Mechanisms of Signal Transduction:

Pctaire1 Phosphorylates *N*-Ethylmaleimide-sensitive Fusion Protein: IMPLICATIONS IN THE REGULATION OF ITS HEXAMERIZATION AND EXOCYTOSIS

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Pctaire1 Phosphorylates *N*-Ethylmaleimide-sensitive Fusion Protein

*IMPLICATIONS IN THE REGULATION OF ITS HEXAMERIZATION AND EXOCYTOSIS**

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Pctaire1, a member of the cyclin-dependent kinase (Cdk)-related family, has recently been shown to be phosphorylated and regulated by Cdk5/p35. Although Pctaire1 is expressed in both neuronal and non-neuronal cells, its precise functions remain elusive. We performed a yeast two-hybrid screen to identify proteins that interact with Pctaire1. *N*-Ethylmaleimide-sensitive fusion protein (NSF), a crucial factor in vesicular transport and membrane fusion, was identified as one of the Pctaire1 interacting proteins. We demonstrate that the D2 domain of NSF, which is required for the oligomerization of NSF subunits, binds directly to and is phosphorylated by Pctaire1 on serine 569. Mutation of this phosphorylation site on NSF (S569A) augments its ability to oligomerize. Moreover, inhibition of Pctaire1 activity by transfecting its kinase-dead (KD) mutant into COS-7 cells enhances the self-association of NSF. Interestingly, Pctaire1 associates with NSF and synaptic vesicle-associated proteins in adult rat brain. To investigate whether Pctaire1 phosphorylation of NSF is involved in regulation of Ca^{2+}-dependent exocytosis, we examined the effect of expressing Pctaire1 or NSF phosphorylation mutants on the regulated secretion of growth hormone from PC12 cells. Interestingly, expression of either Pctaire1-KD or NSF-S569A in PC12 cells significantly increases high K^+-stimulated growth hormone release. Taken together, our findings provide the first demonstration that Pctaire1 phosphorylation of NSF regulates the ability of NSF to oligomerize, implicating an unexpected role of this kinase in modulating exocytosis. These findings open a new avenue of research in studying the functional roles of Pctaire1 in the nervous system.

Originally identified as a Cdc2-like kinase (1, 2), Pctaire1 is ubiquitously expressed in mammalian tissues and is particularly abundant in terminally differentiated cells such as postmitotic neurons (1–3). In contrast to classical Cdks,[2] which depend on cyclins for activation, Pctaire1 is not activated by cyclins. We have reported previously that Pctaire1 interacts with the Cdk5 activator p35, and phosphorylation by Cdk5/p35 enhances the kinase activity of Pctaire1 (4). An alternative regulatory mechanism to inhibit the activity of Pctaire1 through protein kinase A has also been proposed (5). Similar to Cdk5, Pctaire1 is not involved in the regulation of cell cycle progression (1, 3). To date, the precise functions of Pctaire1 remain elusive. Recent studies indicate that Pctaire1 regulates neurite outgrowth in Neuro-2A cells, whereby overexpression of the kinase-dead (KD) mutant of Pctaire1 leads to enhanced neurite outgrowth (5).

As a first step to explore the functional roles of Pctaire1 in the nervous system, we have performed a yeast two-hybrid screen to identify proteins that interact with Pctaire1 in adult brain. Our study reveals that Pctaire1 interacts with *N*-ethylmaleimide-sensitive fusion protein (NSF), a cytosolic protein that is essential for vesicular transport and fusion of synaptic vesicles with presynaptic membrane (6, 7). NSF is recruited to membrane through adaptor proteins known as soluble NSF attachment proteins (SNAPs), which bind to membrane-bound SNAP receptors (SNAREs). The hexameric NSF acts as a molecular chaperone to hydrolyze ATP and alters the conformation of SNARE complex, disassembles it, and recycles SNARE monomers for subsequent membrane fusion (8). Each NSF monomer is comprised of three domains: an N-terminal domain (amino acids 1–205) that is responsible for the interaction with α-SNAP and SNAREs; and two homologous ATP-binding domains, D1 (amino acids 206–477), in which the hydrolytic activity is associated with NSF-driven SNARE complex disassembly, and D2 (amino acids 478–744), which is believed to be responsible for hexamer formation (9, 10). The activity of NSF is tightly regulated by post-translational modifications to ensure its proper function during membrane fusion.

In this study, we provide evidence that NSF interacts with Pctaire1 and is phosphorylated by Pctaire1. Phosphorylation of NSF by Pctaire1 on serine 569 attenuates the hexamerization of NSF. The association of Pctaire1 and NSF with components of the molecular machinery required for transmitter release prompted us to explore whether the phosphorylation of NSF by Pctaire1 plays a role in regulating exocytosis. Ca^{2+}-dependent exocytosis of dense core vesicles in PC12 cells has been reported to exhibit properties similar to that of synaptic vesicle release, allowing PC12 cells to be widely adopted for studies on regulated exocytosis (11, 12). Our results with the human growth hormone (hGH) release assay in PC12 cells suggest that the serine phosphorylation of NSF by Pctaire1 plays an important role in regulating the Ca^{2+}-dependent exocytosis. Taken together, the identification of NSF as an interacting protein and substrate for Pctaire1 provides critical insights into how the regulation of NSF phosphorylation by Pctaire1 might impact on its oligomerization and modulate the process of transmitter release.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Complementary DNA (cDNA) encoding mouse Pctaire1, Cdk5, and p35 was subcloned into the vector, pAS2-1

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[2] The abbreviations used are: Cdk, cyclin-dependent kinase; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, soluble NSF attachment protein receptor; hGH, human growth hormone; HA, hemagglutinin; GST, glutathione *S*-transferase; WT, wild type; ATPγS, adenosine 5′-*O*-(thiotriphosphate); KD, kinase-dead.

