Protein Kinase A Phosphorylation of Human Phosphodiesterase 3B Promotes 14-3-3 Protein Binding and Inhibits Phosphatase-catalyzed Inactivation*

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Recent studies confirm that intracellular cAMP concentrations are nonuniform and that localized subcellular cAMP hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) is important in maintaining these cAMP compartments. Human phosphodiesterase 3B (HSPDE3B), a member of the PDE family of PDEs, represents the dominant particulate cAMP-PDE activity in many cell types, including adipocytes and cells of hematopoietic lineage. Although several previous reports have shown that phosphorylation of HSPDE3B by either protein kinase A (PKA) or protein kinase B (PKB) activates this enzyme, the mechanisms that allow cells to distinguish these two activated forms of HSPDE3B are unknown. Here we report that PKA phosphorylates HSPDE3B at several distinct sites (Ser-73, Ser-296, and Ser-318), and we show that phosphorylation of HSPDE3B at Ser-318 activates this PDE and stimulates its interaction with 14-3-3 proteins. In contrast, although PKB-catalyzed phosphorylation of HSPDE3B activates this enzyme, it does not promote 14-3-3 protein binding. Interestingly, we report that the PKA-phosphorylated, 14-3-3 protein-bound, form of HSPDE3B is protected from phosphatase-dependent dephosphorylation and inactivation. In contrast, PKA-phosphorylated HSPDE3B that is not bound to 14-3-3 proteins is readily dephosphorylated and inactivated. Our data are presented in the context that a selective interaction between PKA-activated HSPDE3B and 14-3-3 proteins represents a mechanism by which cells can protect this enzyme from deactivation. Moreover, we propose that this mechanism may allow cells to distinguish between PKA- and PKB-activated HSPDE3B.

Cyclic AMP regulates a diverse array of cellular processes, including intermediary metabolism, vascular and visceral smooth muscle relaxation, hormonal secretion, cytoskeletal organization, as well as transcription, migration, proliferation, and apoptosis (reviewed in Refs.1–3). Although the elements regulating cAMP synthesis have been extensively studied (4, 5), the regulation of cyclic nucleotide phosphodiesterase (PDE)2-mediated hydrolysis of cAMP and its impact on cellular functions have only recently received considerable attention (6–12). Based on their sequence homologies, substrate specificities, and sensitivities to pharmacological inhibitors, mammalian PDEs have been divided into 11 distinct enzyme families (6–12).

Two genes, phosphodiesterase 3A (PDE3A) and PDE3B, encode PDE3 family enzymes (6, 12). PDE3A mRNA is enriched in cells of the cardiovascular system and in oocytes, whereas PDE3B mRNA is abundant in adipocytes, hepatocytes, and cells of hematopoietic lineage (13). Full-length PDE3A and PDE3B contain two N-terminal hydrophobic regions (NHR1 and NHR2) that target these enzymes to the endoplasmic reticulum and perhaps the plasma membrane (13–16). Both PDE3A and PDE3B are substrates of protein kinase A (PKA) or protein kinase B (PKB), and activation of these kinases can result in phosphorylation-mediated activation of these enzymes in some cells (13–16).

A consensus has emerged that protein-protein interactions play a central role in regulating cAMP-mediated signaling. Indeed, it is generally accepted that selective subcellular anchorage of PKA, through interaction with A-kinase anchoring proteins, allows selective coordination of PKA-dependent cellular events (17, 18). Subcellular targeting of certain PDEs also has emerged as a mechanism whereby these enzymes can coordinate various cellular effects of cAMP (17, 18). In this context, several individual variants of the phosphodiesterase 4 (PDE4) family of enzymes interact with proteins including A-kinase anchoring proteins, β-arrestins, and receptor for activated protein kinase C, and these interactions regulate PDE4 subcellular targeting and enzyme activity (17, 18).

Although PDE3 activity can represent a significant fraction of total cAMP hydrolytic capacity in certain cell types (6, 11), little is known concerning how protein-protein interactions coordinate the activity and subcellular targeting of PDE3 enzymes. An HSPDE3B interaction with the insulin receptor in

