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Regulation of Munc-18/Syntaxin 1A Interaction by Cyclin-dependent Kinase 5 in Nerve Endings*

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The Munc-18-syntaxin 1A complex has been postulated to act as a negative control on the regulated exocytotic process because its formation blocks the interaction of syntaxin with vesicle SNARE proteins. However, the formation of this complex is simultaneously essential for the final stages of secretion as evidenced by the necessity of Munc-18's homologues in *Saccharomyces cerevisiae* (Sec1p), *Drosophila* (ROP), and *Caenorhabditis elegans* (Unc-18) for proper secretion in these organisms. As such, any event that regulates the interaction of these two proteins is important for the control of secretion. One candidate for such regulation is cyclin-dependent kinase 5 (Cdk5), a member of the Cdc2 family of cell division cycle kinases that has recently been copurified with Munc-18 from rat brain. The present study shows that Cdk5 bound to its neural specific activator p35 not only binds to Munc-18 but utilizes it as a substrate for phosphorylation. Furthermore, it is demonstrated that Munc-18 that has been phosphorylated by Cdk5 has a significantly reduced affinity for syntaxin 1A. Finally, it is shown that Cdk5 can also bind to syntaxin 1A and that a complex of Cdk5, p35, Munc-18, and syntaxin 1A can be fashioned in the absence of ATP and promptly disassembled upon the addition of ATP. These results suggest a model in which p35-activated Cdk5 becomes localized to the Munc-18-syntaxin 1A complex by its affinity for both proteins so that it may phosphorylate Munc-18 and thus permit the positive interaction of syntaxin 1A with upstream protein effectors of the secretory mechanism.

Many of the key proteins involved in membrane targeting and synaptic vesicle neurotransmitter release have been identified, and a fundamental set of interactions has been defined and placed into a model termed the SNARE hypothesis (1–4). Essential to the SNARE hypothesis is the idea that a complex of proteins is formed from soluble cytosolic proteins and from proteins integral to the synaptic vesicle (termed v-SNAREs) and target membranes (termed t-SNAREs). The soluble proteins include the ATPase *N*-ethylmaleimide-sensitive fusion protein (NSF)[1] and a family of proteins necessary for membrane attachment and activation of NSF termed SNAPs (soluble NSF attachment proteins). The SNAP receptors (*i.e.* the SNAREs) were identified as synaptic vesicle-associated membrane protein (VAMP, also termed synaptobrevin), and the plasma membrane proteins syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) (2, 5). In the SNARE hypothesis, the core of the vesicle docking interaction results from the interaction of v-SNAREs with cognate t-SNAREs via coiled-coil domains to create a protein complex that further recruits cytosolic factors α/β- and γ-SNAP and then NSF to form a 20 S complex. This assemblage of proteins is required for the donor and target membranes to come into close apposition and, upon ATP hydrolysis by NSF, become fusion-competent (4, 5). A specialization imposed on the SNARE hypothesis with regard to neurotransmitter and neurohormone release is the strict Ca^{2+}-dependence of release, which necessitates incorporation of a Ca^{2+} sensor into the fusion machine (1, 3).

Consistent with the SNARE hypothesis, proteins that regulate SNARE complex assembly in either a positive or negative fashion may substantially alter a cell's secretory response. The presence of regulatory proteins is suggested functionally both by the highly controlled nature of neurotransmitter release and by the description of multiple forms of synaptic plasticity that are of presynaptic cause (6). Proteins from two gene families have been identified as key regulators of SNARE complex assembly. These include members of the small GTP-binding family (*e.g.* Rabs) and of the SEC1 family (7, 8). The *SEC1* gene is one of 10 genes identified as essential for the final stages of protein secretion in the yeast, *Saccharomyces cerevisiae* (9). Sec1p protein has sequence similarity to three other yeast proteins, Sly1p, Vps33p (Slp1p), and Vps45p, which are also important for vesicle targeting and fusion (10). The mammalian homologue of yeast Sec1p was initially identified as a syntaxin-binding protein (Munc-18) (11) and independently isolated by two groups and designated n-Sec1 and rbSec1 (12, 13), although they represent identical genes. rbSec1 is expressed in two alternatively spliced isoforms termed rbSec1A and -B, although, no functional differences have been found (14). Recent reports have demonstrated the existence of at least five additional mammalian Munc-18 homologues, with several forms being expressed ubiquitously (15–19).

Sec1p interacts with yeast syntaxins (Sso1p, Sso2p) based on genetic interactions (20), and Munc-18 has been shown to interact with mammalian syntaxins (11–13, 21) particularly with syntaxin isoforms 1a, 2, and 3 but not with isoforms 4 or 5 (15, 21). Munc-18 has not, however, been found to be part of the 20 S SNARE/SNAP/NSF protein assemblage and was thus postulated to be a negative regulator of v- and t-SNARE protein interactions (7, 15, 21). Sec1p protein homologues have also

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[1] The abbreviations used are: NSF, *N*-ethylmaleimide-sensitive fusion protein; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; Cdk, cyclin-dependent kinase.

been reported to be important for vesicle trafficking. For example, Unc-18 (for uncoordinated) (22) and ROP (for Ras opposite) (23) represent neural enriched SEC1 homologues in *Caenorhabditis elegans* and *Drosophila*, respectively. Mutation in the *unc-18* gene in *C. elegans* caused abnormal accumulation of acetylcholine and resistance to acetylcholinesterase inhibitors (22, 24), while ROP overexpression in *Drosophila* reduced the number of spontaneous vesicle fusions in half and significantly decreased evoked responses in response to repetitive stimulation (25). A separate study has shown a *Drosophila* ROP mutant to result in reduced light-evoked synaptic responses (26).

Recent investigations have found that Munc-18, like SNAP-25 and syntaxin, is not restricted to the synaptic region but distributed throughout the axon and soma, suggesting the possibility of additional actions of Munc-18 protein (14). Consistent with this possibility, Munc-18 has recently been reported to interact with members of a family of double C2 domain proteins termed DOC2 (27) and also with cyclin-dependent kinase 5 (Cdk5; also termed PSSALRE and Nclk for neuronal Cdc2-like kinase), to which it can be tightly bound (28). Cdk5, found in adult neural tissue, phosphorylates neurofilament protein and microtubule-associated tau protein (29–33). Cdk5 is a proline-directed kinase that can bind cyclin D but in brain is often associated with and activated by a 35-kDa protein (34). Munc-18 possesses predicted consensus sequences for phosphorylation by protein kinase A, tyrosine kinase, protein kinase C, casein kinase II, and, interestingly, two sites for Cdk5 (12, 35). A recent *in vitro* study demonstrated that phosphorylation by protein kinase C of Munc-18 shifted its binding affinity for recombinant GST-syntaxin fusion protein (36).

The purpose of the present investigation was to define the interactions between Cdk5, Munc-18, and syntaxin that occur at nerve endings and to determine their regulation by Cdk5 kinase activity. Furthermore, we investigated whether Munc-18 is subject to phosphorylation within isolated mammalian secretory nerve endings.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Recombinant pGEX plasmid constructs encoding glutathione *S*-transferase fusion proteins, pGEX-kg-Munc-18 (rat), pGEX-syntaxin $1A_{11}$ (rat), and pGEX-2T-CDK5 (human) and pGEX-2T-P25 (bovine) were gifts of R. Scheller and J. Wang, respectively. The plasmids were individually transformed into *Escherichia coli* strain TG-1. The syntaxin construct used throughout was restricted to the cytoplasmic domain of syntaxin 1A. Antibodies used included a mouse monoclonal and rabbit polyclonal anti-Munc18 (Transduction Laboratories), a mouse monoclonal anti-syntaxin (HPC-1, Sigma), a rabbit polyclonal anti-Cdk5 directed against the carboxyl terminus (Santa Cruz Biotechnology Inc.), and a rabbit polyclonal COOH-terminally directed anti-p35 (Santa Cruz Biotechnology, Inc.). Protein kinase C purified from rat brain (α, β, γ mixture) was obtained from Upstate Biotechnology. $^{32}PO_4$ and [γ-^{32}P]ATP radioactive tracers were obtained from NEN Life Science Products. Olomoucine and iso-olomoucine were from L. C. Laboratories.