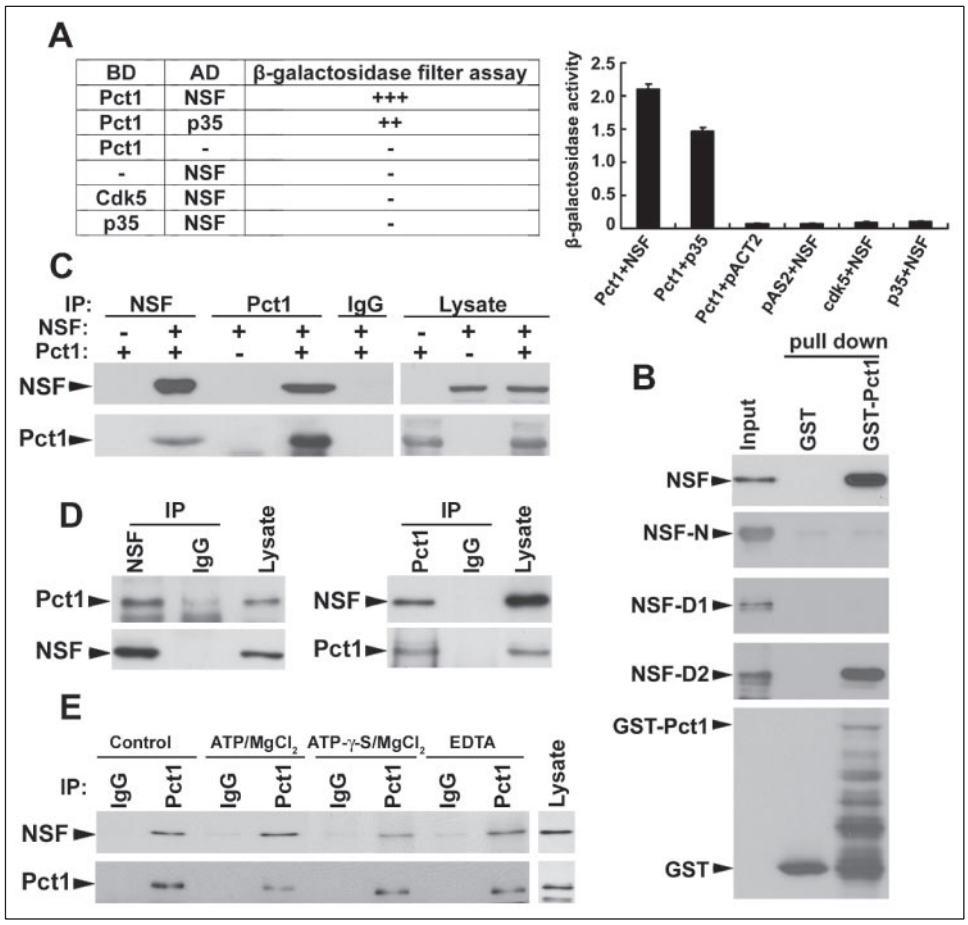

FIGURE 1. **Pctaire1 interacted with the D2 domain of NSF.** *A*, association of NSF with Pctaire1 (*Pct1*) in yeast. The interaction between NSF and Pctaire1, Cdk5, or p35 was examined in yeast by filter assay and β-galactosidase activity assay. *BD*, GAL4 DNA-binding domain; *AD*, transcriptional activation domain. +++, strong interaction; ++, mild interaction; −, no interaction. *B*, Pctaire1 bound to NSF *in vitro*. Recombinant His-tagged proteins encoding for NSF or different domains (NSF-N, NSF-D1, or NSF-D2) were incubated with purified GST-Pctaire1 on glutathione-agarose beads. The bound proteins were analyzed by SDS-PAGE and immunoblotted using NSF or His antibody (*upper panels*). The nitrocellulose membrane was then stripped and blotted with anti-GST antibody (*bottom panel*). *C*, association of Pctaire1 with NSF in COS-7 cells. Pctaire1 and NSF were transfected into COS-7 cells as illustrated, and cell lysates were immunoprecipitated (*IP*) using NSF antibody and immunoblotted with Pctaire1 antibody or vice versa. *D*, association of NSF with Pctaire1 in adult brain. Adult rat brain lysates were immunoprecipitated using monoclonal NSF antibody and immunoblotted with Pctaire1 antibody (*left panels*) or vice versa (*right panels*). The normal IgG was used as a negative control. *E*, the co-immunoprecipitation of NSF and Pctaire1 was independent of the ATP-hydrolyzable condition. Adult rat brain extracts were prepared in different conditions; in the presence of ATP/$MgCl_2$, ATPγS/$MgCl_2$ or EDTA, and the lysates were co-immunoprecipitated with Pctaire1 antibody and immunoblotted with NSF antibody.

(Clontech). Full-length, partial cDNA fragments and point mutations (*e.g.* serine 569 to alanine (S569A)) of mouse NSF were amplified by PCR and subcloned into different expression vectors including pGEX-6P-2, pETH-32, pcDNA3-His_6, and pcDNA3-3HA. pXGH5 containing the hGH gene was a gift from Prof. Masami Takahashi (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan), and synaptotagmin antibody was generously provided by Prof. Benjamin Peng (The Hong Kong University of Science and Technology). Pctaire1 antibody was raised against the peptide GEAPTRVAPGELRSIR, and NSF antibody was purchased from Calbiochem. Antibodies specific for synapsin I and synaptophysin were purchased from Cell Signaling Technology (Beverly, MA) and Chemicon (Temecula, CA), respectively. Antibodies against SV-2 and α-tubulin were from Sigma, whereas antibodies specific for HA, His, and syntaxin1 (HPC-1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser/Thr-Pro (MPM-2) antibody was from Upstate Biotechnology (Lake Placid, NY).

Yeast Two-hybrid Screen—Yeast two-hybrid screen was performed following the Matchmaker two-hybrid screen protocol (Clontech). The NSF clone isolated from the two-hybrid screen encoded the protein that lacked the five amino acids at the N terminus. Subsequent two-hybrid interaction analyses were carried out by co-transformation of plasmids encoding the GAL4 DNA-binding (pAS2-1) and activation (pACT2) domains into yeast. An *o*-nitrophenyl-β-D-galactopyranoside-based β-galactosidase activity assay was performed according to the manufacturer's protocol (Clontech).