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2 The abbreviations used are: PDE, phosphodiesterase; HSPDE3B, human cyclic nucleotide phosphodiesterase 3B; PKA, protein kinase A; PKB, protein kinase B; GST, glutathione S-transferase; WT, wild type; DTT, dithiothreitol; IBMX, 3-isobutyl-1-methylxanthine; aa, amino acid; TEMED, N,N,N′,N′-tetramethylethylenediamine; PIPES, 1,4-piperazinediethanesulfonic acid; P38K, phosphokinatide 3-kinase γ; PKC, protein kinase C; CIAP, calf intestinal alkaline phosphatase; C, catalytic; F/I, forskolin/IBMX; MA, membrane-associated; DN, dominant negative.
human adipocytes has been reported (19). Recently, rat adipocyte PDE3B was reported to interact with caveolin-1 (20) placing this enzyme in lipid rafts in these cells (20, 21). Disruption of an interaction between the murine PDE3B and phosphoinositide 3-kinase γ (PI3Kγ) (22), likely coordinated by one of its regulatory subunits p87PIKAP (PI3Kγ adapter protein of 87 kDa) (23), reduced cardiomyocyte contractility (22, 23). Of more immediate relevance to the studies reported here, insulin was reported previously to stimulate a PI3K-dependent interaction between murine PDE3B and 14-3-3β in 3T3-L1 adipocytes (24). Binding of 14-3-3 proteins to numerous proteins, usually following their phosphorylation, allows 14-3-3 proteins to act as regulators, adaptors, or scaffolds for these proteins (25). A PKC-dependent phosphorylation of HSPDE3A also stimulates association of HSPDE3A with 14-3-3 proteins (26).

In this study, we report that PKA-mediated phosphorylation of HSPDE3B at Ser-318 activates this enzyme and promotes its interaction with 14-3-3 proteins. Although PKA is also shown to phosphorylate HSPDE3B at two other sites, Ser-73 and Ser-296, these events neither activated nor promoted HSPDE3B interactions with 14-3-3 proteins. Although PKB activated HSPDE3B, this kinase did not promote 14-3-3 protein binding of HSPDE3B or influence the effects of PKA. Taken together, our data are consistent with the novel hypothesis that PKA-activated, but not PKB-activated, HSPDE3B interacts with 14-3-3 proteins and that this selective protein-protein interaction protects the PKA-activated HSPDE3B from phosphatase-mediated deactivation in cells.

EXPERIMENTAL PROCEDURES

Two-hybrid Screen—Mass screening for protein-protein interactions were carried out using a modification of the “interaction trap” methodology described by Fields and Song (27) using the Y860 strain of yeast (Matα ura3::URA3 lexAopADE2 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100) (Dr. C. Boone, University of Toronto, Canada). Briefly, a murine brain expression library in pACT (Clontech) that allowed expression of HSPDE3B constructs at Ser-318 activates this enzyme and promotes its interaction with 14-3-3 proteins. Although PKA is also shown to phosphorylate HSPDE3B at two other sites, Ser-73 and Ser-296, these events neither activated nor promoted HSPDE3B interactions with 14-3-3 proteins. Although PKB activated HSPDE3B, this kinase did not promote 14-3-3 protein binding of HSPDE3B or influence the effects of PKA. Taken together, our data are consistent with the novel hypothesis that PKA-activated, but not PKB-activated, HSPDE3B interacts with 14-3-3 proteins and that this selective protein-protein interaction protects the PKA-activated HSPDE3B from phosphatase-mediated deactivation in cells.

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Expression and Characterization of Glutathione S-Transferase Fusion Proteins—An amino-terminal FLAG-tagged HSPDE3B expression construct (HSPDE3B(AT)) was generated by ligating an EcoRI-HSPDE3B-digested cDNA (base pairs 1–1548 inclusively) into EcoRI-digested pCMV tag 2B vector. A carboxyl-terminal FLAG-tagged HSPDE3B expression construct (HSPDE3B(CT)) was generated by ligation of a PstI-digested fragment of HSPDE3B containing base pairs 1795–3269, inclusively into the PstI-digested pCMV tag 2C. For use in 14-3-3 pulldown experiments, recombinant 14-3-3 (β or η) or HSPDE3B fragments were expressed as glutathione S-transferase (GST) fusion proteins in E. coli. A pGEX-2T plasmid containing human 14-3-3β was a gift from Dr. Q. Medley (Dana Farber Cancer Institute, Harvard University), and the 14-3-3-η and HSPDE3B-GST fragments were expressed using pGEX-5X-3 (Amersham Biosciences). E. coli (BL21(DE)) expression was induced with 1 mM isopropyl-1-thio-D-galactopyranoside at 30 °C for 3 h. Bacterial cell pellets were collected by centrifugation (6000 × g, 15 min) and lysed by sonication in phosphate-buffered saline supplemented with 1% Triton X-100, 0.02% sodium azide, 100 μM dithiothreitol (DTT), 10 μg/ml lysozyme, 5 mM benzamidine, 1 mM EDTA, 2 μg/ml leupeptin, 5 μg/ml bestatin, 2 μg/ml aprotinin, 10 μg/ml antipain, 10 μM E-64, 2 μg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride. Filtered protein lysates were loaded on reduced glutathione (GSH)-Sepharose 4B (Amersham Biosciences), washed, and eluted with 5 mM free GSH in 10 mM Tris, pH 8.0, and GSH was removed using a Amicon® Centri-con® centrifugation filters (Millipore). GST fusion protein yields and purities were assessed by SDS-PAGE.

