Exocytosis, a critical process for neuronal communication and hormonal regulation, involves several distinct steps including MgATP-dependent priming (which involves the synthesis of phosphatidylinositol 4,5-bisphosphate). Type I phosphatidylinositol phosphate kinases (PIPKIs) were purified biochemically as a priming factor. PIPKI consists of three domains: the N-terminal region, the central kinase domain, and the C-terminal region. Three isoforms (α, β, and γ) of PIPKI have been identified, and each is alternatively spliced at the C-terminal region. In the present study, we conducted a structure/function analysis of PIPKIs in the priming of exocytosis, and we found that recombinant PIPKIα and PIPKIγ had priming activity. However, an unexpected finding of these results was that PIPKIβ did not prime exocytosis. The N- or C-terminal region of PIPKIα and PIPKIγ was not required for priming, which indicates that the central kinase domain is sufficient for this process. Alternative splicing in each isoform did not affect the isoform specificity in priming. Priming activity by isoforms is strongly correlated with their phosphatidylinositol phosphate kinase activity because PIPKIα and PIPKIγ had higher kinase activity than PIPKIβ. These results suggest that PIPKIα and PIPKIγ are the critical priming factors for exocytosis; it also suggests that the levels of phosphatidylinositol phosphate kinase activity in producing phosphatidylinositol 4,5-bisphosphate specify the function of PIPKI isoforms in priming.

Neurotransmitter exocytosis is regulated by many cytosolic and membrane proteins (1–3). A reconstituted exocytosis assay using permeabilized PC12 cells has proved very effective in the molecular dissection of exocytosis (4–14). This assay revealed two kinetically distinct processes: MgATP-dependent priming and Ca2+-dependent triggering (4). Purification of priming factors identified PIPKI1 and phosphatidylinositol transfer protein (6, 7). Ca2+-dependent activator protein for secretion was identified as a triggering factor (5). The recognition of a role for PIPKI and phosphatidylinositol transfer protein has led researchers to hypothesize that PIP2 generation by PIPKI and phosphatidylinositol transfer protein, along with membrane-associated phosphatidylinositol 4-kinase (15), is a key component of exocytosis.

Molecular cloning has identified three isoforms of PIPKI: α, β, and γ (16–18). Two different research groups, working independently, have simultaneously given the same isoforms different names (“PIPKIα” and “PIPKIβ”) (16, 17). In this article, we will follow the nomenclature of Loijens and Anderson (17). PIPKIγ is expressed primarily in the brain (18), where it is concentrated at the synapse of the neurons (19); PIPKIα and PIPKIβ, by contrast, are expressed ubiquitously (16, 17). The in vivo function of PIPKIγ has been investigated recently through the generation of PIPKIγ knock-out mice. The mice die after birth, and their vesicle trafficking at the synapse is severely disrupted (20).

Each PIPKI isoform is alternatively spliced at the C-terminal region (Refs. 16 and 17 and the present study). The splicing sequence (residues 636–661) in PIPKIγ is critical for binding to talin (21, 22), which is a component of focal adhesion plaques. It was found that only the longer isoform of PIPKIγ concentrates at focal adhesion points of the cell via a talin link, where it enhances the PIP kinase activity of PIPKIγ. What has not been examined, however, is whether this alternative splicing also regulates the priming activity of PIPKI.

Overexpression of PIPKI isoforms in COS7 cells results in massive actin polymerization (16, 23); this suggests that PIPKIs may also be involved in the regulation of actin cytoskeleton, in addition to their role in membrane trafficking. Overexpression of PIPKIα, but not of PIPKIβ, has effects on the endocytosis of the epidermal growth factor receptor (24). Similar isoform specificity may exist in PIPKI-mediated priming: Aikawa and Martin (25) found that the transfection of PC12 cells with PIPKIα or PIPKIγ, but not with PIPKIβ, reversed the ARF6 transfection-mediated inhibition of priming. However, the authors of that study could not draw a conclusion about this isoform specificity, given that they found that the expression level of transfected PIPKIβ in PC12 cells was lower than it was in other isoforms (25). It is unclear what determines these potential isoform specificities. In this article, we show that bacterially expressed recombinant PIPKIs exhibit priming activity. Using these recombinant PIPKIs, we have addressed the isoform specificity as well as the mechanism that underlies it. We also attempted to determine whether alternative splicing in PIPKIs affects their priming activity, and whether the central kinase domain of PIPKIs is sufficient for the priming of exocytosis.
EXPERIMENTAL PROCEDURES