Cell Culture and Transfection—COS-7 and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum plus antibiotics. PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with heat-inactivated horse serum (6%, v/v), heat-inactivated fetal bovine serum (6%, v/v) plus antibiotics and maintained at 37 °C in a humidified atmosphere with 7.5% CO_2. Transient transfection was carried out using Lipofectamine PLUS reagents (Invitrogen) according to the manufacturer's instructions.

Protein Purification, Protein Extraction, Western Blot, Immunoprecipitation, and in Vitro Phosphorylation Analysis—The recombinant GST-Pctaire1 and His_6-tagged NSF and its mutants were purified following the instructions of the manufacturer (Amersham Biosciences) and by nickel-nitrilotriacetic acid-agarose affinity chromatography (Qiagen) (13), respectively. Protein extracts from cells and brain tissues were prepared using different lysis buffers supplemented with various protease inhibitors. COS-7 cells were lysed in lysis buffer A (in mM: Tris, pH 7.6, 20; NaCl, 150; EDTA, 1; EGTA, 1; NaF, 1) with 0.5% Nonidet P-40. Brain tissues were homogenized in lysis buffer B (in mM: Tris, pH 8, 50; NaCl, 150; EGTA, 2; dithiothreitol, 1; NaF, 5) with 1% Nonidet P-40, 0.25% sodium deoxycholate. HEK 293T cells were lysed in lysis buffer C (in mM: Tris, pH 7.5, 25; NaCl, 50; β-glycerophosphate, 20; EDTA, 1; dithiothreitol, 1) with 0.1% Nonidet P-40. Subcellular fractions of adult rat brain were prepared as described previously (14, 15). For co-immunoprecipitation analysis, 100 μg of COS-7 cell lysates were incubated with the corresponding antibody (2 μg) at 4 °C overnight and then incubated with 40 μl of protein G-Sepharose at 4 °C for 1 h. The samples were washed with buffer A and resuspended in SDS sample buffer, and co-immunoprecipitated proteins were detected using Western blot analysis. *In vivo* co-immunoprecipitation studies using brain lysates were performed as described previously (4, 16–18). Active Pctaire1 was immunoprecipitated from adult rat brain lysates with Pctaire1 antibody in radioimmune precipitation assay buffer and then