Mammalian Cell Culture—HEK293T (herein 293T) or NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM l-glutamine, and 1 g/liter d-glucose.

Heterologous Expression of HSPDE3B Constructs—A cDNA encoding HSPDE3B (Dr. V. C. Manganiello, National Institutes of Health) was cloned into the mammalian expression vector pCMV-Tag2C (Stratagene) using BamHI. This construct was used to express HSPDE3B. In experiments in which HSPDE3B was expressed heterologously, transfections were carried out with amounts of plasmid to limit overexpression of HSPDE3B to levels not exceeding 4-fold those of endogenous HSPDE3B. With full-length HSPDE3B, levels of expression were determined by PDE activity assays. Point mutations that allowed substitution of alanine (Ala) for serine (Ser) at positions 73, 295, 296, or 318 within HSPDE3B were generated using the Quick-Change site-directed mutagenesis kit (Clontech) according to the manufacturer’s protocol. Plasmids encoding wild type (WT), membrane-associated (MA), or dominant negative (DN) PKB were provided by Dr. D. Alessi (University of Dundee, Dundee, Scotland, UK). HSPDE3B constructs were transiently expressed in 293T or NIH 3T3 cells following transfection using FuGENE 6 (Roche Applied Science).

In Vitro Phosphorylation of HSPDE3B—GST- or FLAG-tagged proteins were purified by conventional approaches
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using GSH-Sepharose or M2-agarose. HSPDE3B proteins were incubated with recombinant PKA catalytic subunit or activated recombinant PKBβ (Upstate Biotechnology, Inc., Lake Placid, NY) in a buffer without or with 200 μM ATP or 200 μM ATP supplemented with [γ-32P]ATP (10 μCi per reaction; 3000 Ci/mmol stock) for 1 h at 30 °C. For reference, reactions were carried out with neither kinase nor ATP, with kinase or ATP alone, or with both ATP and kinase. At the completion of these reactions, proteins were subjected to SDS-PAGE (10–12% gels).

Treatment of Cells with Pharmacological Agents and Cell Processing—Confluent cultures of 293T or NIH 3T3 cells were incubated at 37 °C for 20 min in serum-free media containing forskolin (1–100 μM; Calbiochem), 3-isobutyl-1-methylxanthine (IBMX; 10–100 μM; Sigma), or vehicle (MeSO, 0.2% v/v; Fisher). When used, protein kinase inhibitors, H89, Bis-1, E-64, 2,5-bis(trifluoromethyl)phenyl imidazole (IBMX; 10–100 μM; Sigma), or vehicle (MeSO, 0.2% v/v; Fisher), were added 30 min prior to the test agent. At the end of the incubation period, cells were either flash-frozen until use or immediately processed. Treated cell cultures were homogenized with a Tenbroeck tissue grinder in a lysis buffer composed of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 5 mM benzamidine, 100 mM EDTA, 100 μM EGTA, 1% Triton X-100, 2 μg/ml leupeptin, 5 μg/ml bestatin, 2 μg/ml aprotinin, 10 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, and 10 mM sodium glycerophosphate. Cellular debris was removed by centrifugation (1,000 × g; 5 min), and cleared supernatants were used in the experiments. Protein concentrations were measured by the bicinchoninic acid protein assay (Pierce).

Precipitation of Cellular Proteins with Immobilized GST Fusions or M2-Agarose—Interactions between HSPDE3B or its fragments or mutants, with GST-14-3-3 proteins were studied using precipitation by pulldown or immunoprecipitation assays with GST-14-3-3-β, GST-14-3-3-γ or anti-FLAG(M2)-coupled agarose. For immobilized GST pulldown experiments, 50 μl (bed volume) of GSH-Sepharose-4B beads were saturated with GST, GST-14-3-3-β, or GST-14-3-3-γ at 4 °C for 1 h, washed, and incubated for 16 h at 4 °C with cell extracts, prepared as described above. For M2-agarose pulldowns, cell lysates were similarly incubated for 16 h at 4 °C. Following the incubations, Sepharose or agarose beads were centrifuged (6,000 × g; 2 min) and washed repeatedly with 1% Triton X-100-containing lysis buffer. cAMP PDE activities in pulldowns was measured following resuspension of beads in lysis buffer (1:1 volume ratio) using an assay described previously (28). Cilostamide (1 μM) or Ro20-1724 (10 μM) was used to inhibit PDE3 and PDE4 activities, respectively (28). For immunoblotting, pellets were suspended in SDS-PAGE loading buffer. HSPDE3B, as well as other proteins of interest in these pellets, were detected using several antiseras, including a monoclonal antibody specific for HSPDE3B (281K; 1:3000 dilution), a monoclonal antiserum specific for PDE4D (1:4000) (all gifts from Drs. S. Wolda and V. Florio, ICOS Corp., Bothell, WA), or nonspecies selective anti-PDE3B polyclonal antibodies (sc-11835 and sc-11838; Santa Cruz Biotechnology) as we described previously (29). Levels of PKA-phosphorylated HSPDE3B in cells were detected using an antiserum directed at phosphorylated PKA substrates (Upstate Biotechnology, Inc.). Detection of 14-3-3 proteins was carried out by immunoblot analysis using antisera directed against 14-3-3 isoforms (sc-629; pan-reactive; rabbit and goat polyclonals; 1:1000; Santa Cruz Biotechnology).