PC12 Secretion Assay—PC12 cells were maintained in 10-cm dishes with 8 ml of Dulbecco’s modified Eagle’s medium containing 5% calf serum (HyClone), 5% horse serum (HyClone), and 100 units/ml penicillin and streptomycin (Sigma) at 37°C in 9.5% CO2. The secretion assay followed the protocols of previously published work (4). PC12 cells were labeled for 12–20 h with 4 μl of [3H]norepinephrine (NE; 56.4 Ci/mmol; PerkinElmer Life Sciences) in the presence of 0.5 mM ascorbic acid. After washing, the cells were harvested in K glu buffer (20 mM HEPES, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, and 2 mM EGTA) with 0.1% bovine serum albumin. Thirty-minute priming incubations at 30°C contained permeabilized PC12 cells, 2 mM MgATP, and recombinant proteins (or 1.0 mg/ml rat brain cytosol). The cells were recovered by centrifugation, washed with K glu buffer with 0.1% bovine serum albumin, and used for 5-min triggering incubations at 30°C that contained Ca2+ (1.72 mM free Ca2+ concentrations are estimated to be ~1–10 μM) and 0.5 mg/ml rat brain cytosol, which provides the Ca2+-dependent activator protein for secretion required for triggering (5).

Construction of Expression Plasmids—All the expression plasmids were generated using parental plasmid pGex-KG (9, 14). Expression plasmids for full-length PIPKIs and PIPKIa are pGex-mPIPKIa-1 (a long form of PIPKIa, made from IMAGE clone 4503697), pGex-mPIPKIa-8 (a short form of PIPKIa generated by PCR on pGex-mPIPKIa-1 based on the sequence information from IMAGE clones 30932032 and 30693858), pGex-mPIPKIb-3 (a short form of PIPKIb, made from IMAGE clone 3326897), pGex-PIPKIb-4 (a long form of PIPKIb, made from IMAGE clone 5289812), pGex-mPIPKIb-5 (a short form of PIPKIb, made from IMAGE clone 445967), pGex-hPIPKIb-5 (a long form of PIPKIb, made from clone KIAA 0659), and pGex-mPIPKI-1 (PIPKIa, made from IMAGE clone 3672732). IMAGE clone 445967 contained a mutation at residue 12 (S12A), which was corrected by a mutagenesis kit (Stratagene). IMAGE clones were purchased from Invitrogen. KIAA 0589 was a kind gift from Dr. Takahiro Nagase (Kazusa DNA Research Institute). Expression constructs for truncated PIPKIs include pGex-mPIPKIa-3 (encoding residues 1–439 of PIPKIa, C-terminal truncation), pGex-mPIPKIb-4 (residues 32–429 of PIPKIa, N- and C-terminal truncation), and pGex-mPIPKIb-2 (residues of 52–635 of PIPKIa, N-terminal truncation).

Expression and Purification of GST Fusion Proteins—Proteins were expressed in Origami B (DE3) strain (Novagen) or Origami B (DE3) harboring pG-TT2 (Takara) and purified with glutathione-agarose (Sigma, 9, 14). In some cases, we included MgATP washing. Proteins were eluted with an elution buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 1 mM EDTA, and 10 mM glutathione), and the eluted proteins were then concentrated by centrifugation with Nanosep 30 (Pall Gelman). Concentrated proteins (~0.2 mg/ml) were dialyzed with K glu buffer. In some cases, GST fusion proteins were cleaved by thrombin (Roche Applied Science) in a cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 2.5 mM CaCl2). Cleaved proteins were then processed for the secretion assay, similar to the eluted proteins.

Kinase Activity Assays—PKa kinase activity was measured in 50-μl reactions, performed for 11 min at room temperature in a final concentration of 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10 mM MgCl2, 80 μM phosphatidyl-inositol 4-phosphate (Sigma), 50 μM ATP, and 10 μCi of [γ-32P]ATP. The amounts of recombinant proteins used were 0.4 μg for GST and GST-PIPKIs. The reaction was stopped by the addition of 100 μl of 1 N HCl, and lipids were then extracted with 200 μl of chloroform/methanol (1:1). This was followed by further extraction with 80 μl of methanol/1 N HCl (1:1). The extracted lipids were separated using thin layer chromatography plates (Silica Gel 60; Merck) in chloroform/methanol/15 N NH4OH/distilled H2O (90:90:7:22), and the labeled products were detected by autoradiography (16, 17).

RESULTS

Recombinant PIPKIa Primes Exocytosis—First, we re-plated the two-stage assay using permeabilized PC12 cells (4) (Fig. 1). As previously described, incubation of the PC12 cell ghosts with brain cytosol and MgATP at the priming stage enhanced Ca2+-dependent neurotransmitter exocytosis at the triggering stage. Omission of either brain cytosol or MgATP during the priming stage resulted in poor Ca2+-dependent exocytosis during the triggering stage (Fig. 1). Incubation with brain cytosol plus MgATP at the priming stage resulted in an ~3-fold increase in exocytosis at triggering, compared with incubation with MgATP alone at the same stage (Fig. 1) (t10 = 11.3, p < 0.0001). This significant effect provided sufficient resolution to allow us to examine the effect of an individual priming factor.