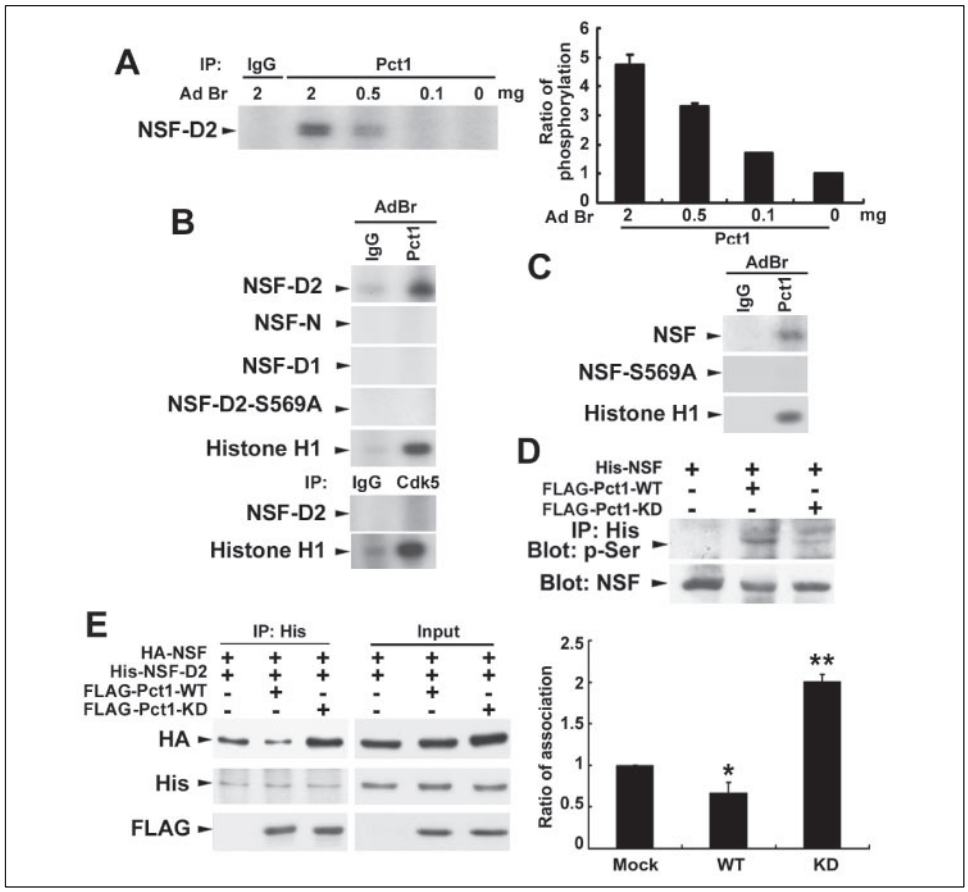

FIGURE 2. **Pctaire1 phosphorylated NSF on serine 569 and reduced its oligomerization.** *A*, NSF-D2 domain was phosphorylated by Pctaire1 (*Pct1*) immunoprecipitated (*IP*) from adult rat brain (*Ad Br*) extracts (of different amounts from 0.1 to 2 mg) using *in vitro* kinase assay. The ratio of the NSF phosphorylation (*right panel*) was shown as the mean of three trials. *B*, active Pctaire1 phosphorylated NSF on serine 569. His-tagged NSF-D1, NSF-N mutant, NSF-D2, and its specific alanine mutant (NSF-D2-S569A) were subjected to the Pctaire1 kinase assay. Histone H1 was used as a positive control. *Bottom panels*, Cdk5 immunoprecipitated from the brain lysate (2 mg) could not phosphorylate serine 569 of NSF *in vitro*. *C*, Pctaire1 phosphorylated the full-length NSF recombinant protein. His_6-tagged recombinant NSF and NSF-S569A mutant proteins were purified and subjected to *in vitro* phosphorylation assay. Histone H1 was used as a positive control. *D*, Pctaire1 phosphorylated NSF in HEK 293T cells. His-NSF was co-transfected with FLAG-tagged Pctaire1-WT or Pctaire1-KD into HEK 293T cells. Cell lysates were immunoprecipitated with anti-His antibody and blotted with proline- directed phospho-serine/threonine antibody or NSF antibody. A protein doublet with the faster migrating form corresponding to the size of NSF was detected in the cells co-transfected with Pctaire1-WT when compared with that of mock and Pctaire1-KD showing that Pctaire1 phosphorylated NSF *in vivo*. *E*, Pctaire1-KD enhanced NSF oligomerization. COS-7 cells were transiently transfected with combinations of HA-tagged NSF, His-tagged D2 domain of NSF, and Pctaire1-WT or Pctaire1-KD. Cell lysates were immunoprecipitated using anti-His antibody and blotted with antibodies against HA, His, or FLAG. Data represent the mean of three experiments, and *error bars* represent the S.E. *, $p < 0.05$; **, $p < 0.01$, significantly different from that of *Mock*.

extensively washed five times with radioimmune precipitation assay buffer. The kinase assay was performed at 30 °C for 30 min in kinase buffer as described previously (19). Two-hundred ng of recombinant proteins were utilized as the substrates in the kinase assay. The samples were then separated on SDS-PAGE and visualized by autoradiography.

Size Exclusion Chromatography and in Vitro Pull-down Analysis—Analytical size exclusion chromatography was performed using a Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences) at 4 °C with a flow rate of 0.5 ml/min. The column was first equilibrated with 10 mM HEPES at pH 7.0, 300 mM NaCl, 2 mM β-mercaptoethanol, 0.5 mM ATP, 0.5 mM $MgCl_2$, 5% glycerol, 0.1% Nonidet P-40, and various protease inhibitors. The pull-down assay was carried out as described previously (4). Briefly, adult brain membrane fraction was prepared as described previously (20), and the pull-down was performed in phosphate-buffered saline supplemented with 5% bovine serum albumin (137 mM NaCl and 2.7 mM KCl in phosphate-buffered saline with no Mg^{2+}, EDTA, and ATP). HEK 293T cells were transfected with FLAG-tagged Pctaire1 or Cdk5, and cell lysates were immunoprecipitated using anti-FLAG antibody, which was immobilized on agarose beads at 4 °C for 2–3 h (Sigma). The immunoprecipitates were then incubated with 200 μg of adult rat brain lysate at 4 °C for 3 h, and the beads were washed extensively with buffer C containing 100 mM NaCl. The samples were then resuspended in SDS sample buffer. Proteins co-immunoprecipitated with FLAG-tagged Pctaire1 and Cdk5 were detected by Western blot analysis.

hGH Release Assay in PC12 Cells—hGH release assays in PC12 cells were carried out at 37 °C as described previously (21, 22). Forty-eight hours after transfection, PC12 cells were washed with a low K^+ solution and maintained in the low K^+ solution (5.6 mM) for 30 min. To measure the low or high K^+-evoked hGH secretion, the cells were incubated either in the same solution (low K^+) or in the high K^+ solution (56 mM) for another 10 min. The culture medium was collected, and the amount of hGH was measured using the hGH enzyme-linked immunosorbent assay kit (Roche Applied Science). Student's *t* test was used for statistical analysis, and the results are presented as mean ± S.E.

RESULTS AND DISCUSSION

NSF Interacted with Pctaire1—A positive clone encoding NSF was identified to interact with Pctaire1 based on the yeast two-hybrid screen of an adult brain library. The ~2.2-kb cDNA clone encodes NSF that lacks the first five amino acids at the N terminus. The full-length NSF cDNA was cloned by PCR, and the interaction of full-length NSF with Pctaire1 in yeast was confirmed (Fig. 1*A*). Results obtained with the β-galactosidase activity assays revealed that the interaction of Pctaire1 with NSF was stronger than that observed with p35, which was reported previously to associate with Pctaire1 (Ref. 4; Fig. 1*A*). On the other hand, NSF failed to interact with Cdk5 and p35 in yeast. We further validated the interaction between NSF and Pctaire1 using *in vitro* GST pull-down assays. GST-Pctaire1 protein was immobilized on glutathione-agarose beads and incubated with recombinant His-NSF. His-NSF bound specifically to GST-Pctaire1 but not the GST control (Fig. 1*B*). To map the specific regions of NSF that interacted with Pctaire1, recombinant proteins encoding various domains of NSF were generated, and their ability to interact with Pctaire1 was analyzed using pull-down assays. Our results indicated that the D2 domain of NSF (NSF-D2) strongly interacted with Pctaire1 (Fig. 1*B*).

The interaction between Pctaire1 and NSF was then examined in COS-7 cells that were transiently transfected with Pctaire1 and NSF cDNA constructs. We found that NSF or Pctaire1 antibody could immunoprecipitate NSF and Pctaire1 from the total lysates of COS-7