Statistical Analysis—Some of the data describing protein–protein interactions are shown as representative immunoblots. In all cases, data consistent with that shown in the representative immunoblot were obtained in at least three additional separate experiments. Numerical data are presented as means ± S.E. and are from at least four independent experiments. Statistical differences were assessed using unpaired analysis of variance, with a Tukey post hoc test, or unpaired Student’s t test, as appropriate, with a value of p < 0.05 considered statistically significant.

Materials—Yeast and bacteria culture reagents were obtained from Difco (yeast nitrogenous base without amino acids), Fisher (peptone, tryptone, and dextrose), Qbiogene (complete supplement mixture −His −Leu −Ade −Trp), and Sigma (adenine, histidine, leucine, tryptophan, and o-nitrophenyl-β-d-galactopyranoside). Restriction enzymes, TaqDNA polymerase, Superscript Moloney murine leukemia virus-reverse transcriptase, isopropyl 1-thio-β-d-galactopyranoside, Dulbecco’s modified Eagle’s medium, RPMI 1640, antibiotic/antimycotic solution, trypsin-EDTA, bovine serum albumin, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, and fetal bovine serum were from Invitrogen. Plasmid purification was achieved using QIAprep spin columns, and MIDI columns were from Qiagen. [3H]cAMP (25 Ci/mmol), 5′-[14C]AMP (590.4 mCi/mmol), [γ-32P]ATP (3000 Ci/mmol), and enhanced chemiluminescence reagents (Western Lightning) were from PerkinElmer Life Sciences. DTt, Triton X-100, EDTA, EGTA, Pipes, acrylamide, bisacrylamide, TEMED, and ammonium persulfate were purchased from ICN Biomedicals. Sodium azide, Tris, trisodium citrate dihydrate, citric acid monohydrate, HEPES, NaCl, and Tween 20 were from Fisher. Benzamidine-HCl, leupeptin hemisulfate, aprotinin, phenylmethylsulfonyl fluoride, and G418 antibiotic were purchased from Calbiochem. All other reagents, salts, and enzymes were obtained from Sigma.

RESULTS

Identification of HSPDE3B-interacting Proteins—To identify potential HSPDE3B-interacting proteins, we screened a yeast two-hybrid mouse brain cDNA library with HSPDE3B fragments as bait. “Bait-1” encoded amino acids 2–90, and “bait-2” encoded amino acids 257–711 of HSPDE3B, respectively. No interacting proteins were isolated when bait-2 was used. In contrast, multiple clones of a brain 14-3-3 protein, 14-3-3ζ (GenBankTM accession number NM-0011738), were isolated using precipitation by pulldown or immunoprecipitation assays with GST-14-3-3 proteins (28). Levels of PKA-phosphorylated HSPDE3B in cells were detected using an antiserum directed at phosphorylated PKA substrates (Upstate Biotechnology, Inc.). Detection of 14-3-3 proteins was carried out by immunoblot analysis using antisera directed against 14-3-3 isoforms (sc-629; pan-reactive; rabbit and goat polyclonals; 1:1000; Santa Cruz Biotechnology).
have promoted the interaction of bait-1 with 14-3-3 in yeast, bait-1 encodes several basophilic Ser/Thr kinase sites (30), and this fragment was phosphorylated by the PKA catalytic (C) subunit in an in vitro kinase assay (Fig. 1E).

In Vitro and in Vivo Association of Purified HSPDE3B and GST-14-3-3 Proteins Is Stimulated following PKA Phosphorylation of HSPDE3B—Using a GSH-based adsorption assay (herein the “GST-14-3-3 pulldown”), purified HSPDE3B interacted with either immobilized GST-14-3-3 or GST-14-3-3 but not with immobilized native GST. Consistent with an important regulatory role for HSPDE3B phosphorylation in regulating this interaction, prior in vitro incubation of HSPDE3B with the C-subunit of PKA and ATP, but not the C-subunit alone, increased markedly these interactions (Fig. 2, A and B). In contrast, although in vitro incubation of HSPDE3B with purified recombinant-activated PKB and ATP increased HSPDE3B activity by ~90 ± 15% (n = 3), it did not promote HSPDE3B binding to GST-14-3-3 proteins (Fig. 2B). Overall these data are consistent with the idea that HSPDE3B and 14-3-3 proteins interact directly in vitro and that phosphorylation of HSPDE3B by PKA, but not PKB, promoted this direct interaction. The modest HSPDE3B binding caused by ATP alone (Fig. 2A) was likely because of the presence of trace amounts of kinase activity in the purified HSPDE3B.