We expressed full-length mouse PIPKIa as a GST fusion protein (GST-mPIPKIa-1) in Escherichia coli. The purified protein was eluted with glutathione and dialyzed with the same buffer used in the secretion assay (see “Experimental Procedures”) (Fig. 2A). We questioned whether the recombinant PIPKIa alone (i.e. without brain cytosol) could significantly prime exocytosis. Purified GST-mPIPKIa-1 and MgATP were introduced to permeabilized PC12 cells at the priming stage, and their effects on exocytosis at the triggering stage were examined. Recombinant PIPKIa exhibited dose-dependent priming activity and could prime up to 60% above the control (Fig. 2B), whereas purified GST had no effect. The dose required for priming was similar to the required dose of partially purified native PIPKI from erythrocytes (7), suggesting...
that recombinant PIPKIα and native PIPKI are similarly active in priming. We were able to conclude that PIPKIα is indeed an important factor for priming and that recombinant PIPKIs are useful tools for the study of the structure/function relationship of PIPKI in priming of neurotransmitter exocytosis.

**Isoform Specificity in Priming of Exocytosis and the Role of Alternative Splicing**—Molecular cloning revealed three isoforms of PIPKIs, and each isoform is alternatively spliced. In PIPKIγ, the C terminus is alternatively spliced (shown as D in Fig. 3A) (18), and this splicing regulates binding to the focal adhesion protein, talin (21, 22). This binding in turn augments the PIP kinase activity of PIPKIγ. The C terminus of PIPKIβ is similarly spliced (shown as C in Fig. 3A) (17). In contrast, human PIPKIα is alternatively spliced at the region in the immediate proximity of the central kinase domain (shown as A in Fig. 3A). We found a nearly identical splicing in mouse PIPKIα by searching the mouse expressed sequence tag database (Fig. 3B). In addition, we found a novel splicing site in human PIPKIβ (shown as B in Fig. 3A). We compared splicing site A in (human and mouse) PIPKIα and splicing site B in PIPKIβ by aligning the sequences of PIPKIα and PIPKIβ; we found that these two splicing sites exist at similar positions (Fig. 3B). Thus, the region in the immediate proximity of the central kinase domains of both PIPKIα and PIPKIβ is alternatively spliced by a similar mechanism; this finding suggests that there is a functional significance.

To examine isoform specificity in the priming of exocytosis and investigate the role of alternative splicing for this process, we generated a short (i.e. lacking splicing site A) form of PIPKIs, long (i.e. containing splicing site B) and short (i.e. lacking splicing site B) forms of PIPKIβ, and long (i.e. containing splicing site D) and short (i.e. lacking splicing site D) forms of PIPKIγ (Fig. 4, A and B). As a negative control, we generated recombinant PIPKIα (Fig. 4, A and B) because native PIPKII was previously shown to exhibit no priming activity (7). As expected, recombinant PIPKIα (GST-mPIPKIα-1) had no priming activity (Fig. 4C). In contrast, the short form of recombinant PIPKIs (GST-mPIPKIs-8) exhibited significant priming, as did the long form of PIPKIα (GST-mPIPKIα-1) (Fig. 4C). Furthermore, both the short (GST-mPIPKIγ-3) and long (GST-mPIPKIγ-5) forms of PIPKIγ exhibited similar priming. Unexpectedly, neither the short (GST-mPIPKIβ-3) nor long (GST-mPIPKIβ-4) form of PIPKIβ exhibited priming activity (Fig. 4C). Thus, alternative splicing in each isoform does not appear to affect the priming of exocytosis. Furthermore, PIPKIα and PIPKIγ, but not PIPKIβ, appear to be the priming factors for exocytosis.

**Isoform Specificity Is Determined by PIP Kinase Activity**—Our finding of unexpected isoform specificity in priming raised two possibilities. First, the generation of PIP2 alone may be an inadequate explanation for the priming actions of PIPKIs (assuming that they all have comparable PIP kinase activity). The second possibility is that differences in the degree of kinase activity among PIPKI isoforms may underlie the differences in priming. To further examine these two possibilities, we compared the kinase activity of the recombinant PIPKIs using phosphatidylinositol 4-phosphate as a substrate. Thin layer chromatography was used to separate the PIPKI phos-