Expression and Purification of Recombinant Proteins—Recombinant GST fusion proteins were expressed in *E. coli* (TG-1) and subsequently purified by glutathione linked to Sepharose 4B (Pharmacia Biotech Inc.) as described (37, 38). Host bacteria were lysed using a French press at 1200 p.s.i. Removal of the GST moiety from the purified fusion protein, when necessary, was accomplished by thrombin (human, Sigma) treatment (0.2 NIH unit/μl, 4 h, 22 °C) in phosphate-buffered saline followed by the addition of phenylmethylsulfonyl fluoride (175 μg/ml) to inhibit thrombin action, centrifugation, and collection of the supernatant. Expressed fusion proteins were generally used immediately following purification for *in vitro* protein/protein interactions or protein phosphorylation studies. Induction of appropriate recombinant protein expression and its subsequent purification was verified by SDS-PAGE and Coomassie Blue staining or by Western blotting and probing with specific antibodies.

Determination of Protein Interactions in Vitro Using GST Fusion Protein—Protein interactions were determined by incubation of syntaxin 1A, Cdk5, p25, and Munc-18 fusion proteins (individually or combinations) with an individual GST fusion protein, which was immobilized on glutathione-Sepharose 4B beads. Proteins were incubated in protein binding buffer with end-to-end rotation for 1 h or overnight at 4 °C. Protein binding buffer contained 4 mM Hepes/NaOH (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 3.5 mM $CaCl_2$, 3.5 mM $MgCl_2$, and 0.5% Nonidet P-40. Following incubation, protein bound to the GST fusion protein was collected by centrifugation of the samples and washed three more times in binding buffer. Controls for each experiment consisted of analysis of fusion protein binding to GST protein alone immobilized on glutathione beads and elimination of binding through inclusion within the reaction of a 2–5-fold molar excess of a GST-free form of the fusion protein. In each case, protein bound on the beads and the final wash supernatant were dissolved in SDS-sample buffer, boiled and subjected to SDS-PAGE, and Western blotted onto nitrocellulose (0.2-μm Protran, Schleicher & Schuell), which was probed with different antibodies. ECL (Amersham Corp.) was performed according to the manufacturer's instructions, and chemiluminescence was quantitated with a GS-250 Molecular Imager (Bio-Rad) or visualized by x-ray film. The EC_{50} in saturation binding experiments was defined as half-maximal binding between two proteins with concentrations of the reactants kept below the point where significant nonspecific binding to GST immobilized on beads occurred. Quantitation of protein binding was based on pixel intensity obtained by phosphor imaging as above. To facilitate comparison between experimental determinations, data for each protein concentration were generally expressed as a percentage of the value observed upon binding saturation.

Analysis of Cdk5 Protein Kinase Activity—Determination and monitoring of Cdk5 kinase activity utilized either purified Cdk5 and p25 fusion proteins or native Cdk5 immunoprecipitated with polyclonal anti-Cdk5 and protein A-agarose beads (Pierce) from rat brain homogenate. The rat brain sample was prepared by homogenization of whole brain in 5 ml of lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40, 40 mM sodium pyrophosphate, 100 mM sodium fluoride, 175 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The homogenate was then left on ice for 30 min, after which it was centrifuged at 100,000 $\times$ *g* for 30 min. A 1-ml aliquot of the resulting supernatant was used for Cdk5 immunoprecipitation after preclearing with 60 μl of a 50% slurry of protein A-agarose beads. Protein kinase activity of aliquots of immunoprecipitated protein and Cdk5/p25 fusion protein (1 μM) were determined using a Cdc2 kinase assay kit (Upstate Biotechnology, Inc.) by following incorporation of ^{32}P radiotracer (0.025 μCi/μl, 3000 Ci/mmol) into a histone H1 peptide (22 μM) from bovine calf thymus containing predicted Cdc2 phosphorylation sites. Incubation of reactants was carried out for 15 min at 30 °C. Labeling was quantitated following reaction termination via binding of the histone substrate to phosphocellulose P81 paper, which was then extensively rinsed in 0.75% phosphoric acid and liquid scintillation-counted. Background controls included no added kinase. Specificity of tracer incorporation to Cdk5 activity was tested by inclusion of protein kinase C inhibitor peptide (RFARKGALRQKNV; 5 μM), protein kinase A inhibitor peptide (TYADFIASGRTGRRNAI; 0.5 μM), and a calmodulin-dependent protein kinase inhibitor (R24571; 5 μM) in the reaction mixture. In addition, kinase activity was tested for sensitivity to the highly specific Cdk5 inhibitor olomoucine (25 μM) or the much less active analog iso-olomoucine (25 μM). Labeled phosphate incorporation into Munc-18 fusion protein by both immunoprecipitated Cdk5 and purified Cdk5/p25 kinase activity was also determined by subjecting reaction samples to SDS-PAGE followed by autoradiography.

Modulation of Protein Interactions by Protein Kinase-mediated Phosphorylation—GST-Munc-18 purified from bacterial lysates by immobilization onto glutathione-Sepharose 4B beads was eluted off the beads in buffer containing 10 mM reduced glutathione and 50 mM Tris-HCl (pH 8.0) at room temperature for 10 min. The eluate was then dialyzed (Slide-a-lyzer, Pierce, 10-kDa cut-off) overnight at 4 °C against buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 6.25 mM β-glycerol phosphate, 10 mM $MgCl_2$, 100 μM $CaCl_2$. The sample was recovered, and protein concentration was determined (Bio-Rad protein assay). GST-Munc-18 was then phosphorylated with either protein kinase C (0.42 μg/ml) or Cdk5 immunoprecipitated from rat brain according to the manufacturer's instructions (Upstate Biotechnology). Protein kinase C reactions included Ca^{2+} (1 mM), phosphatidylserine (83.3 μg/ml), and diglyceride (8.3 μg/ml) activators along with a protein kinase A inhibitor peptide (R24571, 3.3 μM). Phosphate incorporation was determined by inclusion of [γ-^{32}P]ATP radiotracer (0.017–0.025 μCi/μl, 3000 Ci/mmol) during the kinase reaction, which was followed by SDS-PAGE, extraction of the Munc-18 protein band from the gel, and liquid scintillation counting of each sample. Analysis of effects of Munc-18 phos-