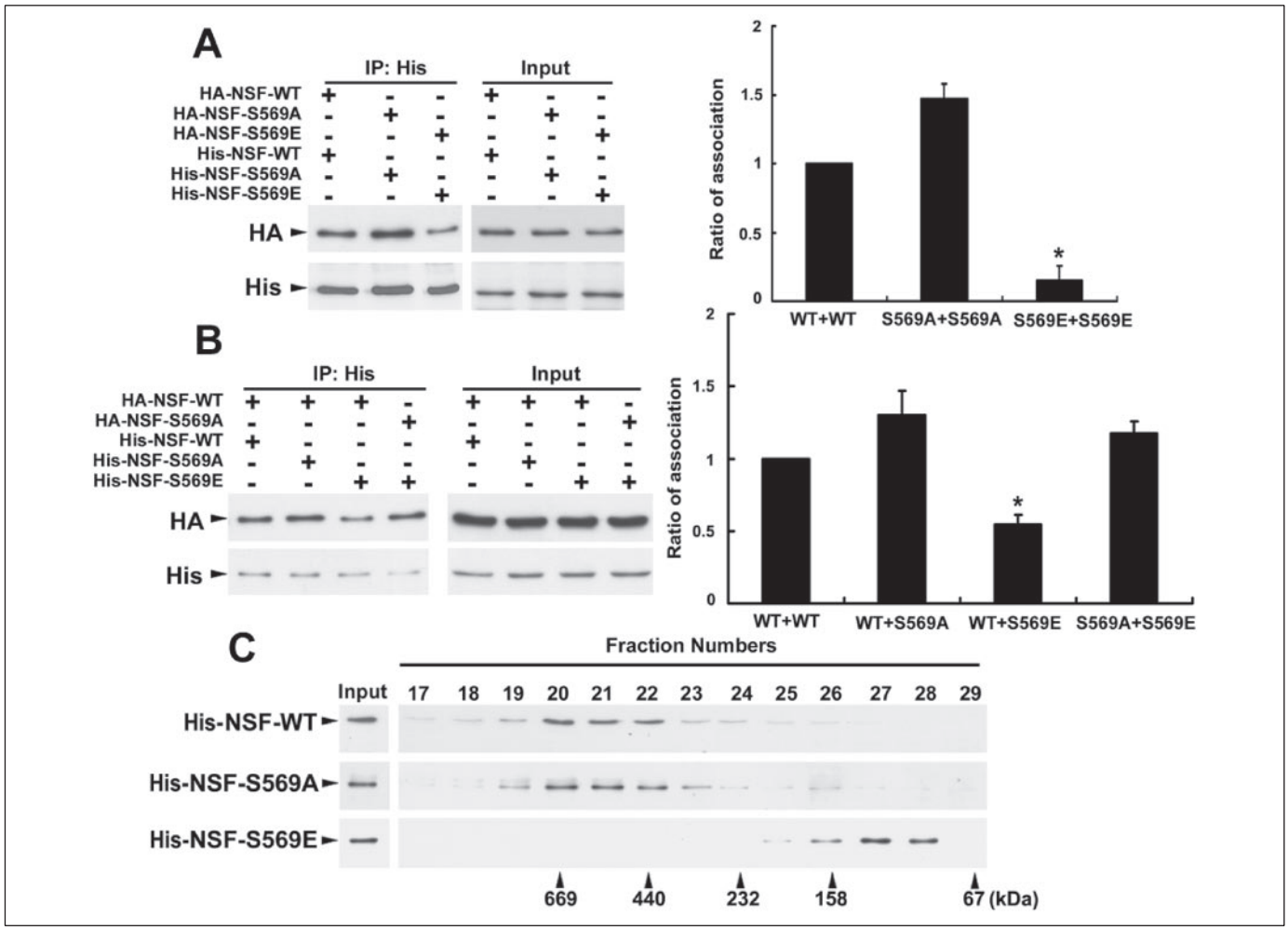

FIGURE 3. **Phosphorylation of NSF on serine 569 regulated its oligomerization.** *A* and *B*, oligomerization of NSF-WT with its phosphorylation mutants, NSF-S569A or NSF-S569E. COS-7 cells were transiently transfected with HA-tagged and His-tagged of NSF (wild type, S569A, or S569E) as illustrated. Cell lysates were immunoprecipitated using anti-His antibody and blotted with anti-HA or anti-His antibody. Data represent the mean of three experiments, and *error bars* represent the S.E. *, $p < 0.05$, significantly different from that of NSF-WT control. *C*, size exclusion chromatography analysis of recombinant protein encoding His_6-NSF and its phosphorylation mutants, NSF-S569A and NSF-S569E. His_6-tagged recombinant proteins were purified by nickel-nitrilotriacetic acid-agarose affinity chromatography and then separated by size exclusion chromatography. The amount of NSF eluted in different fractions was detected by Western blot analysis. The molecular size of corresponding fractions was calibrated by specific protein marker as indicated.

cells overexpressing both cDNA constructs (Fig. 1*C*). Pctaire1 could be immunoprecipitated from whole brain lysate using the anti-NSF antibody, and similarly, NSF was immunoprecipitated by the antibody against Pctaire1 (Fig. 1*D*). Thus, the *in vivo* interaction between Pctaire1 and NSF was confirmed in adult rat brain. It has been reported that the interaction of NSF with some of its binding proteins is dependent on the nucleotide-binding status of NSF (16–18). To examine whether the interaction between NSF and Pctaire1 is dependent on the ATP-hydrolyzable condition, the co-immunoprecipitation experiment was carried out under different conditions, *i.e.* in the presence of ATP/$MgCl_2$ (ATP-hydrolyzable condition), ATPγS/$MgCl_2$ (ATP non-hydrolyzable condition), or EDTA (Mg^{2+} chelating condition). The interaction of Pctaire1 and NSF apparently remained stable under these three conditions (Fig. 1*E*).