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**Fig. 3. Analysis of alternative splicing in C-terminal regions of PIPKIs.** A, schematic representation of PIPKI isoforms. The sites of alternative splicing (labeled A–D) in C-terminal regions of PIPKIs are underlined. B, sequence alignment of splicing site A in PIPKIα and splicing site B in PIPKIβ. Sequences of human and mouse PIPKIα and PIPKIβ are aligned (h, human; m, mouse). Residues that are identical in at least three sequences are highlighted. The number of the last residue of each PIPKI sequence is indicated on the right. Alternative splicing site A found in human and mouse PIPKIα (Ref. 17 and this study) and splicing site B found in mouse PIPKIβ in this study are underlined.
phospholipid products. We found dramatic differences in PIP kinase activity among isoforms of PIPKI (Fig. 7). Both GST-mPIPKI/H9251 and GST-mPIPKI/H9253 exhibited strong kinase activity. In contrast, GST-mPIPKI/H9252 showed much lower kinase activity. Control experiments using GST alone did not show any kinase activity. These results indicate a strong correlation between PIP kinase activity and the priming of exocytosis; this suggests that the lack of priming activity by PIPKI/H9252 is due to its relatively poor kinase activity. Our results also support the hypothesis that PIP2 generation is the critical element for priming.

**DISCUSSION**

Two PIPKIs of 68 and 90 kDa were partially purified as priming factors for neurotransmitter exocytosis in permeabilized PC12 cells, with the 90-kDa protein having the major activity (7). Molecular cloning of PIPKI (16–18) followed this finding; it is worth recalling that priming by recombinant PIPKI has never been demonstrated. In addition, a structure/function analysis of PIPKIs has been unavailable. The present study has succeeded in demonstrating the priming activity of recombinant PIPKI\(\alpha\) and PIPKI\(\gamma\). Our results suggest that the partially purified 68-kDa protein corresponds to PIPKI\(\alpha\), whereas the 90-kDa protein corresponds to PIPKI\(\gamma\). The results also imply that the predominant activity of the 90-kDa PIPKI, in the course of purification from brain cytosol (7), is due to the fact that the presence of PIPKI\(\gamma\) in the brain is greater than that of PIPKI\(\alpha\), rather than the higher priming activity of PIPKI\(\gamma\). Furthermore, we have shown that the kinase domain of PIPKI\(\alpha\) (and probably PIPKI\(\gamma\)) alone is sufficient for priming. Unexpectedly, we found that PIPKI\(\beta\) had little or no priming activity, compared with PIPKI\(\alpha\). This result could be attributed to the lower PIP kinase activity of PIPKI\(\beta\), compared with PIPKI\(\alpha\) and PIPKI\(\gamma\) (Fig. 7).

Similar isoform specificity has been suggested in other func-
structure/function of PIPKI isoform in priming of exocytosis

...isoform specificity remains after the cleavage of GST. GST-cleaved PIPKια, but not GST-cleaved PIPKιβ, primed NE exocytosis. Error bars indicate S.E. (n = 8–10).

FIG. 6. Central kinase domain of PIPKια or PIPKιγ is sufficient for priming. A, the schematic representation of truncated proteins of PIPKια and PIPKιγ. B and C, for comparison, the truncated GST-PIP Kια and GST-PIP Kιγ proteins were tested with GST (negative control) and GST full-length PIPKια or PIPKιγ (positive control). Error bars indicate S.E. (n = 6–9).

...isoforms are alternatively spliced. We found a novel splicing site just in the immediate proximity of the central kinase domain in PIPKιβ, which is nearly identical to the site in PIPKια (Fig. 3). We tested the function of these splicing sites in PIPKια and PIPKιγ, as well as splicing in the C terminus of PIPKιγ, in the regulation of their priming activities. However, our permeabilized secretion assays did not reveal any such regulation by alternative splicing. This result supports our finding that the central kinase domain alone is sufficient to prime exocytosis. But this does not exclude the possibility that the splicing may be critical for secretion from intact cells.

Our results support the hypothesis that PIP2 generation is the critical step in the priming of exocytosis. The generated PIP2 may serve as the signal for neurotransmitter release by interacting with the potential Ca2+ sensors for exocytosis (such as synaptotagmin (9–12, 26) and Ca2+-activating proteins (5, 27)). The localization of generation of PIP2, however, remains to be determined. The original hypothesis was that PIP2 is generated at the vesicles where major phosphatidylinositol phosphate 4-kinase activity is observed (15). However, recent experiments using the GFP-PH (pleckstrin homology) domain, which specifically binds to PIP2, suggest that PIP2 is located mainly at the plasma membrane (25, 28). The hypothesis that PIP2 is generated at the plasma membrane is supported by the revelation that transfected PIPKι attaches to the plasma membrane (29). Our active recombinant PIPKι isoforms will be a useful tool in future investigations of the localization of PIP2 generation in permeabilized PC12 cells.

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