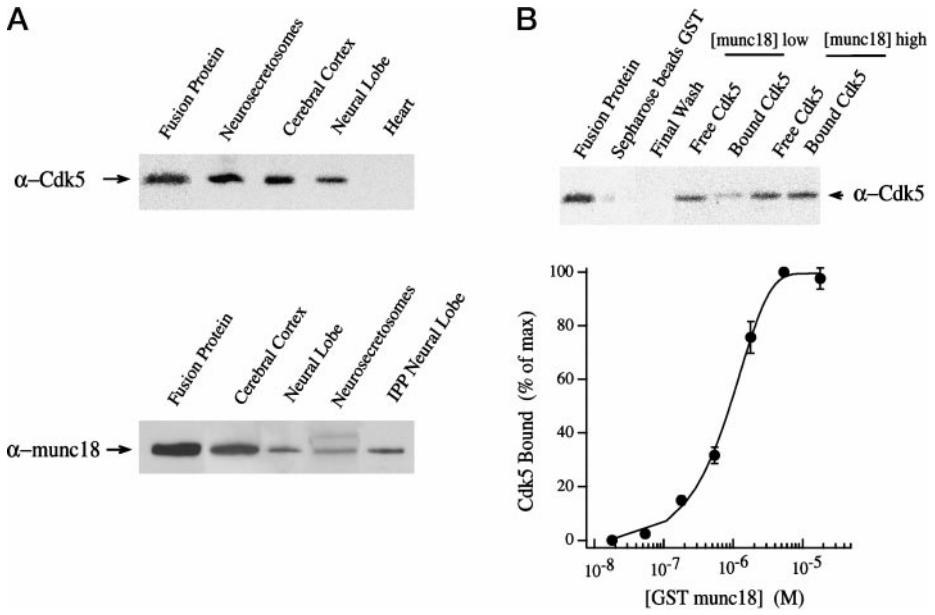

FIG. 1. **Distribution of Cdk5 and Munc-18 to neuroendocrine nerve endings and Cdk5 interaction with GST-Munc-18.** *A*, immunoblot following SDS-PAGE probed with polyclonal antibody against Cdk5 (*top*) and Munc-18 (*bottom*), demonstrating the presence of Cdk5 and Munc-18 immunoreactivity in rat cerebral cortex, neural lobe, and isolated peptidergic neural lobe nerve endings, *i.e.* neurosecretosomes (0.5 μg of protein/lane). Immunoreactivity comigrated on SDS-PAGE with appropriate bacterially expressed GST fusion proteins (Cdk5 or Munc-18), which had been thrombin-treated to release the GST moiety. Polyclonal antisera against Munc-18 incubated with neural lobe lysate immunoprecipitated protein (*IPP Neural Lobe*) immunoreactive to monoclonal Munc-18 antibody. *B*, GST-Munc-18 binding of Cdk5 in neural lobe lysates. Increasing concentrations of recombinant GST-Munc-18 linked to glutathione-Sepharose beads were incubated with aliquots of neural lobe lysate and then collected and extensively washed. Bound Cdk5 was detected folowing SDS-PAGE by Western blotting and reaction with a horseradish peroxidase-linked secondary antibody, and chemiluminescence was quantitated by a PhosphorImager (Bio-Rad) (mean ± S.E., $n = 5$). *Upper displays* representative raw data at both low (0.185 μM) and high (1.85 μM) Munc-18 concentrations and demonstrate a lack of immunoreactivity in either a glutathione-Sepharose beads-GST sample or a final wash of low concentration GST-Munc-18 pellet.

phate incorporation on binding to syntaxin 1A were performed using nonradioactive ATP and by rebinding phosphorylated Munc-18 to glutathione-Sepharose 4B beads (1 h, room temperature) following the kinase reaction. The glutathione-Sepharose-linked GST-Munc-18 was then centrifuged and washed extensively with protein binding buffer. The phosphorylated Munc-18 fusion protein at a concentration of 200 nM was next incubated with increasing concentration of syntaxin $1A_{11}$ fusion protein with rotational agitation for 30 min at 4 °C in protein binding buffer. Following incubation, samples were centrifuged, pellets were extensively washed, and protein was bound to beads solubilized in SDS-sample buffer and subjected to SDS-PAGE and Western blotted. Blots were probed with monoclonal anti-syntaxin followed with a horseradish peroxidase-linked secondary antibody. Immunoreactive syntaxin bound to GST-Munc-18 was visualized with ECL and quantitated by phosphor imaging analysis. Nonphosphorylated Munc-18 incubated with syntaxin 1A served as control. GST alone was not phosphorylated by the above kinases, and no Cdk5 phosphorylation consensus sequences occur in syntaxin $1A_{11}$. Over the concentration range examined, no binding of GST to syntaxin $1A_{11}$ was observed.

RESULTS

Cyclin-dependent Kinase 5 Interaction with Munc-18—Cyclin-dependent kinase 5- and Munc-18 protein-specific antibodies were used on immunoblots to demonstrate localization of the respective proteins within lysates of the mammalian neurohypophysis (*i.e.* neural lobe). Immunoreactivity within the lysates was found to comigrate with immunoreactivity to the respective recombinant bacterially expressed GST fusion proteins for Cdk5 or Munc-18 that had been thrombin-digested to release the GST moiety (Fig. 1*A*). Further localization of Cdk5 and Munc-18 to nerve endings was demonstrated by their continued presence in a preparation of purified isolated nerve endings (>90% peptidergic nerve endings) termed neurosecretosomes. Both Munc-18 and Cdk5 antibody were also capable of immunoprecipitating the respective proteins from neural lobe homogenates.

To evaluate a potential interaction between Munc-18 and Cdk5, we next utilized GST-Munc-18 fusion protein attached to glutathione-Sepharose 4B beads in an *in vitro* binding assay that included neural lobe lysate. Incubation of the GST-Munc-18 bead complex at increasing concentration with the nerve ending lysate was followed by collection and extensive washing of the pellet to remove unbound protein and then elution and analysis by Western blotting of bound Cdk5. Binding of Cdk5 was found to be saturable with a 50% effective concentration (EC_{50}) of 0.89 μM, as determined by analysis of quantitative phosphor imaging of immunoreactivity (Fig. 1*B*). Specificity of binding was demonstrated by lack of binding of Cdk5 to GST protein adhered to glutathione-Sepharose 4B when used at a concentration corresponding to the highest used for GST-Munc-18 protein. Carryover of free Cdk5 was unlikely, since analysis of the final wash supernatants exhibited no detectable Cdk5 immunoreactivity. Analysis of the amount of Cdk5 remaining within the supernatant following incubation demonstrated that even at saturating concentrations of GST-Munc-18, at most only approximately 50% of the total Cdk5 immunoreactivity within a sample was bound.

Catalytically active Cdk5 purified from bovine brain extracts exists as a heterodimer of Cdk5 and a regulatory and neural specific subunit termed p25 (34, 39). Subsequent reports on a bovine cDNA clone for the 25-kDa Cdk5 activator protein have revealed that it is derived from a larger 35-kDa protein precursor, suggesting that the native form of the heterodimer consists of Cdk5 and a 35-kDa protein, although the p25 fragment was found fully competent for Cdk5 activation (29, 34). We therefore attempted to determine if the Cdk5 bound to the GST-Munc-18 fusion protein following incubation with the neural lobe lysate also contained the p35/p25 activator protein. Analysis of immunoblots demonstrated both Cdk5 and p35 immunoreactivity in washed glutathione-Sepharose 4B GST-Munc-18 pellets that had previously been incubated with the nerve ending lysate (Fig. 2*A*). In additional experiments, Cdk5 was directly immunoprecipitated from neural lobe lysate and

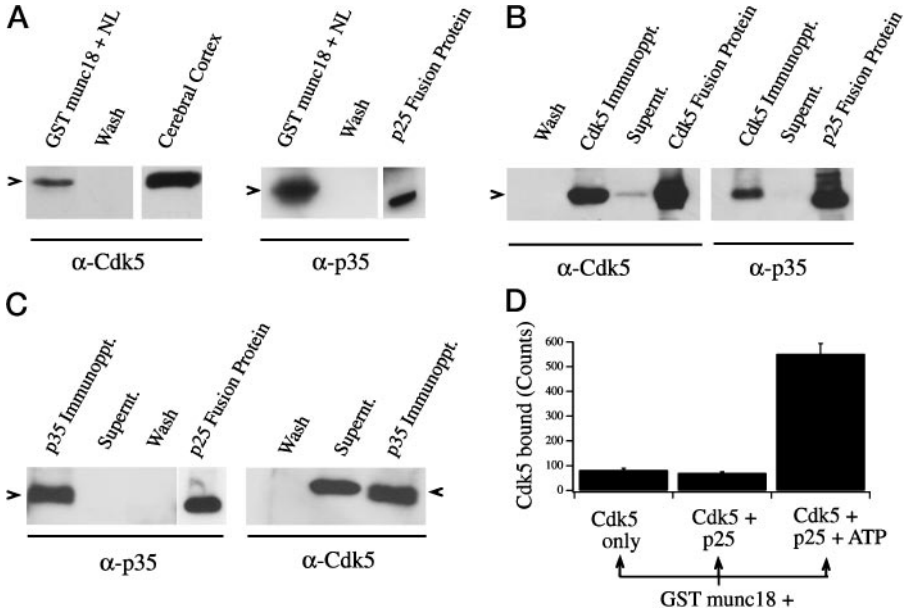

FIG. 2. **Cdk5 interaction with p35 and binding to Munc-18.** *A*, GST-Munc-18 (2 μM) immobilized on glutathione-Sepharose beads was incubated (1 h at 4 °C) with lysate aliquots prepared from neural lobe and then collected and extensively washed. Bound Cdk5 and p35 protein were visualized by SDS-PAGE and immunoblotting. Rat cerebral cortex and bacterially expressed p25 fusion protein were electrophoresed as co-migration controls. Antibodies (polyclonal α-Cdk5 and α-p35) bound were detected using a horseradish peroxidase-linked secondary antibody and chemiluminescent development. *B* and *C*, immunoprecipitation of Cdk5 or p35 from neural lobe lysate co-precipitates p35 or Cdk5 immunoreactivity, respectively. Supernatant (*supernt.*) and wash fractions verify immunoprecipitation and wash efficiency. Immunoprecipitation used polyclonal Cdk5 or p35 antibody and protein A-Sepharose. Precipitated protein was visualized by SDS-PAGE and immunoblotting. *D*, ATP enhanced binding of Cdk5-p25 fusion proteins to GST-Munc-18. Glutathione-Sepharose-immobilized GST-Munc-18 (0.25 μM) was incubated (1 h at 4 °C) with Cdk5 (0.25 μM) with or without p25 (0.25 μM) fusion protein and with or without ATP (1 mM) and subsequently collected, washed, subjected to SDS-PAGE, and Western blotted. CDK5 bound was determined by probing with anti-CDK5, and immunoreactivity was quantitated ($n = 3$) as above. *Arrowheads* indicate migration position of 33–35-kDa protein standards. All data are representative of at least three determinations.

