Ca\textsuperscript{2+}-dependent Phosphorylation of Syntaxin-1A by the Death-associated Protein (DAP) Kinase Regulates Its Interaction with Munc18*

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Syntaxin-1 is a key component of the synaptic vesicle docking/fusion machinery that binds with VAMP/synaptobrevin and SNAP-25 to form the SNARE complex. Modulation of syntaxin binding properties by protein kinases could be critical to control of neurotransmitter release. Using yeast two-hybrid selection with syntaxin-1A as bait, we have isolated a cDNA encoding the C-terminal domain of death-associated protein (DAP) kinase, a calcium/calmodulin-dependent serine/threonine protein kinase. Expression of DAP kinase in adult rat brain is restricted to particular neuronal subpopulations, including the hippocampus and cerebral cortex. Biochemical studies demonstrate that DAP kinase binds to and phosphorylates syntaxin-1 at serine 188. This phosphorylation event occurs both in vitro and in vivo in a Ca\textsuperscript{2+}-dependent manner. Syntaxin-1A phosphorylation by DAP kinase or its S188D mutant, which mimics a state of complete phosphorylation, significantly decreases syntaxin binding to Munc18-1, a syntaxin-binding protein that regulates SNARE complex formation and is required for synaptic vesicle docking. Our results suggest that syntaxin is a DAP kinase substrate and provide a novel signal transduction pathway by which syntaxin function could be regulated in response to intracellular [Ca\textsuperscript{2+}] and synaptic activity.

Neurotransmitter release from presynaptic nerve terminals requires calcium-triggered exocytosis of synaptic vesicles, a process that involves a series of protein-mediated docking and fusion events between the membranes of synaptic vesicles and the presynaptic terminal (1, 2). The synaptic vesicle-associated protein synaptobrevin (VAMP,\textsuperscript{1} v-SNARE) interacts with two plasma membrane-associated proteins (t-SNAREs), SNAP-25, and syntaxin, to form a stable SNARE core complex (4–7). Considerable evidence indicates that the SNARE complex is a biochemical intermediate essential for a late step in the membrane fusion process (2, 8–11). In neurons, the final stages of vesicle priming and membrane fusion are strictly Ca\textsuperscript{2+}-dependent (12). Presynaptic exocytosis is characterized by a fast response with a very short delay between excitation and secretion (13) and by limited release with only a small percentage of morphologically docked vesicles completing fusion upon Ca\textsuperscript{2+} influx (14). These properties suggest that the molecular basis of SNARE protein-protein interactions that mediate the final steps of synaptic vesicle docking/fusion are tightly and finely regulated.

Second messenger regulation of protein interactions within the exocytotic apparatus, for example, by protein phosphorylation or dephosphorylation, may be one mechanism by which cellular events could affect SNARE protein function and mediate synaptic plasticity (8). Although the time course between action potential arrival at the nerve terminal and the resultant vesicle fusion is too short for protein phosphorylation to exact a direct and acute effect during a single round of vesicle exocytosis, protein kinases, and phosphatases may have significant effects on subsequent neurotransmitter release events in an activity or Ca\textsuperscript{2+}-dependent manner. Activation of protein kinases in presynaptic terminals, particularly CaMKII, PKA, and PKC, has been shown to correlate with transmitter release (15–28). A number of SNAREs and their regulatory proteins have been reported as potential substrates in vitro for protein kinases (29–43). For example, in vivo phosphorylation of synapsin, which is dependent on intracellular [Ca\textsuperscript{2+}], has been shown to affect synaptic vesicle availability at release sites and control the kinetics of vesicle pool turnover (44–46). It is reasonable to speculate that the phosphorylation/dephosphorylation state of synaptic proteins that mediate vesicle exocytosis is critical to the regulation of the biochemical pathways leading from vesicle docking to neurotransmitter release. Thus, identifying protein kinases and phosphatases that modulate the assembly/disassembly of the fusion machinery, particularly kinases and phosphatases functionally regulated by synaptic activity or intracellular [Ca\textsuperscript{2+}], could offer valuable insights to the molecular mechanisms underlying synaptic transmission and plasticity.