Phosphorylation of NSF by Pctaire1 Reduced Its Oligomerization—As Pctaire1 is a Cdk-related serine/threonine kinase (1), we speculated that Pctaire1 might utilize a preferential phosphorylation consensus sequence, (S/T)P*X*(K/H/R), similar to that of other Cdks. Two proline-directed serine/threonine residues were identified in NSF, *i.e.* serine 40 and serine 569. We showed that active Pctaire1 could phosphorylate the D2 domain, but not the N-terminal or D1 domain, of NSF in a dose-dependent manner (Fig. 2, *A* and *B*). These findings, together with the localization of serine 40 to the N-terminal domain, suggest that serine 569 within the D2 domain is a primary phosphorylation site for Pctaire1. As expected, mutation of serine 569 to alanine (S569A) abolished the phosphorylation of D2 domain by active Pctaire1 (Fig. 2*B*). The inability of another Cdk, Cdk5, to phosphorylate NSF confirmed the specificity of Pctaire1 phosphorylation on NSF (Fig. 2*B*). The ability of Pctaire1 to phosphorylate NSF was confirmed by *in vitro* phosphorylation assay using recombinant His_6-tagged NSF protein purified from bacteria. Although the wild type recombinant NSF protein, which was shown previously to exist as a hexamer (23), could be phosphorylated by active Pctaire1, the phosphorylation was not observed with the NSF-S569A mutant protein (Fig. 2*C*). To provide evidence that NSF is phosphorylated by Pctaire1 *in vivo*, NSF was co-transfected with either Pctaire1-WT or its kinase-dead (Pctaire1-KD) mutant (K194A; generated by mutating the key residue involved in the catalytic process of phosphorylation (24)) into HEK 293T cells. NSF protein was co-immunoprecipitated, and its phosphorylation status was examined by Western blot analysis using proline-directed phospho-serine/threonine antibodies. Phosphorylation of NSF was only observed in cells that were co-transfected with Pctaire1-WT (Fig. 2*D*) but not with Pctaire1-KD.

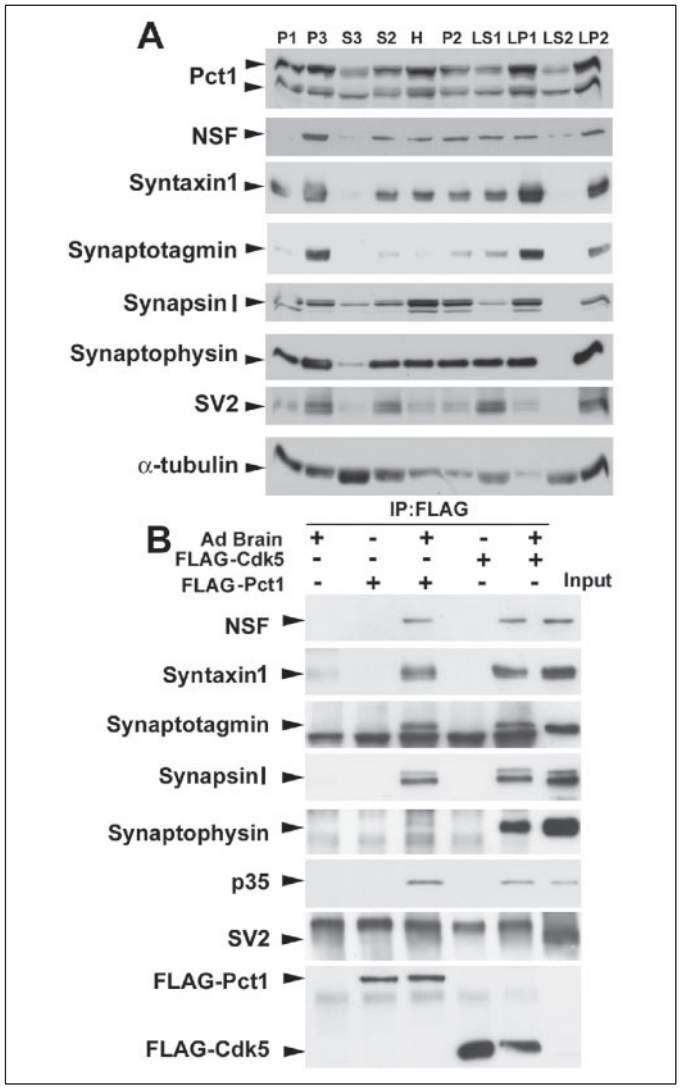

FIGURE 4. **Association of Pctaire1 with synaptic vesicle-associated proteins.** *A*, localization of Pctaire1 (*Pct1*) and NSF to the synaptosomal fraction. Subcellular fractions of adult rat brain were obtained by differential centrifugation and extraction and subjected to Western blot analysis using various markers of SNARE and synaptic vesicle-associated proteins (including syntaxin1, synaptotagmin, synapsin I, synaptophysin, and SV2). *P1*, nuclear pellet and debris; *P3*, light membranes (Golgi, endoplasmic reticulum); *S3*, cytosolic fraction; *S2*, cytosol plus light membranes; *H*, whole brain; *P2*, crude synaptosomal fraction; *LS1*, synaptosomal cytosol; *LP1*, synaptosomal membrane fraction; *LP2*, synaptic-vesicle-enriched fraction; *LS2*, LS1 minus LP2. *B*, association of Pctaire1 with synaptic vesicle-associated proteins. Overexpressed FLAG-tagged Pctaire1 or Cdk5 in 293T cells were immunoprecipitated (*IP*) and then incubated with adult (*Ad*) brain membrane fraction. The panels depict Western blot analysis for NSF, syntaxin1, synaptotagmin, synapsin I, synaptophysin, SV2, and p35 (as control). Immunoblotting with anti-FLAG antibody detected the overexpressed Pctaire1 and Cdk5 proteins. *Input*, adult brain membrane fraction.

The D2 domain of NSF that interacted directly with Pctaire1 has been reported to be required for the hexamerization of NSF (25). Thus, we determined whether the interaction of NSF-D2 with Pctaire1 and/or phosphorylation by Pctaire1 could regulate the oligomerization of NSF. HA-tagged NSF and His-tagged D2 domain of NSF were transfected into COS-7 cells with Pctaire1-WT or Pctaire1-KD. Cell lysates were immunoprecipitated using anti-His antibody and blotted with anti-HA antibody. In the presence of Pctaire1-KD, there was an increase of HA-tagged NSF protein detected in the co-immunoprecipitated complex compared with control. Conversely, in the presence of Pctaire1-WT, a reduced level of HA-tagged NSF was detected in the co-immunoprecipitated complex (Fig. 2*E*). This finding suggests that alteration of Pctaire1 activity is able to regulate the oligomerization of NSF.