washed extensively, and the precipitate and final wash were analyzed for p35 immunoreactivity. Fig. 2*B* demonstrates the presence of p35 along with Cdk5 in the precipitate, but not the final wash, demonstrating that a Cdk5 and p35 interaction was present within the nerve ending lysate. Complementary experiments in which p35 was immunoprecipitated from the neural lobe lysate and the precipitate was probed for Cdk5 and p35 immunoreactivity resulted in a similar conclusion (Fig. 2*C*).

Comparison of the Cdk5 with the p35 immunoprecipitation data demonstrated a heterodimer complex of p35 and Cdk5 proteins with p35 being of limited abundance. Further studies were therefore performed to determine if formation of a heterodimer protein complex was required prior to interaction of Cdk5 with Munc-18. Initial experiments found that preincubation of lysate of the neural lobe with recombinant p25 (3 μM for 1 h at 4 °C) increased by 33% from control (*i.e.* no added p25) the binding during a subsequent incubation (1 h at 4 °C) of lysate Cdk5 to GST-Munc-18 (10 μM). These data indicate that the level of Cdk5 bound to Munc-18 was, in part, determined by available p25 and its interaction with Cdk5. No direct interaction between recombinant p25 and Munc-18 proteins has been found (see below). We next investigated whether the enhanced Cdk5/GST-Munc-18 interaction was a direct result of p25 interaction with Cdk5 or resulted from p25 activation of kinase activity and was therefore ATP-dependent. To test these possibilities, the effects of p25 fusion protein (0.25 μM; soluble p35 has not been expressed successfully in bacteria) and ATP on the binding interaction between Cdk5 (0.25 μM) and GST-Munc-18 (0.25 μM) fusion proteins *in vitro* were determined. As shown in Fig. 2*D*, the addition of recombinant p25 resulted in no significant enhancement of the interaction between Cdk5 and GST-Munc-18. In comparison, the addition of ATP (1 mM) together with the recombinant p25 protein resulted in a significant ($p < 0.05$, $n = 6$) enhancement of Cdk5 bound to the washed glutathione-Sepharose 4B-linked GST-Munc-18 compared with the Cdk5 alone control. These data are consistent with an increase in the level of interaction resulting from conditions that support formation of the active heterodimer kinase (*i.e.* the presence of p25 or p35 and ATP) and increased substrate turnover. Thus, the increased binding of Cdk5 to Munc-18 in the neural lobe lysate supplemented with recombinant p25 probably resulted from an increased formation of heterotrimer complex and the presence of ATP in the lysate.

Cyclin-dependent Kinase 5 Phosphorylation of Munc-18—Munc-18 possess consensus phosphorylation sequences for a variety of protein kinases including two (Ser^{158}, Thr^{574}) for the proline-directed kinase Cdk5. Cdk5 immunoprecipitated from nerve ending lysates demonstrated enzymatic activity as measured by labeled phosphate incorporation into a histone peptide (Fig. 3*A*). Phosphorylation activity was inhibited by 73% (mean, $n = 3$) by the specific Cdk5 inhibitor olomoucine (25 μM; reported $IC_{50} = 3$ μM (39)). A less active olomoucine analog, iso-olomoucine ($IC_{50} > 1$ mM) was found to have limited inhibitory effects (~15%) on the immunoprecipitated kinase activity. Protein A-agarose beads alone following incubation with the neural lobe lysate served as control samples and demonstrated low kinase activity of nonspecifically adhered proteins.

Of particular interest is whether Munc-18 may serve as a phosphorylation substrate for Cdk5 and if a phosphorylation event may serve to regulate the strength of the interaction between Munc-18 and syntaxin. To evaluate these possibilities, we incubated GST-Munc-18 fusion protein with Cdk5 immunoprecipitated from a neural lobe lysate in the presence of [γ-^{32}P]ATP. Subsequent gel electrophoretic separation and autoradiography demonstrated radiotracer incorporation into Munc-18 protein (Fig. 3*B*). Labeled phosphate incorporation into Munc-18 was greatly reduced by inclusion of 50 μM olomoucine during the reaction but not of 50 μM iso-olomoucine.