In the current study, we have used the C-terminal half of syntaxin-1A as a bait to screen a human brain cDNA library via the yeast two-hybrid selection technique. We have isolated one cDNA encoding the C-terminal domain of DAP kinase (47), a calcium/calmodulin-dependent serine/threonine protein kinase (48). DAP kinase was first reported as a potential mediator of γ-interferon-induced cell death (47). However, the native substrates of DAP kinase and the molecular pathways underlying DAP kinase-mediated signal transduction remain unclear. Examination of the tissue distribution of DAP kinase mRNA...
demonstrated that it is predominantly expressed in brain and lung. DAP kinase mRNA is widely distributed in the embryonic brain and gradually declines in postnatal expression. Interestingly, the adult expression of DAP kinase is restricted to particular neuronal subpopulations that are important in memory and learning, such as the hippocampus and cerebral cortex, suggesting that DAP kinase-mediated signal transduction pathways might be implicated in neural functions related to synaptic transmission or plasticity (49–50). Our current yeast two-hybrid cloning results and biochemical studies demonstrate that DAP kinase binds to and phosphorylates neurosyntxin-1A. This phosphorylation event occurs both in vitro and in vivo in a Ca2+−dependent manner. Phosphorylation of syntxin-1A at Ser-188, or its mutation to S188D, which mimics a state of complete phosphorylation, significantly decreases syntxin binding to Munc 18-1, a syntxin regulatory protein that controls SNARE complex formation and vesicle docking (51–53). Our results suggest that syntxin is a Ca2+−dependent phosphorylation target of DAP kinase and that syntxin phosphorylation at Ser-188 could be a mechanism for regulation of syntxin binding properties in response to elevated intracellular [Ca2+]i and synaptic activity.

**EXPERIMENTAL PROCEDURES**

**Isolation of DAP Kinase**—The yeast two-hybrid system was used to isolate proteins that interact with syntxin-1A. The C-terminal half (CT, 181–288) of rat syntxin-1A cDNA was inserted in-frame into a pGAT9 bait vector containing the GAL4 DNA binding domain (Clontech). Yeast two-hybrid screens of a human brain cDNA library in vector pACT1 (Clontech) containing the GAL4 activation domain were performed and evaluated according to the protocols described for the MATCH-MAKER yeast two-hybrid system (Clontech). Positive clones were selected on plates lacking leucine, tryptophan, and histidine with 5 mM 3-aminotriazole and confirmed by filter assay for β-galactosidase activity.

**Fusion Protein Construction, Preparation, and Purification—**HA-DAP kinase cDNA was kindly provided by A. Kimchi (47), and purified DAP kinase catalytic domain (KD, amino acids 1–285) by Dr. Martin Wattersen (54). DAP kinase (KD-CalM) was produced by subcloning the region of the DAP kinase cDNA that encodes amino acids 1–320, corresponding to the catalytic domain and calmodulin binding regulatory domain, into the pASK-IBA3 (Sigma) expression vector at the BsoI sites. DAP kinase K42A and syntxin S188A/S188D mutants were generated using the QuikChange™ XL site-directed mutagenesis kit (Stratagene). Full-length syntxin-1A (FL, syntxin-1A/TM1–190), syntaxin-1A C-terminal-half (CT) (181–264), SNAP-25, VAMP-2, Munc 18-1, and DAP kinase C-terminal (CT) (1157–1431) were subcloned into GST fusion protein vector pGEX-4T-1 (Amersham Biosciences) or His-tagged fusion protein vector pET28 (Novagen). Fusion proteins were prepared as crude bacterial lysates by mild sonication in phosphate-buffered saline (50 mM sodium phosphate, pH 7.4, 140 mM NaCl, plus protease inhibitors of 1 mM phenylmethylsulfonyl fluoride, 1 μM/ml leupeptin, 2 μM/ml aprotinin) with 1% Triton X-100. His-tagged fusion proteins were purified by binding to Ni2+−charged nickel-nitrilotriacetic acid agarose columns (Qiagen) and eluted with 500 mM imidazole in phosphate-buffered saline. GST fusion proteins were purified by binding to glutathione-Sepharose beads (Amersham Biosciences) in TBS buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.1% Triton X-100, plus protease inhibitors), and eluted by 15 mM reduced glutathione (in 50 mM Tris-HCl, pH 8.0) or cleaved from the GST tag by incubation with biotinylated thrombin (Novagen) at a final concentration of 0.1 units/ml at room temperature for 4 h. Biotinylated thrombin was removed from the reaction by using streptavidin–agarose beads (Novagen). The eluates were dialyzed in a 10,000-molecular weight cutoff dialysis cassette (Pierce) against TBS and concentrated using Centricon-10 filtration units (Amicon).