To investigate if PKA-mediated phosphorylation of HSPDE3B also promoted 14-3-3 binding in cells, we used a cell line that expressed HSPDE3B endogenously, namely HEK293T, “293T.” Incubation of 293T cells with a combination of forskolin and IBMX (F/I) markedly increased HSPDE3B binding to GST-14-3-3β and, when tested by immunoprecipitation of HSPDE3B, the amount of 14-3-3β that associated with HSPDE3B (Fig. 2, C and D). Consistent with a role for PKA in coordinating the effects of F/I, prior incubation of cells with a PKA inhibitor (H89, 10 μM) ablated this effect (Fig. 2D). Addition of a PKC inhibitor (8 μM Bis-1; Fig. 2D) or a PI3K inhibitor (10 μM LY294002; data not shown) did not alter F/I-induced HSPDE3B binding to GST-14-3-3β. Addition of either the exchange proteins activated by cAMP-selective activator (10 μM; 8-(4-chlorophenylthio)-2’-O-methyl-cAMP), insulin (100 nM; data not shown), or of an activator of conventional PKCs (100 nM PMA; Fig. 2D) did not impact HSPDE3B binding to GST-14-3-3β. Taken together, these data are consistent with the idea that endogenous 293T HSPDE3B interacts with 14-3-3 proteins in a PKA-dependent manner and that this interaction is independent of activation, or inhibition, of exchange proteins activated by cAMP, insulin-dependent signaling, PKC, PI3K, or PKB.

An Amino-terminal Fragment of HSPDE3B Interacts with GST-14-3-3β—A FLAG-tagged HSPDE3B amino-terminal fragment, containing amino acids 1–518, HSPDE3B(AT), bound to GST-14-3-3β in a PKA-dependent manner in 293T cells (Fig. 3). In contrast, a FLAG-tagged HSPDE3B carboxy-terminal fragment comprising amino acids 519–1112, HSPDE3B(CT), did not (Fig. 3). These data indicate that 14-3-3 proteins interacted with HSPDE3B through sequences contained within the amino-terminal domain of HSPDE3B.

An in silico analysis (30) identified several potential PKA phosphorylation consensus sites within HSPDE3B(CT), and three of these (Ser-73, Ser-296, and Ser-318) were selected for study. PKA phosphorylated the equivalent site in murine PDE3B (MMPDE3B-Ser-73), and the PKA-dependent in vitro phosphorylation of a GST-HSPDE3B chimera encoding amino acids 2–90 of HSPDE3B, GST-HSPDE3B chimera was expressed in E. coli, purified, and incubated with PKA (10 units/ml) in the presence or absence of [γ-32P]ATP (200 μM; 10 μCi/reaction) at 30 °C for 1 h. Reaction products were resolved by SDS-PAGE, and the gel was stained, dried, and subjected to autoradiography. A representative stained gel and autoradiogram are shown.

The GST-14-3-3 pulldown detected a single band of ~35 kDa when purified HSPDE3B or GST-PDE3B was incubated with PKA (Fig. 4A). This band was not detected when GST-14-3-3, GST-PDE3B, or GST was incubated with PKA (Fig. 4A). The presence of this band was consistent with the idea that PKA phosphorylation of HSPDE3B is required for its interaction with 14-3-3 proteins.
in MMPDE3B (Ser-273) activated the enzyme (31) and was suggested to promote its association with 14-3-3 in murine adipocytes (24).

All of our data were consistent with the idea that PKA-mediated phosphorylation of HSPDE3B at Ser-318 coordinated 14-3-3 binding. Thus, although F/I treatment of cells expressing an S318A mutant form of FLAG-HSPDE3B(AT) did not promote 14-3-3β binding of this protein, F/I treatment of 293T cells expressing HSPDE3B(AT) forms encoding S73A, S295A, or S296A mutants did promote their interactions with 14-3-3β (Fig. 4A). Omitting effects because of differences in the responses of cells expressing these constructs to F/I treatments, endogenous HSPDE3B expressed in these cells interacted with GST-14-3-3β identically, irrespective of the HSPDE3B(AT) fragments expressed (Fig. 4A). Similar results were obtained when HSPDE3B(AT) constructs were expressed in NIH 3T3 cells even though these cells do not express MMPDE3B endogenously (not shown).