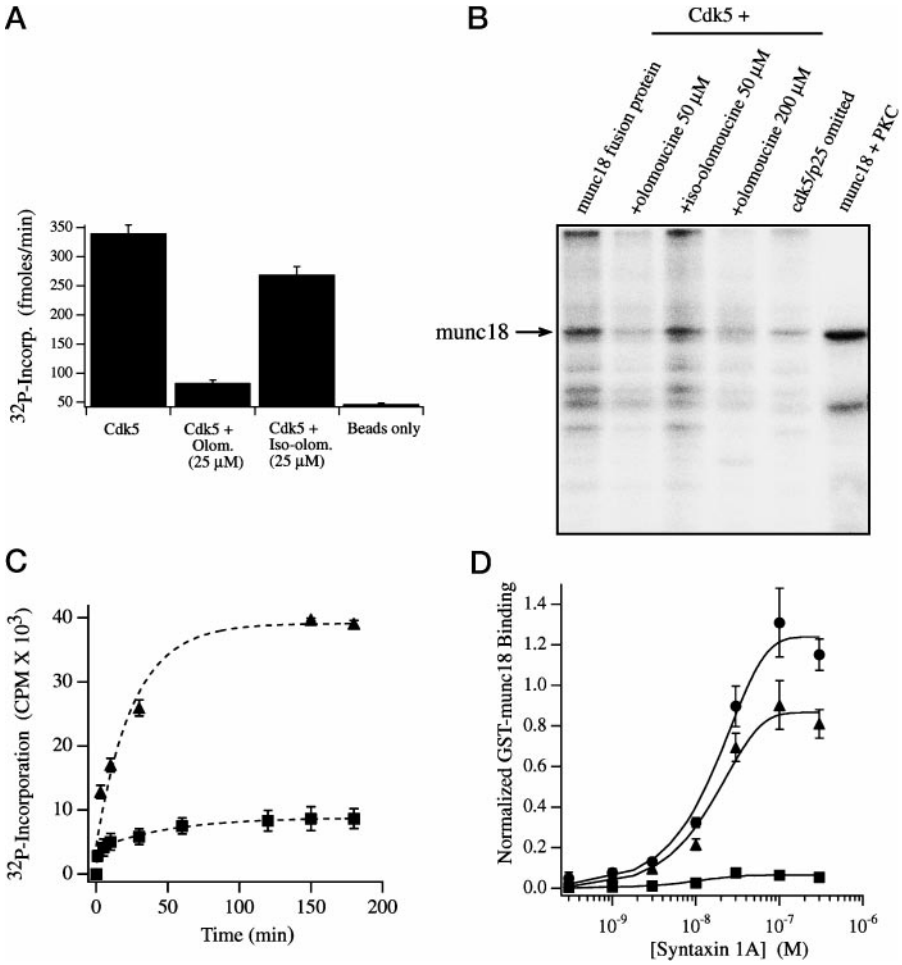

FIG. 3. **Cdk5-mediated phosphorylation of Munc-18 inhibits interaction of Munc-18 with syntaxin.** *A*, Cdk5 catalytic activity immunoprecipitated from neural lobe lysates and its inhibition by olomoucine (25 μM), a specific Cdk5 inhibitor. Immunoprecipitation used a Cdk5-specific polyclonal antibody and protein A-Sepharose. Catalytic activity of precipitated Cdk5 aliquots and of Sepharose A bead controls that had similarly been incubated with neural lobe lysate were determined in reaction buffer by measuring ^{32}P incorporation from [γ-^{32}P]ATP into a histone H1 substrate peptide. The histone H1 peptide was subsequently bound to phosphocellulose P81 paper, extensively rinsed, and counted (see "Experimental Procedures"). Iso-olomoucine (25 μM) was used to confirm pharmacological specificity of olomoucine action to Cdk5 inhibition. Data shown represent the mean ± S.E., $n = 9$). *B*, effect of immunoprecipitated Cdk5 on phosphorylation of Munc-18. Munc-18 fusion protein (0.5 μM) was incubated in reaction buffer for 15 min in the presence of Cdk5 and [γ-^{32}P]ATP (0.9 μCi, 3000 Ci/mmol). Olomoucine or iso-olomoucine were added during the reaction as indicated. Following incubation, samples were subjected to SDS-PAGE, electroblotted onto nitrocellulose, and autoradiographed using a high intensity phosphor imaging screen. A sample excluding Cdk5 from the reaction solution was included as control. The last lane shows comigration of Cdk5 radiolabeled product with Munc-18 fusion protein phosphorylated *in vitro* by purified protein kinase C. *C*, comparison of the time course and extent of protein kinase C (*filled triangles*) and Cdk5 (*filled squares*) phosphorylation of GST-Munc-18 (50 nM) *in vitro*. Kinase-mediated phosphorylation reactions were carried out (see "Experimental Procedures") at 30 °C on 1 μM GST-Munc-18 in the presence of [γ-^{32}P]ATP. Samples were then subjected to SDS-PAGE and Coomassie-stained, and the band corresponding to the Munc-18 protein was cut from the gel and subjected to scintillation counting. *Dashed lines* represent least squares exponential fits to the data ($n = 3$). *D*, effect of protein kinase C and Cdk5 phosphorylation of GST-Munc-18 (200 nM) on interaction with syntaxin 1A fusion protein. Protein kinase C (*filled triangles*) and Cdk5 (*filled squares*) phosphorylation reactions were run for 180 min under conditions shown in *C* prior rebinding to glutathione-Sepharose beads, washing, and initiation of binding interactions with syntaxin 1A. Control (*filled circles*) samples were treated identically but without pretreatment with a protein kinase. Following incubation in binding buffer (1 h), samples were collected, washed, subjected to SDS-PAGE, and Western blotted. Blots were probed with anti-syntaxin and horseradish peroxidase-linked secondary antibody, and chemiluminescence was quantitated by phosphor imaging (mean ± S.E., $n = 3$). Data were normalized with respect to the Munc-18 binding observed at 1 μM syntaxin 1A under control conditions.

Olomoucine used at a 4-fold higher concentration resulted in no further decrease in labeling than 50 μM olomoucine or than that seen under control incubations with [γ-^{32}P]ATP but lacking the immunoprecipitated Cdk5. The Cdk5-phospholabeled Munc-18 co-migrated with ^{32}P-Munc-18 labeled *in vitro* using the catalytic subunit of purified protein kinase C. A further series of experiments compared the effects of Cdk5 and protein kinase C phosphorylation of GST-Munc-18 on a subsequent *in vitro* binding interaction with recombinant syntaxin. Fig. 3*C* compares the time course and levels of ^{32}P incorporation into Munc-18 (1 μM) for reactions containing [γ-^{32}P]ATP with immunoprecipitated Cdk5 or purified protein kinase C. An approximately 4-fold greater level of ^{32}P incorporation was observed in the protein kinase C reactions, with the stoichiometry (mol of phosphate/mol of Munc-18) being 0.64 ± 0.09 and 2.85 ± 0.02 (mean ± S.E., $n = 3$) for Cdk5 and protein kinase C phosphorylation, respectively. All phosphorylation reactions were run for 180 min to assure complete phosphorylation of Munc-18 prior to initiation of binding reactions. As shown in Fig. 3*D*, phosphorylation of GST-Munc-18 by immunoprecipitated Cdk5 strongly reduced the interaction between the syntaxin and GST-Munc-18 proteins compared with that observed under control conditions (no added kinase activity) or to phosphorylation by protein kinase C. The GST moiety was found not to be subject to phosphorylation by native (*i.e.* immunoprecipitated) or recombinant p25-activated Cdk5 kinase activity.

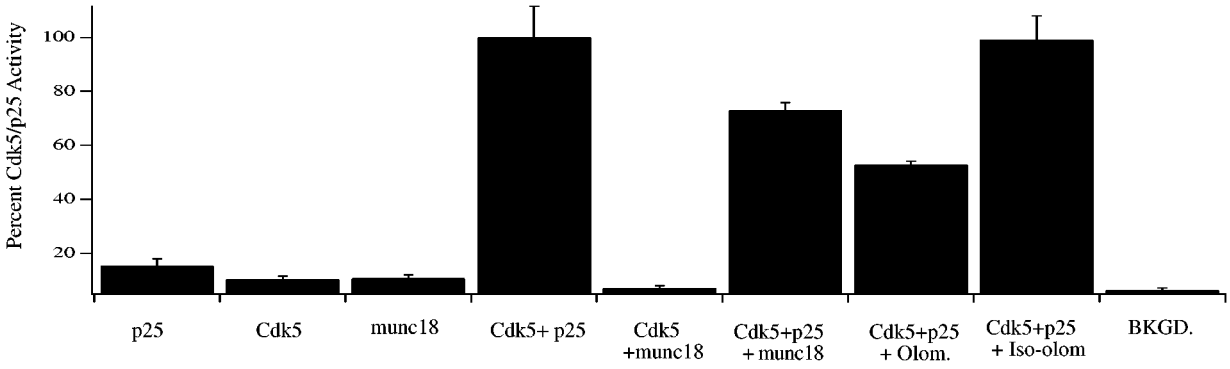

FIG. 4. **Effect of recombinant p25 and Munc-18 on catalytic activity of Cdk5 fusion protein.** Kinase activity was determined in reaction buffer by measuring ^{32}P incorporation from [γ-^{32}P]ATP into Cdk5 histone H1 substrate peptide (mean ± S.E., $n = 3$) as described under "Experimental Procedures." Protein components (p25, 1 μM; Cdk5, 1 μM; Munc-18, 1 μM) were added to the reaction buffer as indicated as were olomoucine (25 μM) and iso-olomoucine (25 μM). Background (*BKGD.*) represents no added protein.

Although a prior report has indicated an activation of Cdk5 purified from bovine brain by Munc-18 (28), we found no activation of recombinant Cdk5 kinase activity, measured by phosphate incorporation into the histone substrate, by recombinant Munc-18 (Fig. 4). Recombinant Cdk5, p25, or Munc-18 purified protein fractions alone gave only slight kinase activity above background controls, probably resulting from nonspecific kinase carry-over during purification. In contrast, a mixture of recombinant Cdk5 and p25 proteins resulted in a considerable enhancement (>5-fold) of kinase activity that was inhibited by approximately 50% by olomoucine but not inhibited by iso-olomoucine, as monitored by labeling of the histone peptide substrate. The addition of Munc-18 to Cdk5 or to a Cdk5-p25 combination failed to stimulate Cdk5 kinase activity.