**In Vitro Binding**—Approximately 1 μg of GST fusion proteins were bound to glutathione-Sepharose beads (Amersham Biosciences) in TBS buffer, incubated at 4 °C for 1 h with constant agitation, and washed with TBS to remove unbound proteins. Glutathione-Sepharose beads coupled with similar amounts of GST fusion proteins were added to the full-length HA-tagged DAP kinase lysates from transfected HEK293T cells or His-DAP-kinase-CT lysates from bacteria and incubated with gentle mixing for 3 h at 4 °C. The beads were washed three times with TBS buffer, and bound proteins were eluted in 30 μl of SDS-PAGE sample buffer and electrophoresed on 10–20% SDS-Tricine gradient gel. Bound HA-DAP-kinase or His-DAP-kinase-CT was detected by anti-DAP kinase antibody (Sigma) or monoclonal anti-His7-T7-tag antibody (Novagen), respectively, and GST fusion proteins were detected by anti-GST antibody (Amersham Biosciences). Horseradish peroxidase-conjugated secondary antibodies and ECL (Amersham Biosciences) were used to visualize the bands.

**Transfection of HEK293T Cells—**HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Invitrogen) and 0.5% l-glutamine. Syntaxin-1A cDNA was inserted into the HindIII site of the pcDNA3.1 vector (Invitrogen). HEK293T cells were then transfected with the full-length of ENA using LipofectAMINE 2000 (Invitrogen), according to the instruction manual. After 48 h, the cells were harvested and solubilized in TBS buffer with 1% Triton X-100, 5 mM EDTA, and protease inhibitors. Cell lysates were centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was used for in vitro binding and immunoprecipitation studies.

**Synaptosome Preparation—**Rat brain synaptosomes were prepared by differential and discontinuous Percoll gradient centrifugation and solubilized as described (55). Synaptosome fractions were isolated as described (56). Briefly, after Percoll-gradient sucrose centrifugation, the synaptosomes were washed once in medium M (0.32 mM sucrose, 1 mM KH2PO4, 0.1 mM EDTA, pH 7.5), resuspended and homogenized in medium L (1 mM KH2PO4, 0.1 mM EDTA, pH 8.0), and incubated at 4 °C for 1 h. The resulting suspension was layered over 5 ml of 1× sucrose in medium L and centrifuged for 30 min at 96,300 × g in an SW27 rotor. The supernatant was mixed to homogeneity and centrifuged again for 14 h at 25,000 × g. The supernatant was collected as synaptosomal, and the pellets were homogenized in medium L and applied to a gradient of 7 ml of 1.2, 1.0, 0.8, 0.6, and 0.4 m sucrose in medium L and centrifuged for 90 min at 68,000 × g in an SW27 rotor. Bands at each interface were collected and washed once with medium L in a Ti50 rotor (45 min at 106,500 × g). The synaptosomal was dialyzed against medium L and centrifuged at 140,000 × g in a Ti50 rotor for 1 h to separate any remaining synaptosomal vesicles. Soluble proteins in each fraction were determined by BCA protein assay (Pierce) against a bovine serum albumin standard.

**Immunocytochemistry of Cultured Hippocampal Cells—**Rat hippocampal neuron cultures were prepared at a density of 100,000 cells/ml on polyornithine- and fibronectin-coated glass chamber slides (Lab-Tek) from hippocampi dissected from E18 rat embryos as described (57). Cultures were plated on a glial bed in Neurobasal (Invitrogen) supplemented with 1× B27 and 100× l-glutamine, with half-feed changes every 3–4 days. Neurons were prepared for immunofluorescent confocal microscopy on DIV11 following fixation in 4% paraformaldehyde, solubilization in 0.1% Triton X-100, and blocking in 1% bovine serum albumin. Antibodies against syntaxin-1 (from M. Takahashi) and DAP kinase (from R.-H. Chen, Ref. 58) were detected using tagged fluorescent anti-mouse and anti-rabbit antibodies (Jackson).