We further examined the possibility that phosphorylation on serine 569 of NSF could regulate its ability to oligomerize. Two phosphorylation mutants of NSF, *i.e.* the phosphorylation-deficient mutant (serine to alanine, S569A) and the phosphomimetic mutant (serine to glutamic acid, S569E), were generated. COS-7 cells were transiently transfected with HA-tagged and His-tagged (wild type, S569A or S569E mutant) NSF. Cell lysates were immunoprecipitated using anti-His antibody and blotted with anti-HA antibody. As predicted, the anti-His antibody could immunoprecipitate the HA-tagged NSF protein. The oligomerization of NSF-S569A increased, whereas that of NSF-S569E decreased, when compared with wild type NSF (Fig. 3*A*). These results suggest that phosphorylation of NSF by Pctaire1 on serine 569 inhibited its oligomerization. Similar results on the regulation of NSF oligomerization were observed when the individual mutants were expressed together with the wild type (Fig. 3*B*); that is, the self-association of NSF was attenuated in cells co-transfected with NSF-S569E and NSF-WT, similar to the results obtained with NSF-S569E alone. Interestingly, the reduced oligomerization of NSF was not observed when the cells were co-transfected with both S569E and S569A mutants, suggesting that the effect of overexpressing NSF-S569A mutant is dominant compared with that of NSF-S569E (Fig. 3*B*). Taken together, these findings suggest that the oligomerization of NSF might be regulated by the phosphorylation status of NSF on serine 569.

To further characterize whether the phosphorylation of NSF on serine 569 is critical in regulating the hexamerization of NSF, purified His_6-NSF protein (WT) and its phosphorylation mutants (S569A or S569E) were subjected to size exclusion chromatography followed by SDS-PAGE and Western blot analysis for NSF. The predicted molecular mass of hexameric NSF is ~510 kDa. We found that in the absence of Pctiare1 both NSF-WT and S569A were eluted in fractions corresponding to a higher molecular mass (~440–669 kDa). Interestingly, the phosphomimetic form of NSF (NSF-S569E) was eluted in subsequent fractions corresponding to a lower molecular mass (~67–158 kDa), suggesting that a negative charge at position 569 might hinder the assembly of the hexameric NSF.

Binding of Pctaire1 to Synaptic Vesicle-associated Proteins—To further characterize the interaction between Pctaire1 and NSF, we examined the subcellular localization of these proteins in brain. Pctaire1 as well as NSF was shown to be concentrated in synaptosomal membrane fraction (LP1) and synaptic vesicle-enriched fraction (LP2), similar to that of the SNAREs, such as syntaxin1, synaptotagmin, or synaptic vesicle-associated proteins including synapsin I and synaptophysin (Fig. 4*A*). The co-localization of Pctaire1 and NSF with these proteins to the specific subcellular fractions suggests that Pctaire1 might regulate the mobilization of vesicles through the phosphorylation of NSF. To further explore the involvement of Pctaire1 in synaptic vesicle functions, we performed a pull-down analysis and found that Pctaire1 interacted with a number of synaptic vesicle-associated proteins *in vivo*. For example, components of SNAREs, such as syntaxin1 and synaptotagmin, could be pulled down from rat brain membrane fraction by FLAG-tagged Pctaire1 (Fig. 4*B*). Synapsin I and p35 (a Cdk5 activator, which was demonstrated previously to interact with Pctaire1 *in vivo*) could also be pulled down by Pctaire1 (Fig. 4*B*) (4). The immunoprecipitations were specific because other synaptic vesicle proteins such as synaptophysin as well as SV2 (Fig. 4*B*) could not be detected in the co-immunoprecipitated complex. Because direct interaction between Pctaire1 and syntaxin1 was not detected in adult brain by *in vivo* co-immunoprecipitation (data not shown), it is possible that Pctaire1 interacts directly with

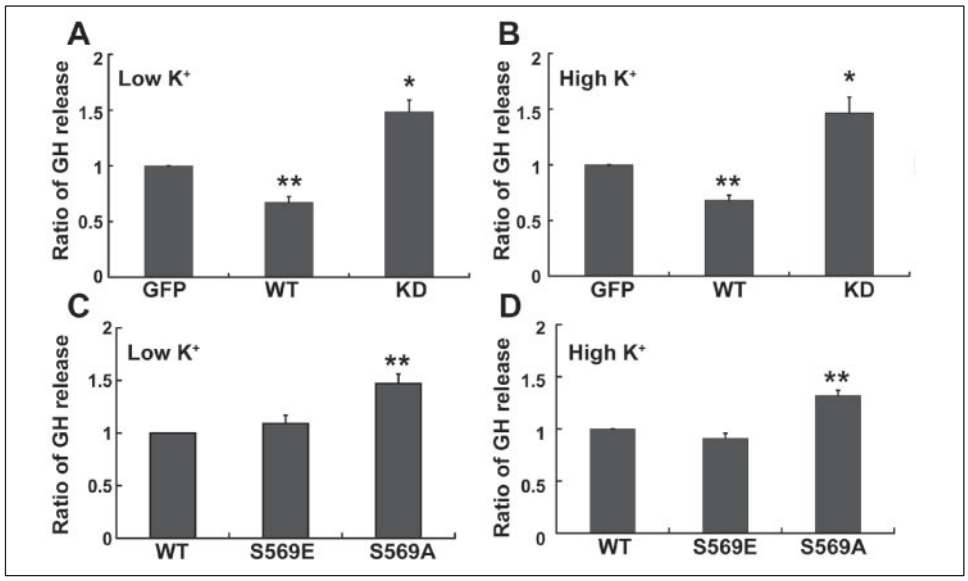

FIGURE 5. **Pctaire1 activity and phosphorylation of NSF regulated both low and high K^+-evoked hGH release in PC12 cells.** *A* and *B*, expression constructs encoding hGH and Pctaire1-WT or Pctaire-KD were transfected into PC12 cells. Culture medium and cell lysates were collected, and the hGH in culture medium was assayed by hGH enzyme-linked immunosorbent assay. The ratio of hGH release in PC12 cells expressing Pctaire1-WT or Pctaire1-KD was compared with that of green fluorescent protein (*GFP*) control under low K^+ (*A*) and high K^+ (*B*) conditions. The mean of five experiments is depicted, and *error bars* represent the S.E. **, $p < 0.01$; *, $p < 0.05$ compared with green fluorescent protein. *C* and *D*, PC12 cells were transfected with various NSF expression constructs (WT, S569A, or S569E). The ratio of hGH release in PC12 cells expressing S569A or S569E was compared with that of NSF wild type under both low K^+ (*C*) and high K^+ (*D*) conditions. The mean of five experiments is depicted and *error bars* represent the S.E. **, $p < 0.01$; *, $p < 0.05$, significantly different from that of NSF-WT.

