestinal mucosa, and kidney.
Using a standard curve generated by immunoblotting various amounts of recombinant rat CRHSP-24 (data not shown) and comparing it with the level of expression in a cell lysate, it was estimated that CRHSP-24 represents \(0.01\%\) of total acinar cell protein.

Immunoblotting was also conducted following two-dimensional electrophoresis of \(^{32}P\)-labeled proteins from control and CCK-stimulated acini (Fig. 4B). Overlaying the autoradiograph of \(^{32}P\)-labeled CRHSP-24 with the film from the immunoblot revealed that the antiserum recognized all of the phosphorylated isoforms of CRHSP-24. Furthermore, the pattern of the CCK-induced alkaline shift in CRHSP-24 seen by \(^{32}P\) labeling was readily apparent by immunoblotting, indicating that the affinity of the antiserum for CRHSP-24 was not altered by the phosphorylation state of the protein.

Similar to the subcellular distribution of the protein detected by Western blotting, immunohistochemical localization of CRHSP-24 using affinity-purified antibodies showed a diffuse pattern of cytosolic staining that was most intense in the basal cytoplasm (Fig. 5). Little or no staining was observed in nuclei and secretory granules (Fig. 5). Specificity of CRHSP-24 staining was evidenced by the total loss of fluorescence seen following preincubation of the antibody with a molar excess of antigen (data not shown). A similar diffuse cytosolic localization of CRHSP-24 was also detected in nerve growth factor-differentiated PC-12 cells (data not shown). The overall pattern of CRHSP-24 localization was unchanged by a 30-min KCl depolarization of PC-12 cells or CCK treatment of acini (data not shown).

**Immunosuppressants Define a Role for Calcineurin in CRHSP-24 Dephosphorylation**—As CRHSP-24 was initially identified as a calcineurin substrate in acinar cells (15), the effects of the immunosuppressants CsA, FK506, and rapamycin to influence its phosphorylation were examined by immunoblotting following isoelectric focusing (Fig. 6). Treatment of acinar cells with CsA (1 \(\mu M\)) or FK506 (10 \(nM\)) either alone or in combination with rapamycin (2 \(\mu M\)) had no effects on CRHSP-24 phosphorylation. In contrast, treatment with 1 \(nM\) CCK or the calcium ionophore ionomycin (data not shown) stimulated a marked shift in CRHSP-24 to its three more alkaline isoforms, indicative of its dephosphorylation. The CCK-induced dephosphorylation was seen as early as 30 s and was sustained for up to 30 min in the presence of agonist (data not shown). Pretreatment of cells with CsA or FK506 prior to CCK fully inhibited the dephosphorylation of CRHSP-24, whereas rapamycin pretreatment had no effects. Conversely,
excess rapamycin completely reversed the effect of FK506 to inhibit the dephosphorylation of CRHSP-24, but was unable to block the inhibitory effects of CsA. These results are in accordance with previous studies demonstrating that FK506 and rapamycin are ligands for the same intracellular receptor, yet act on different enzymes within cells, namely calcineurin and RaFT1, respectively (40).

Previously, a resistance of acinar cells to calcineurin inhibition by FK506 was reported (15). This resistance was speculated to be due to a reduced concentration of FKBP12 in acini. Subsequently, it was shown that rapamycin, which also acts through FKBP12, is a potent and specific inhibitor of p70 S6 kinase in acini (41). Furthermore, p70 S6 kinase inhibition by rapamycin was reversed by pretreatment with excess FK506, demonstrating that these compounds are ligands for the same intracellular binding protein, but act on different signaling pathways in acini. Whether the FK506 resistance was due to a different source of the compound, its nonspecific binding to buffer components or lab ware, its hydrophobic aggregation in aqueous solution when used at micromolar concentrations, or a reversal of the FK506-FKBP12-calcineurin complex upon preparation of the lysates is uncertain. The present results are consistent with the effects of rapamycin and FK506 on p70 S6 kinase activity in acini and, furthermore, are in close agreement with the pharmacological characteristics of these compounds in immune cells. Collectively, these data strongly support a role for calcineurin in regulating CRHSP-24 phosphorylation in acini.