Interestingly, trace amounts (~5%) of the HSPDE3B(AT) S318A, S73A/S318A, or S296A/S318A mutants were recovered in GST-14-3-3β pulldowns when 293T cells were incubated with F/I (Fig. 4A). These findings were consistent with data from unrelated studies in which we show that HSPDE3B(AT) can dimerize with endogenous full-length HSPDE3B in 293T cells.3 Indeed, when S318A mutants of HSPDE3B(AT) were expressed in NIH 3T3 cells, a cell type that does not express MMPDE3B, these constructs were not detected in GST-14-3-3β pulldowns (not shown).

**Full-length HSPDE3B Variants Encoding an S318A Mutation Do Not Bind GST-14-3-3β—**Incubation of 293T cells expressing full-length S73A (not shown) or S296A HSPDE3B mutants with F/I promoted their interactions with GST-14-3-3β (Fig. 4B). In contrast, F/I treatment of cells expressing an HSPDE3B S318A mutant did not (Fig. 4B). Again, these data are consistent with the idea that Ser-318 is the sole relevant phospho-acceptor site that promotes PKA-dependent interactions between HSPDE3B and GST-14-3-3β. In addition to representing the HSPDE3B phosphorylation site responsible for coordinating HSPDE3B/14-3-3β binding (Fig. 4) and enzyme activation (Table 1), by using an antiserum directed against phosphorylated PKA substrates we found that Ser-318 may also represent a

3 D. R. Raymond and D. H. Maurice, unpublished observations.
major site of PKA phosphorylation of HSPDE3B in cells incubated with F/I (Fig. 5). Because it is currently unknown if this antiserum reacts with similar affinity to all phosphorylation consensus sequences, a more detailed analysis will be required to quantify the absolute levels of phosphate at each of these sites. Although in silico analysis (30) identified other potential PKA consensus sites within HSPDE3B, our data are consistent with the idea that only Ser-318 was required for 14-3-3 binding in 293T cells.

**PKB Does Not Promote HSPDE3B/14-3-3 Interactions**—A previous report proposed that an insulin-promoted, PKB-dependent phosphorylation of MMPDE3B at Ser-279 and Ser-302 (residues equivalent to Ser-295 and Ser-318 in HSPDE3B) activated this enzyme and promoted its interaction with 14-3-3 in murine adipocytes (24). In marked contrast to this earlier report, although our data unequivocally confirm that PKB can activate HSPDE3B (see above), they are completely inconsistent with the idea that phosphorylation of HSPDE3B by PKB at Ser-295, or any other site, promotes binding of this enzyme with 14-3-3. Similarly, our data are inconsistent with the idea that PKB-mediated actions on HSPDE3B alter the ability of PKA phosphorylation at Ser-318 to promote 14-3-3 binding (Fig. 6). Indeed, expression of wild type PKB (WT PKB), a membrane-targeted and constitutively activated PKB (MA PKB), or a dominant negative and “kinase-dead” PKB (DN PKB) in 293T cells did not alter either the basal levels of endogenous HSPDE3B binding to GST-14-3-3 in these cells nor the ability of F/I treatment to promote this binding (Fig. 6). Consistent with their state of activation, immunoblot analysis with a phospho-PKB antiserum indicated that a large fraction of the heterologously expressed wild type or activated PKBs were phosphorylated and that the DN PKB, which is kinase-dead was not (not shown). Similarly, because F/I treatment promoted S295A/HSPDE3B binding to 14-3-3 (Fig. 4A), it is also unlikely that Ser-295 was involved in coordinating the PKA-dependent binding of HSPDE3B to 14-3-3 in these cells.

**Binding of the PKA Phosphorylated and Activated HSPDE3B to 14-3-3 Alters Its Susceptibility to Phosphate-catalyzed Dephosphorylation and Inactivation**—Because our data showed that PKA phosphorylation of Ser-318 in HSPDE3B activated this enzyme and promoted its binding to 14-3-3, we hypothesized that this interaction might alter the susceptibility of this fraction of HSPDE3B to be dephosphorylated and inactivated by phosphatases. Our data are completely consistent with this novel idea. Indeed, although a 20-min incubation of M2-agarose-purified HSPDE3B from F/I-treated cells with calf intestinal alkaline phosphatase (CIAP) resulted in substantial dephosphorylation (Fig. 7) and enzyme inactivation (Table 2), this identical CIAP treatment of the PKA-activated, GST-14-3-3-bound HSPDE3B did not result in substantial dephosphorylation (Fig. 7) or enzyme inactivation (Table 2). Because PKB phosphorylation of HSPDE3B did not promote GST-14-3-3 binding (Fig. 2), nor influence the extent of 14-3-3 binding of this enzyme caused by F/I (Fig. 6), it is highly unlikely that PKB-activated HSPDE3B would be similarly protected from phosphate-mediated dephosphorylation and inactivation. A scheme depicting these concepts is presented in Fig. 8.