NSF and co-immunoprecipitates with some of the SNARE proteins. Consistent with our previous observation that Pctaire1 activity can be regulated by Cdk5/p35, a majority of the synaptic proteins that interact with Pctaire1 (Fig. 4*B*) could also be pulled down by FLAG-tagged Cdk5. These results are consistent with the notion that Pctaire1 is potentially involved in membrane trafficking events.

Effect of NSF Phosphorylation by Pctaire1 on Ca^{2+}-dependent Exocytosis—NSF plays a critical role in the process of exocytosis. According to the NSF-SNAP-SNARE hypothesis, the hexameric NSF uses energy from ATP hydrolysis to dissociate SNARE complexes after membrane fusion, separating syntaxin, SNAP-25, and vesicle-associated membrane protein and allowing the individual SNARE proteins to be recycled for subsequent rounds of fusion (26). It is possible that Pctaire1 is a component of the NSF-SNAP-SNARE complex and plays a regulatory role in Ca^{2+}-dependent exocytosis through the phosphorylation of NSF. We therefore examined the effect of overexpressing Pctaire1 and phosphorylation-deficient NSF mutant on SNARE-mediated vesicle fusion using the hGH release assay. PC12 cells were transiently transfected with plasmid pXGH5 encoding hGH and different expression constructs. In transfected PC12 cells, hGH is concentrated in large dense core vesicles, and upon high K^+-induced depolarization, cells undergo Ca^{2+}-dependent exocytosis. The release of dense core vesicles in PC12 cells under basal and stimulated conditions was determined by measuring the amount of hGH upon exposure to low and high K^+ conditions, respectively. As shown in Fig. 5, *A* and *B*, inhibition of Pctaire1 activity by overexpression of Pctaire1-KD increased the hGH secretion significantly in both low and high K^+-stimulated conditions. Moreover, expression of NSF-S569A also significantly increased the hGH release in both conditions (low and high K^+) when compared with that of NSF-WT or the NSF-S569E (Fig. 5, *C* and *D*). Nonetheless, it is interesting to note that whereas overexpression of Pctaire1-WT significantly reduced hGH release (Fig. 5, *A* and *B*), overexpression of NSF-S569E had no effect on hGH secretion (Fig. 5, *C* and *D*). This finding suggests that Pctaire1 may also affect hGH release via a NSF-independent pathway. However, elevation in hGH secretion following overexpression of both Pctaire1-KD and NSF-S569A indicated that Pctaire1-mediated phosphorylation of NSF at least constitutes one of the mechanisms by which Pctaire1 regulates hGH secretion.

During the events of membrane fusion, the activity of NSF could be regulated by different mechanisms, including the reversible inactivation by *S*-nitrosylation and *N*-ethylmaleimide, extent of ATP hydrolysis, and association with SNAP-SNARE complex, as well as oligomerization and phosphorylation status (27–32). It has been reported that tyrosine phosphorylation of NSF can regulate its ATPase activity as well as its affinity for α-SNAP (32). Furthermore, phosphorylation of NSF by a serine/threonine kinase, protein kinase C, negatively regulates the function of NSF. Protein kinase C phosphorylates NSF on serine 237 in the catalytic D1 domain *in vitro*; accordingly, mutation of serine 237 to glutamic acid attenuates the binding of NSF to SNAP-SNARE complexes (27). In this study, we provide evidence that NSF can also be phosphorylated and regulated by another serine/threonine kinase, Pctaire1. Whereas phosphorylation of the D2 domain by Pctaire1 on serine 569 reduces the oligomerization of NSF, inhibition of Pctaire1 activity enhances the ability of NSF to self-associate. Based on the crystal structure of NSF, serine 569 is likely to be exposed, being located on the interface of NSF monomers in the hexameric complex (9). It is reasonable to speculate that phosphorylation of S569 could result in some conformational change that would affect the self-association of NSF. Although the precise effect of this phosphorylation on the conformation of NSF remains to be determined, findings from this study suggest that Pctaire1 affects the oligomerization of NSF through the phosphorylation of the D2 domain.

Pctaire1 activity can be regulated by Cdk5/p35, which is known to play a pivotal role in neurotransmission through the phosphorylation of various synaptic proteins (33–36). Although Cdk5 does not directly interact with or phosphorylate NSF *in vitro* (data not shown), Cdk5/p35 associates with Pctaire1 and regulates its activity. Our findings in the present study reveal that Pctaire1 not only interacts with NSF in adult rat brain but also with a number of synaptic proteins including components of the SNARE complex. It would therefore be interesting to examine whether Pctaire1 and Cdk5 exist as components of a large protein complex and together play an important role in phosphorylating a repertoire of protein substrates to ensure proper transmitter release from synaptic vesicles. The present study reveals a potential novel role of Pctaire1 phosphorylation in modulating NSF-mediated exocytosis. Recent studies of NSF function in regulated secretion suggest that NSF serves to prime synaptic vesicles and rearrange the SNARE complex for Ca^{2+}-triggered fusion after docking. This rearrangement is required to maintain the readily releasable pool of synaptic vesicles (37–39), whereas regulation of NSF phosphorylation by Pctaire1 might modulate

the regulated secretion. Further studies on Pctaire1 functions in neurotransmitter release might shed light on its roles in neurons during development.

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