In Situ Munc-18 Phosphorylation—While kinase-mediated phosphorylation of Munc-18 decreases its interaction with syntaxin, it has not yet been shown that Munc-18 exists as a phosphoprotein *in situ*. To determine if Munc-18 exists as a phosphoprotein *in situ*, we compared the pattern of Munc-18 immunoreactivity for control and alkaline phosphatase-treated brain Munc-18 immunoprecipitates on blots following two-dimensional gel electrophoresis (isoelectric and SDS-PAGE). As shown in Fig. 5, multiple Munc-18 immunoreactive spots migrating at 67 kDa, but which differed in isoelectric point, were observed in control immunoprecipitates. Importantly, dephosphorylation of the immunoprecipitated protein by alkaline phosphatase prior to two-dimensional gel electrophoresis resulted in a loss of reactivity of two spots at the acidic end concomitant with an increase of Munc-18 immunoreactivity toward the basic end of the isoelectric gel, thereby demonstrating that a proportion of Munc-18 *in situ* exists as phosphoprotein. Specificity of the immunoreactive signal on the blots for Munc-18 was demonstrated by a complete loss of immunoreactive signal upon incubation of the Munc-18 primary antibody with excess Munc-18 fusion protein prior to probe of the blots.

Syntaxin Interaction with Cyclin-dependent Kinase 5 and Munc-18—We next examined whether syntaxin, which has been reported to interact with a number of the proteins of the exocytotic mechanism including Munc-18 (21), was capable of interacting with Cdk5. Initial experiments evaluated binding using GST-syntaxin 1A attached to glutathione-Sepharose 4B beads and incubated with cytosol from neural lobe. Analysis of Cdk5 immunoreactivity within the pelleted GST-syntaxin and its comparison with GST protein run as control demonstrated that syntaxin exhibited a specific interaction with cellular Cdk5 (Fig. 6*A*). Further analysis aimed at defining the properties of the binding interaction were performed *in vitro* using a constant concentration of GST-Cdk5 fusion protein (200 nM)

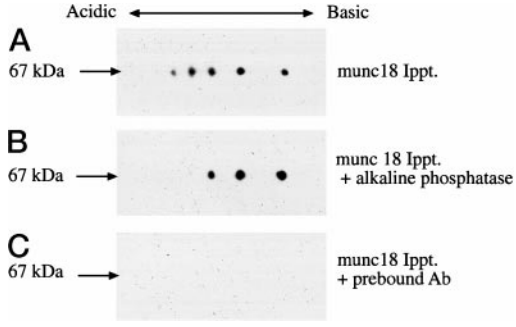

FIG. 5. **Evaluation of phosphorylation state of *in situ* Munc-18.** Comparison of immunoreactive patterns for Munc-18 immunoprecipitated from a high speed supernatant (100,000 × *g*, 30 min, 4 °C) of rat brain lysate (4.2 mg/ml protein) and subjected to isoelectric focusing, SDS-PAGE separation, and Western blotting. *A*, control pattern of Munc-18 immunoreactivity; *B*, immunoreactive pattern when immunoprecipitated Munc-18 was treated with alkaline phosphatase (0.27 unit/μl, 3 h, 37 °C) prior to PAGE separation. *C*, loss of immunoreactivity by treatment of anti-Munc-18 antibody with Munc-18 fusion protein prior to probing the blot. The data shown are representative of three separate experiments.

that was incubated with increasing concentration of syntaxin 1A fusion protein. Binding of syntaxin to GST-Cdk5 was found to be saturable with an EC_{50} value of 0.53 μM ($n = 5$, Fig. 6*B*). The addition of p25 fusion protein (200 nM) to the binding reaction was found to be without significant ($p > 0.05$, $n = 3$) effect on the GST-Cdk5 interaction with syntaxin (*open symbol* in Fig. 6*B*), although the presence of ATP (2 mM) resulted in a 2-fold increase of syntaxin bound (118,183 ± 15,777 counts for control *versus* 208,826 ± 22,002 counts with ATP, $n = 3$).

Formation and Disassembly of a Cdk5-p25, Munc-18, Syntaxin Protein Complex—Based on our results demonstrating that Munc-18 can interact with Cdk5 and with syntaxin and that Cdk5 can itself interact with syntaxin, we attempted to determine if these proteins might associate *in vitro* into a stable heteroligomeric protein complex. An initial series of experiments was performed using GST-p25 fusion protein linked to glutathione-Sepharose 4B beads that was incubated in a sequential manner with each of the proteins forming the putative protein complex. Following incubation of GST-p25 with each individual fusion protein, the mixture was centrifuged and extensively washed prior to incubation with the next fusion protein. Immunoblot analysis of the pellets and final washes from each reaction demonstrated that each additional protein component was present in the pellet but not in the final wash following its incubation reaction (Fig. 7*A*). The data also demonstrate that GST-p25 fails to directly interact with

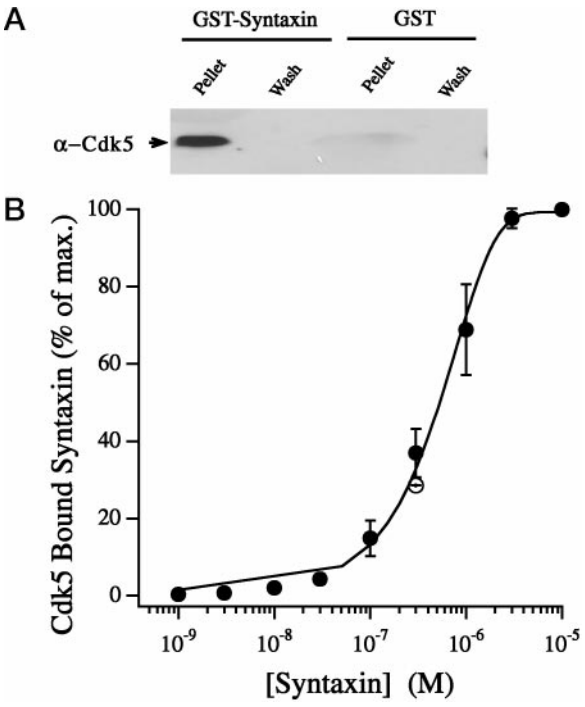

FIG. 6. **Syntaxin binding of Cdk5.** *A*, immunoblot of glutathione-Sepharose-immobilized GST-syntaxin 1A fractions following incubation (1 h at 4 °C) with aliquots of precleared rat brain soluble protein fraction and probed with anti-Cdk5 antibody. Immobilized Cdk5 protein (pellet) and that remaining in the final wash were evaluated and compared with an identical procedure performed using immobilized GST protein as control for nonspecific binding. *B*, GST-Cdk5 binding of syntaxin 1A fusion protein. Increasing concentrations of recombinant syntaxin 1A were incubated (1 h at 4 °C) with a fixed concentration (200 nM) of GST-Cdk5 linked to glutathione-Sepharose beads and then collected and extensively washed. Bound syntaxin 1A was detected following SDS-PAGE by Western blotting and reaction with a horseradish peroxidase-linked secondary antibody, and chemiluminescence was quantitated by phosphor imaging (mean ± S.E., $n = 5$). Control reactions with glutathione-Sepharose-linked GST (200 nM) alone exhibited no detectable syntaxin 1A binding.

Munc-18 and that the addition of Munc-18 in this experiment results via its interaction with Cdk5, which interacts with the GST-p25 fusion protein. Overall, these data are supportive of the formation of a heteroligomeric complex containing Cdk5, Munc-18, syntaxin 1A, and GST-p25.

To further confirm that such a heteroligomeric protein complex can form *in vitro*, we have performed sequential series of binding reactions culminated by immunoprecipitation of one of the initial reactants and probing for co-immunoprecipitated proteins. Fig. 7*B* shows results from an experiment in which GST-p25 linked to glutathione-Sepharose beads and Cdk5 was combined in an initial reaction as were glutathione-Sepharose GST-Munc-18 and syntaxin. Following these reactions, the samples were centrifuged, and pellets were washed extensively and then each subjected to thrombin cleavage of the GST moiety to release the heterodimeric complexes into the supernatant. Combination and incubation of the two supernatants was followed by the addition of Cdk5 antibody and immunoprecipitation with protein A beads. Pellets were extensively washed, subjected to SDS-PAGE, Western blotted, and probed for coprecipitating syntaxin and p25 immunoreactivity. Control experiments omitted Cdk5 protein during the reaction but were similarly subjected to immunoprecipitation with Cdk5 antibody or treated with protein A beads. The data demonstrate the presence of both syntaxin and p25 in the immunoprecipitated pellet when all four proteins were present and an absence of immunoreactive signal on blots from the control experiments. The presence of syntaxin in this immunoprecipitate could only occur if formation of the heteromultimeric complex consisting of all four proteins had occurred.