### DISCUSSION

Many proteins involved in cellular signaling are promiscuous, regulating several distinct events simultaneously (33, 34).

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**TABLE 1**

**Impact of PKA-induced phosphorylation of HSPDE3B on enzyme activity**

| HSPDE3B | Homogenate vehicle | Homogenate F/I | M2-agarose ip vehicle | M2-agarose ip F/I |
|---------|--------------------|----------------|------------------------|--------------------|
| Mock    | 36 ± 3             | 50 ± 2         | 17 ± 0                 | 13 ± 1             |
| S296A   | 93 ± 5             | 125 ± 2        | 153 ± 6                | 183 ± 5            |
| S318A   | 99 ± 1             | 102 ± 0        | 146 ± 4                | 142 ± 4            |
| S296/318A | 90 ± 2           | 93 ± 2         | 101 ± 12               | 106 ± 7            |

* Data signify significant differences between vehicle and F/I-treated cells, p < 0.05. Similar data were obtained in three separate experiments.
Although the events that allow coordination of the multiple actions of signaling proteins are as yet poorly understood, dynamic regulation of protein phosphorylation has emerged as an important point of control (33, 34). In this study, we have confirmed that phosphorylation of HSPDE3B regulates this enzyme activity in cells, and we have uncovered a mechanism that may allow cells to discriminate between the PKA- and the PKB-activated populations of this enzyme.

As described in the Introduction, cells express only one HSPDE3B variant, and this enzyme can be targeted to both the endoplasmic reticulum and, perhaps to a lesser extent, to the plasma membrane of cells (21, 35–36). However, there is currently a paucity of information regarding the proteins with which these potentially separate populations of HSPDE3B might interact and on the impact such protein-protein interactions might have on HSPDE3B activity. Indeed, whereas PDE3B in rodent or human adipocytes were reported to interact with the insulin receptor and/or with caveolin-1, and the rodent enzyme was shown to interact with p87PKA-AP in heart, the impact of these events on PDE3 activity and on cellular events regulated by the hydrolysis of cAMP by this enzyme are as yet poorly understood.

Previously, the rat adipocyte PDE3B was reported to interact with 14-3-3β in an insulin- and PI3K activation-dependent manner. Indeed, in this earlier study, it was suggested that phosphorylation of Ser-279 or Ser-302, sites equivalent to Ser-295 and Ser-318 in HSPDE3B, can be targeted from CIAP-dependent dephosphorylation. 293T cells were transiently transfected with plasmids encoding FLAG-tagged HSPDE3B. After 24 h, transfected cells were incubated with Me2SO (0.2% v/v) or F/I (100 μM each) for 20 min. Following these incubations, cells were processed for either M2-agarose or GST-14-3-3 pulldowns. In these experiments, 0.2 mg of 293T cell lysate from each incubation was added either to M2-agarose (20-μl bed volume) or to 14-3-3-GST-coupled GSH-Sepharose (50-μl bed volume) and processed as described (see “Experimental Procedures”). Following these pulldowns, resuspended pellets were equally divided such that half was incubated with CIAP buffer and the other half incubated with this buffer supplemented with CIAP (1 unit) for 20 min at 37 °C. Reaction products were processed for immunoblot analysis. Samples were first probed with an antibody for phosphorylated PKA substrates (4) and subsequently with the M5 anti-FLAG antiserum (8). Amounts of immunoreactive proteins were quantified by densitometric analysis. Similar data were obtained in three separate experiments.

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14-3-3 Proteins and HSPDE3B Activation

TABLE 2
Impact of phosphatase treatment on HSPDE3B activity

Note that 293T cells were transiently transfected with a plasmid encoding a FLAG-tagged HSPDE3B. After 24 h, cells were either treated with vehicle (0.2% Me2SO, v/v) or forskolin and IBMX (F/I, 100 μM each) for 20 min, rinsed, and lysed, and the lysates were used in M2-agarose precipitation, or GST-14-3-3 pulldown experiments (see “Experimental Procedures”). Pellets from the pulldowns were either incubated with nothing or 5 units/ml CIAP for 20 min at 37 °C. Following these incubations, cAMP PDE activity was determined using volumes of the reaction products from 14-3-3 pulldowns that gave roughly similar basal levels of activity.

| Additions | M2-agarose | 14-3-3β |
|-----------|------------|---------|
|           | CIAP       | cAMP PDE activity | % control | CIAP       | cAMP PDE activity | % control |
| Vehicle   | —          | 38 ± 4   |          | —          | 14 ± 2         |          |
| F/I       | —          | 49 ± 1   | 29       | —          | 81 ± 6         | 479       |
| F/I       | +          | 33 ± 3   | −13      | +          | 67 ± 0         | 379       |