**CRHSP-24 Phosphorylation**—The alkaline shift of CRHSP-24 seen following a stimulated increase in intracellular calcium is completely inhibited by pretreatment with CsA or FK506 and is perfectly reproduced in vitro by calcineurin treatment (15), suggesting that at least 3 of its serine residues are susceptible to dephosphorylation by the enzyme. The absence of any additional more alkaline forms of native CRHSP-24 on immunoblot analysis also suggests that 1 of the phosphoserines is not dephosphorylated by calcineurin. Six of the 14 serine residues of CRHSP-24 are located within the first 60 amino acids, with the sequence surrounding serines 30 and 32 forming a consensus site for potential phosphorylation by a number of protein kinases, including the proline-directed kinases. Currently, the specific serine residues that are phosphorylated on CRHSP-24 as well as the specific CRHSP-24 kinase(s) are being investigated.

The regulated phosphorylation of CRHSP-24 is similar to that of NFAT in T cells. Both proteins are maximally phosphorylated on serine residues in resting cells and rapidly dephosphorylated by calcineurin following a rise in free cellular calcium. Like NFAT, CRHSP-24 appears to be phosphorylated by a constitutively active kinase in acini. This is supported by metabolic labeling, showing that under basal conditions, CRHSP-24 will incorporate large amounts of $^{32P}$ during a 1-h incubation of cells with radiolabeled orthophosphate. Furthermore, $^{32P}$ labeling in the presence of CsA has little effect on the basal phosphorylation of CRHSP-24 (data not shown), indicating that it is also constitutively dephosphorylated by a serine phosphatase other than calcineurin. These results denote a high basal turnover rate of phosphate on CRHSP-24 and also underscore that its phosphorylation is a highly regulated process in acini.

**Concluding Remarks**—Based on amino acid homologies, CRHSP-24 may play a role as a transcriptional or translational regulatory protein in acinar cells. However, in preliminary experiments, no nucleotide binding activity of either the native or recombinant CRHSP-24 protein has been detected. It should also be noted that nucleotide binding of the cloned PIPPIN protein was not demonstrated in its initial characterization, but rather was reported as a putative property of the molecule (35). In addition, the presence of CRHSP-24 in the soluble fraction of acinar cell lysates seen by immunoblotting, together with its diffuse immunolocalization within cells and absence from the nuclei of acini and PC-12 cells, argues against a transcriptional role for the protein. Alternatively, CsA-mediated calcineurin inhibition in acinar cells was associated with a significant decrease in calcium-stimulated amylase secretion (15). Moreover, both CCK-stimulated secretion and CRHSP-24 dephosphorylation were inhibited over a similar concentration range of CsA (15). As CCK is a major physiological regulatory hormone of exocrine pancreas with effects on all aspects of acinar cell metabolism, including secretion, transcription, and translation, a role for CRHSP-24 in one of these processes is highly likely. Clearly, the elucidation of the functional significance of CRHSP-24 in acini will provide valuable insight into the physiological and biochemical mechanisms of Ca$^{2+}$/calcineurin-mediated signal transduction in mammalian cells.