The protein interaction data thus support the formation of a multimeric protein complex in the absence of ATP. On the other hand, the Cdk5 phosphorylation experiments demonstrated phosphorylation of Munc-18 weakens the interaction between Munc-18 and syntaxin. Therefore, we next investigated whether the addition of ATP to the preformed multimeric protein complex induced complex disassembly. For these experiments, the complex was formed by co-incubation of Cdk5 with p25 immobilized as a GST fusion protein on glutathione-Sepharose 4B followed by the sequential addition of Munc-18 and syntaxin fusion proteins as described above. The multimeric protein complex on the glutathione-Sepharose 4B beads was then washed extensively and incubated (15 min at 30 °C) with or without ATP (1 mM). Following incubation, syntaxin (Fig. 7*C*) and Cdk5 immunoreactivity in the pellet and supernatant was analyzed by immunoblotting. Those reactions including ATP were found to contain significantly greater amounts of syntaxin or Cdk5 (data not shown) in the supernatant than in those lacking ATP. These data suggest a model whereby the presence of ATP supports Cdk5 phosphorylation activity and dissociation of the multimeric complex, likely via phosphorylation and conformational rearrangement of Munc-18.

DISCUSSION

The family of proteins related to yeast Sec1p is essential for maintenance of normal secretory activity as evidenced by the missorting of secretory products and the accumulation of secretory vesicles with mutations of Sec1p in yeast or the homologous proteins in *C. elegans* (Unc-18) and *Drosophila* (ROP) (9, 22, 24–26). In part, the necessity of the Sec1-related proteins is believed to result from their direct and high affinity interaction with members of the t-SNARE family of syntaxin proteins and from the control by this complex of a v- and t-SNARE protein interaction required for vesicle fusion (7, 15, 21). The present study focused on elucidating the regulatory control of protein interactions of the mammalian Sec1 homologue Munc-18 (also termed n-Sec1, rbSec1) at nerve endings with specific examination of the role of the proline-directed cyclin-dependent kinase 5 to which Munc-18 has been reported to bind (28). We have shown here that bacterially expressed Munc-18 fusion protein interacts with Cdk5 found in nerve ending lysates. This Cdk5 has bound its neural specific p35 activator protein and has therefore been rendered catalytically active. Our data further demonstrate that Munc-18 is subject to phosphorylation by p35-activated Cdk5 and that this phosphorylation significantly reduces Munc-18 binding to syntaxin *in vitro*. We also report here that in addition to their interaction with Munc-18, Cdk5 and the p35-Cdk5 complex are capable of interacting with syntaxin. Last, we demonstrate that a stable protein complex composed of p35, Cdk5, Munc-18, and syntaxin fusion proteins can be formed *in vitro* and that dissociation of the complex can be initiated by the addition of ATP. We interpret these data as consistent with a secretory model in which the interaction between Munc-18 and syntaxin 1A at mammalian nerve endings is under regulatory control by the association and phosphorylation of Munc-18 by p35-activated Cdk5. This phosphorylation of Munc-18 would then promote its dissociation from syntaxin and allow the formation of the heterotrimeric 7 S core complex, which is composed of syntaxin, SNAP-25, and VAMP proteins.

The dissociation of the hydrophilic Munc-18 from the integral membrane syntaxin protein is probably under strict regulatory control based on a reported 1000-fold higher binding affinity of Munc-18 than VAMP for syntaxin and of the absence

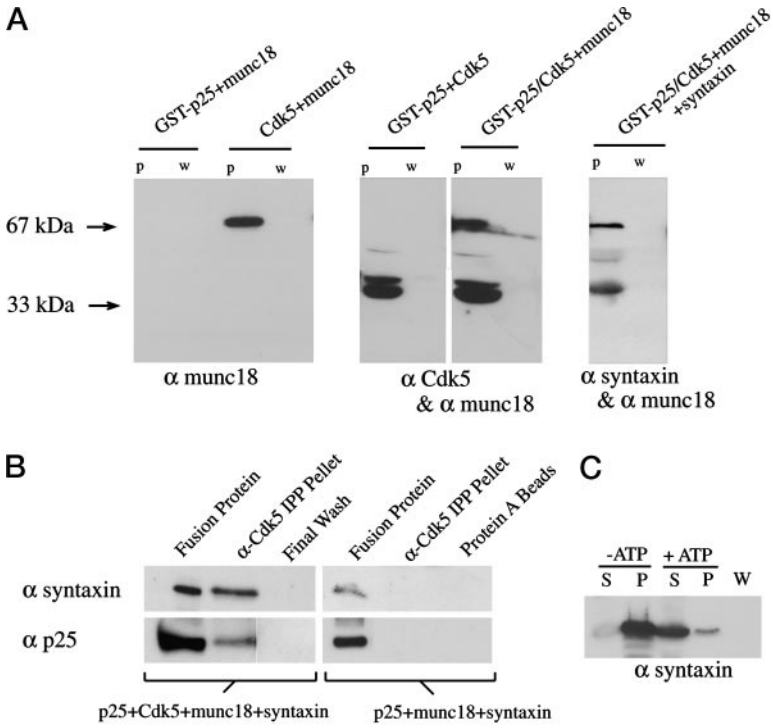

FIG. 7. **Formation of a protein complex from GST-p25 together with Cdk5, Munc-18, and syntaxin.** *A*, sequential binding of Cdk5, Munc-18 and syntaxin to GST-p25 (each protein used at 1 μM) to form a heterooligomeric protein complex. GST-p25 immobilized to glutathione-Sepharose beads was incubated (1 h, 4 °C) in binding buffer with the protein indicated and then collected and washed extensively prior to the addition and incubation with the next indicated protein component. Aliquots removed from the sample and final wash following each incubation were then analyzed with respect to the proteins bound by SDS-PAGE and immunoblotting. Protein-specific antibody used to probe the blots is indicated (below), and bound proteins were visualized by horseradish peroxidase-linked antibody and chemiluminescence. Initial lanes demonstrate binding controls showing absence of Munc-18 binding to GST-p25 and capability of Cdk5 binding to Munc-18 (each protein used at 1 μM). *B*, heteromultimeric protein complex formation following incubation of Munc-18-syntaxin 1A and Cdk5-p25 heterodimeric protein complexes. *Left*, heterodimeric protein complexes were formed by incubation of appropriate fusion proteins (glutathione-Sepharose-linked GST-Munc-18 plus syntaxin 1A and glutathione-Sepharose-linked GST-p25 plus Cdk5; 500 nM each) followed by centrifugal collection and extensive washing of the pellets. Pellets were then treated with thrombin to release the dimeric complexes into the supernatant. The supernatants were then combined and incubated for 1 h in binding buffer, after which Cdk5 antibody was added and a immunoprecipitation protocol was followed. Immunoprecipitated pellets were washed repetitively, and samples were subjected to SDS-PAGE and Western blotted. Blots were then probed for syntaxin or p25 immunoreactivity. *Right*, control experiments omitted Cdk5 protein, and immunoprecipitation was performed both with and without added Cdk5 antibody. *C*, dissociation of the formed multimeric protein complex by ATP. Multimeric protein complex was formed as in *A*, washed extensively, divided into two equal fractions, and then incubated (15 min at 30 °C) with or without ATP (1 mM) in binding buffer. Samples were then centrifuged, and syntaxin in the supernatant (*S*) or remaining bound within the protein pellet (*P*) with or without ATP was determined by SDS-PAGE and immunoblot analysis. Analysis of the supernatant for free syntaxin prior to ATP treatment (*W*) is also shown.