![Image](https://via.placeholder.com/150)

**FIGURE 8.** Model depicting HSPDE3B binding to 14-3-3 proteins and the impact of this event on PKA- or PKB-mediated activation. A scheme is presented that summarizes the data obtained in our analysis of the interaction of HSPDE3B and 14-3-3 proteins. The scheme depicts how PKA-phosphorylated HSPDE3B is protected from phosphatase-mediated dephosphorylation/inactivation compared with unbound PKA- or PKB-phosphorylated HSPDE3B in cells.

ing with HSPDE3B in cells that was not sensitive to H89 or the activation of PKB in our experiments. Examples of similar 14-3-3 interactions that were not dependent on protein phosphorylation have been reported (37–38). Whether phosphorylation of Ser-13 and Ser-73 in cells plays a role in organizing cellular HSPDE3B complexes at a step not investigated in our studies remains possible and will form the basis of future work in our laboratory. From our site-directed mutagenesis studies we identified a dominant role for one HSPDE3B residue, namely Ser-318, in coordinating the direct, PKA-dependent, interaction between HSPDE3B and 14-3-3 proteins. Indeed, binding of the S318A mutant of HSPDE3B was not increased when cells were incubated with cAMP-elevating agents. Our data also rule out a direct role for phosphorylation of either of the other HSPDE3B PKA phospho-acceptor sites (Ser-73 and Ser-296) or the PKB phospho-acceptor residues (Ser-295) in coordinating PKA-dependent HSPDE3B binding to 14-3-3β. Indeed, HSPDE3B, in which multiple PKA sites were mutated, interacted with immobilized GST-14-3-3β in a manner consistent with the singular importance of Ser-318. Also, because the interaction between the S295A-HSPDE3B mutant used in our studies and 14-3-3β was stimulated by activation of PKA, this site is unlikely to have contributed to 14-3-3 binding. Because expression of a constitutively activated PKB or of a kinase-dead dominant negative PKB had no impact on basal or PKA-dependent HSPDE3B-14-3-3 protein binding, we suggest that HSPDE3B does not interact with 14-3-3 proteins in a PKB-dependent fashion in 293T cells. In unpublished data, we have similarly shown that PKB does not stimulate HSPDE3B binding to 14-3-3 proteins in either the pre-B acute lymphoblastic leukemia cell line (REH) or U937 cells, a human cell line established from a diffuse histiocytic lymphoma that displays several monocytic characteristics.

Our data obviating the possible involvement of PKB in fostering HSPDE3B binding to 14-3-3β are unequivocal. Indeed, we found no evidence to support a role for PKB in mediating either basal levels of HSPDE3B binding to 14-3-3β or in promoting association between these proteins in response to increases in cellular cAMP. In relation to insulin-mediated stimulation of RNPDE3B binding to 14-3-3β in adipocytes (24), it may be significant that insulin can activate adenylyl cyclase in several cell types and that this latter effect was PI3K-inhibitor-sensitive (39). Whether insulin stimulated RNPDE3B binding to 14-3-3β in rat adipocytes through an insulin-mediated activation of adenylyl cyclase, thus involving PKA, or whether a direct effect of PI3K was involved, which was not tested in our study, remains to be established. Perhaps the use of a site-directed mutation-based approach similar to that employed here, rather than the use of high concentrations of phosphorylated peptides, as was done in the earlier work (24), would be beneficial in future studies.

To our mind, the most interesting finding of this study relates to our demonstration that the 14-3-3β-bound, PKA-activated form of HSPDE3B was protected from phosphatase-catalyzed inactivation. Indeed, 14-3-3-bound HSPDE3B was less sensitive to phosphatase-based inactivation when compared with unbound PKA-activated HSPDE3B or with PKB-activated HSPDE3B. Of course, the PKB-activated HSPDE3B was not 14-3-3-bound. Indeed, we suggest that this difference in phosphatase-catalyzed inactivation of the PKA- versus PKB-activated forms of HSPDE3B may represent a novel mechanism by which cells can differentiate activation of HSPDE3B by these two kinases (Fig. 8). Similarly, earlier works by others (40, 41) have shown that 14-3-3 protein binding to some, but not all, phosphorylated binding partners reduces their rate of dephosphorylation. Ongoing studies in our laboratory are aimed at assessing the impact that 14-3-3 protein-mediated “protection” against dephosphorylation of the PKA-activated enzyme, but not that activated by PKB, may have on intracellular cAMP.

4 L. S. Wilson and D. H. Maurice, unpublished observations.
levels and the signaling events associated with HSPDE3B activation in cells.

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