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**REFERENCES**

1. Klee, C. B., Ren, H., and Wang, X. (1998) *J. Biol. Chem.* **273**, 13367–13370
2. Klee, C. B., Draetta, G. F., and Hubbard, M. J. (1997) *Adv. Enzymol. Relat. Areas Mol. Biol.*** **61**, 149–200
3. Cohen, P. (1989) *Ann. Rev. Biochem.* **58**, 453–508
4. Kincaid, R. (1993) *Adv. Second Messenger and Phosphoprotein Res.* **27**, 1–23
5. Mumby, M. C., and Walter, G. (1993) *Physiol. Rev.* **73**, 673–698
6. Shenolikar, S. (1994) *Annu. Rev. Cell Biol.* **10**, 55–86
7. Stemmer, P. M., and Klee, C. B. (1994) *Biochemistry* **33**, 6859–6866
8. Braun, W., Kallen, J., Miko, V., Walkinshaw, M. D., and Wuthrich, K. (1995) *FASEB J.* **9**, 63–72
9. Clipstone, N. A., and Crabtree, G. R. (1992) *Nature* **357**, 695–697
10. O’Keefe, S. J., Tamura, J., Kincade, R. L., Toce, M. J., and O’Neill, E. A. (1992) *Nature* **357**, 692–694
11. Fruman, D. A., Klee, C. B., Bierer, B. E., and Burakoff, S. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3868–3870
12. Beals, R. E., Clipstone, N. A., O’Keefe, S. J., and Crabtree, G. R. (1997) *Genes Dev.* **11**, 824–834
13. Momayez, M., Lumpert, C. J., Kersken, H., Gras, U., Platter, H., Knirks, M. H., and Klee, C. B. (1997) *J. Cell Biol.* **138**, 181–189
14. Hustch, T., Albers, M. W., Schreiber, S. L., and Hohman, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6229–6233
15. Groshegloh, G. E., Wagner, A. C., and Williams, J. A. (1994) *J. Biol. Chem.* **269**, 15111–15117
16. Yakei, J. L. (1997) *Trends Pharmacol. Sci.* **18**, 124–134
17. Nair, A. P., Hahn, S., Banholzer, K., Hirsch, H. H., and Moroni, C. (1994) *Nature* **369**, 239–242
18. Aperia, A., Ibarra, F., Svensson L.-B., Klee, C. B., and Greengard, P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7394–7397C. B.
19. Desdouits, F., Seillane, J. C., Greengard, P., and Girault, J. A. (1995) *Proc.
20. Ankarcrona, M., Dypbukt, J. M., Orrenius, S., and Nicotera, P. (1996) FEBS Lett. 394, 321–324
21. Deleted in proof
22. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995) Cell 83, 463–472
23. Quinlan, E. M., and Halpain, S. (1996) J. Neurosci. 16, 7627–7637
24. Nichols, R. A., Suplick, G. R., and Brown, J. M. (1994) J. Biol. Chem. 269, 23817–23823
25. Mata, M., Merritt, S. E., Gan, G., Yu, G. G., and Holzman, L. B. (1996) J. Biol. Chem. 271, 16888–16896
26. Sugimoto, T., Stewart, S., and Guan, K.-L. (1997) J. Biol. Chem. 272, 29415–29418
27. Williams, J. A., and Yule, D. I. (1993) in The Pancreas, Pathology and Disease (Go, V.-L. W., Dimagno, E. P., Gardner, J. D., Lebenthal, E., Reber, H. A., and Scheele, G. A., eds) pp. 167–189, Raven Press, Ltd., New York
28. Burnham, D. B., and Williams, J. A. (1982) J. Biol. Chem. 257, 10523–10528
29. Wishart, M. J., Groblewski, G. E., Geke, B. J., Wagner, A. C. C., and Williams, J. A. (1994) Am. J. Physiol. 267, G676–G686
30. Groblewski, G. E., Wishart, M. J., Yoshida, M., and Williams, J. A. (1996) J. Biol. Chem. 271, 31502–31507
31. Fernandez, J., DeMott, M., Atherton, D., and Mische, S. M. (1992) Anal. Biochem. 201, 255–264
32. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds (1997) Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York
33. Ohnishi, H., Ernst, S. A., Wys, N., McNiven, M., and Williams, J. A. (1996) Am. J. Physiol. 271, G531–G538
34. Kozak, M. (1986) Cell 44, 283–292
35. Castiglione, D., Scaturro, M., Nastasi, T., Castelli, A., and DiLiegro, I. (1996) Biochem. Biophys. Res. Commun. 218, 390–394
36. Jones, P. G., and Inouye, M. (1994) Mol. Microbiol. 11, 811–818
37. Sobel, A. (1991) Trends Biochem. Sci. 16, 301–304
38. Blackshear, P. J. (1993) J. Biol. Chem. 268, 1501–1504
39. Hu, C., Pang, S., Kong, X., Velleca, M., and Lawrence, J. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3730–3734
40. Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. H. (1994) Cell 78, 35–43
41. Bragado, M. J., Groblewski, G. E., and Williams, J. A. (1997) Am. J. Physiol. 273, C101–C109