of Munc-18 within the 7 S SNARE particle (21, 37). At present, regulation of binding interactions among the several proteins involved in the vesicle recruitment, docking and fusion process are poorly understood, although some have been demonstrated to be modified by phosphorylation. That the phosphorylation state of Munc-18 plays a critical role in regulating the affinity of the Munc-18/syntaxin interaction has been recently demonstrated by an *in vitro* assay with fusion proteins, which evaluated the effects of protein kinase C phosphorylation (36). Our results extend this analysis to demonstrate that native p35-Cdk5 immunoprecipitated from nerve ending lysates, as well as p25-activated recombinant Cdk5, was capable of phosphorylating Munc-18 and thereby reducing a Munc-18/syntaxin 1A interaction. Surprisingly, despite a substantially greater equilibrium level of phosphate labeling of Munc-18 with protein kinase C than with Cdk5, the functional result on syntaxin 1A binding was much greater for Cdk5-phosphorylated Munc-18. Furthermore, our findings present the first evidence that phosphorylation state may be physiologically relevant by demonstrating that a proportion of Munc-18 is sensitive to alkaline phosphatase treatment, and Munc-18 thus exists as a phosphoprotein in intact nerve endings. Munc-18 has been reported to bind to syntaxin isoforms 2 and 3 as well as 1A, although the effects of Munc-18 phosphorylation state on these additional interactions have not yet been evaluated. Regulatory control of Munc-18 binding interactions may not, however, be completely mediated by phosphorylation state, since nitric oxide (NO) has also recently been reported to directly inhibit interaction of Munc-18 with syntaxin *in vitro* (41). In addition, interaction of the yeast Sec1p homologue Sly1p with the t-SNARE syntaxin homologue Sed5p has recently been demonstrated to be regulated by a transient interaction of the Rab protein Ypt1p with Sed5p (8). An additional interaction between Munc-18 and members of a new family of C2 domain proteins termed DOC2 has also recently been described (27). It is thus possible that Munc-18 protein interactions are under multiple regulatory control. Further, as mammalian Munc-18 homologues and splice variants have been reported, there may also occur differential regulation of specific isoforms. For example, rbSec1 bound to bovine adrenal medullary secretory granules interacts with immobilized recombinant syntaxin, while free rbSec1 does not (42), suggesting that additional interactions such as with DOC 2 (27) may regulate conformational state and binding affinity.

Although Cdk5 shows a ubiquitous distribution, its phosphorylation activity is limited to neural tissue, where it is most highly expressed. A prior study had suggested that Munc-18 protein, like p35, was a specific activator of Cdk5 enzymatic activity (28). However, our data argue against a kinase activating function for Munc-18. For example, immunoprecipita-

tion of Cdk5 from neural lobe lysate was found to co-precipitate p35 but not Munc-18, yet that immunoprecipitate did express kinase activity against a histone peptide containing proline-directed phosphorylation consensus sequences. Furthermore, using purified bacterially expressed fusion proteins together with an *in vitro* kinase assay, we have found that Cdk5 enzymatic activity is strongly activated by recombinant p25 but not by recombinant Munc-18. Demonstration that the enzymatic activity being monitored for both the immunoprecipitated and recombinant proteins was specific to Cdk5 kinase activity was provided by the sensitivity of phosphorylation to the specific Cdk inhibitor olomoucine and by the insensitivity to inhibitors of protein kinase C, protein kinase A, and calcium-calmodulin kinase II. Cdk5 has also been reported to associate with cyclin D1 and D3 in fibroblasts and *in vitro*, although binding results in only slight activation of phosphorylation activity (29, 43). Despite its role to activate Cdk5, the p35 protein has no sequence similarity to the cyclins that normally regulate Cdk activity. Furthermore, Cdk5 is unique among the Cdks in that it has not been found to be involved in cell proliferation but rather occurs in postmitotic neurons, where it is believed to control cytoskeletal functions and neurite outgrowth (29, 44). A marked increase in Cdk5 activity *in vivo* occurs during the neurogenic process and coincides with increased p35 levels (34, 45). Our findings that Munc-18 can be phosphorylated by p35- (or p25-) activated Cdk5 *in vitro* and that GST-Munc-18 is capable of a specific interaction with p35-Cdk5 from a neural lobe lysate suggest a functional relationship. The exact sites on Munc-18 phosphorylated by Cdk5 remain undetermined, although two Cdk5 phosphorylation consensus sequences in Munc-18 (Ser^{158}, Thr^{574}) present likely PO_4 sites.

Our findings demonstrating an interaction between Munc-18 with Cdk5 at nerve endings are consistent with a recent report showing the co-purification of Munc-18 (p67) with Cdk5 activity from rat brain and binding of Cdk5 in a rat brain homogenate to p67 immobilized on an affinity matrix (28). However, we report here that only approximately 50% of the immunoreactive Cdk5 present in the nerve ending lysate could be bound to GST-Munc-18, although saturable binding relations between GST-Munc-18 and Cdk5 were found. This upper limit to Cdk5 binding may relate to a limiting amount of p35 protein in the nerve endings and to GST-Munc-18's preferential binding of the catalytically active p35-Cdk5 complex. Our p35 and Cdk5 immunoprecipitation data are largely in support of such a model. For example, immunodepletion of p35 from neural lobe lysate led to only a partial precipitation of the Cdk5 present in the lysate. In comparison, immunoprecipitation of Cdk5 was found to deplete p35 immunoreactivity from the lysate even under conditions where Cdk5 was not completely precipitated. Furthermore, analysis of binding interactions among GST-Munc-18, Cdk5, and p25 fusion proteins *in vitro* demonstrated that the addition of ATP to the incubation to produce a catalytically active p35-Cdk5 protein complex greatly enhanced the level of Cdk5-p35 binding to GST-Munc-18. The data suggest that ATP present in nerve ending lysate likely facilitated activation and thus transient interaction of Cdk5-p35 protein kinase complex to the GST-Munc-18 substrate. Regulation of Cdk5 activity levels may, therefore, be under strict control by the level of expression of p35, similar to that reported for cyclin control of other Cdks.

We have found, in addition to an interaction between Cdk5 and Munc-18, that Cdk5 is capable of establishing a specific and saturable binding interaction with syntaxin 1A of moderate affinity (EC_{50} = 0.52 μM). This adds Cdk5 to an increasing list of cytosolic proteins and membrane proteins that have now been demonstrated to posses an affinity for interaction with syntaxin. These proteins include α-SNAP, VAMP (synaptobrevin), SNAP-25, synaptotagmin, and voltage-gated N type Ca^{2+} channels (37, 46, 40). The interactions have been shown to be largely mediated by assembly of coiled-coil structures with the carboxyl-terminal domain of syntaxin. Whether this specific region is important in Cdk5 binding remains undetermined. Our data also indicate that the ability of Cdk5 to interact with syntaxin was unchanged in the presence of p25 fusion protein, suggesting that Cdk5 does not have to be in an enzymatically active conformational state to bind. One may envision that the interaction of Cdk5 with syntaxin may act to further promote, localize, or stabilize the interaction of Cdk5 or the Cdk5-p35 heterodimer with the Munc-18-syntaxin complex. Indeed, we demonstrate here, using sequential binding reactions of bacterially expressed fusion proteins, that in the absence of ATP, one can assemble *in vitro* a stable protein complex containing the p25, Cdk5, Munc-18, and syntaxin proteins. *In vitro*, Munc-18 has been reported to bind syntaxin in a 1:1 molar ratio (12). These data do not, however, exclude yet larger multimeric complexes, and the stoichiometry of the interactions within the larger protein complex remains to be fully investigated. In addition, it will be of importance to determine the sequence of protein interactions that occur *in situ*. Of particular importance, however, are our findings demonstrating that the addition of ATP to the preformed complex is able to support kinase activity and lead to rapid disassembly of the complex.

This report has focused on regulation of binding interactions by phosphorylation state of the mammalian neural specific Sec1 family member, Munc-18. However, a number of mammalian Sec1/Munc-18 homologues have been identified that are more ubiquitously distributed and exhibit different binding specificity for syntaxin isoforms (*e.g.* Munc 18c) or lack binding to known syntaxins (*e.g.* r-vps33a, r-vps33b, h-vps45) (15–19). Although selectivity in pairing of Munc-18 homologues with syntaxins or syntaxin homologues may be an important determinant of secretory characteristics or specificity to different vesicle transport pathways, it remains unknown whether their binding interactions are similarly under strict regulatory control by Cdk5 phosphorylation.

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