**Coimmunoprecipitation—**Solubilized proteins (100–300 μg) from transfected HEK293T cell lysates or brain homogenates were incubated with either 3 μg of anti-syntaxin-1 (10H5) monoclonal antibody or 3 μg of non-immune mouse IgG (Zymed Laboratories Inc.) as control in 0.5 ml of TBS with 0.1% Triton X-100 and protease inhibitors, and incubated on a microtube rotator at 4 °C for 3 h. Protein A-Sepharose CL-4B resin (2.5 mg) (Amersham Biosciences) was added to each sample, and the incubation continued for an additional 1 h, followed by three washes with TBS/0.1% Triton X-100. Subsequent interaction assays of immunoprecipitated and immobilized protein complexes are described in the section on in vitro binding assays above. For multiple detection with different antibodies, blots were first stripped between applications of different antibodies, blots were first stripped between applications of different antibodies, and final detection was done with different antibodies, without washing between antibodies. For multiple detection with different antibodies, blots were first stripped between applications of different antibodies, and final detection was done with different antibodies, without washing between antibodies. For multiple detection with different antibodies, blots were first stripped between applications of different antibodies, and final detection was done with different antibodies, without washing between antibodies. After washing, blots were incubated with 1/2000 dilution of ECL, and exposed to film.

**Phosphorylation Reactions—**Lysates of transfected HEK293T cells with pDNA-HA-DAP-kinase were immunoprecipitated with 1 μg of anti-HA polyclonal antibodies (Clontech) in 0.5 ml of TBS/0.1% Triton X-100 with protease inhibitors. After 3 h of incubation at 4 °C, protein A-Sepharose CL-4B resin (2.5 mg) was added and washed three times with TBS/0.1% Triton X-100 and once with the kinase assay buffer (50 mM HEPES pH 7.5, 8 mM MgCl2, and 0.5 mM dithiothreitol). Isolated and immobilized DAP kinase full-length or purified DAP kinase catalytic domain (KD) or
KD-CaM domain were then incubated for 30 min at 30 °C in 25–50 μl of reaction buffer containing 5–15 μCi [γ-32P]ATP (1.6–5 pmol) (Amer- sham Biosciences), 100 μM ATP, 0.1 mg/ml bovine serum albumin, and 50 pmol of purified syntaxin-1A. The reactions were terminated by adding SDS-PAGE sample buffer and then heated at 95 °C for 5 min. Aliquots of phosphorylated products were analyzed by 10–20% Tricine SDS-PAGE, stained with Coomassie Blue, and then dried and exposed to x-ray film for autoradiography. For the Ca2+-buffering system, 10 mM EGTA or 10 mM nitrolotriacetic acid was used to produce free Ca2+ buffer (59) as calculated using the max chelator software (version 6.63). For back-phosphorylation studies, HEK293T cells co-transfected with syntaxin-1A and HA-DAP-kinase wild type or K42A mutant were stimulated with 10 μM ionomycin at room temperature for 30 min and then solubilized with 1% Triton X-100. Lysates (2 mg of total protein) were incubated with an anti-stx1 antibody, and the immunoprecipitates were then back-phosphorylated in vitro with anti-HA-immobilized full-length DAP kinase isolated from transfected HEK293 cell lysates. Back-phosphorylation was performed in small volumes (100 μl) with constant agitation throughout the incubation time.

RESULTS

Isolation of DAP Kinase and Its Association with Syntaxin-1A—To elucidate the molecular mechanisms underlying neurotransmitter release and its modulation, we have used the C-terminal-half (181–288) of syntaxin-1A as a bait to screen a library of proteins. The DAP kinase (DAPK) structure and its specific interaction with syntaxin-1A. A. Domain structure of DAPK and its mutants. Human DAPK is a 1431-residue protein that contains an N-terminal kinase domain followed by a CaM binding sequence and a C-terminal syntaxin-binding domain. The K42A and C-terminal truncated (1157–1431) mutants are illustrated schematically. B, Ca2+-independent binding of GST-syntaxin-1A to DAPK in vitro. GST or GST fusion proteins (~1 μg each) were immobilized on glutathione-Sepharose beads and then incubated with HEK293 cell lysates transfected with a construct encoding HA-tagged DAPK full-length construct in the presence of either Ca2+ (500 μM)/CaM (1 μM) or EGTA (2 mM) as indicated. Bound protein complexes were eluted from the matrix, separated by SDS-PAGE, and immunoblotted with anti-DAPK antibody (lower panel). Membranes were then stripped and reprobed with anti-GST antibody (upper panel). C, binding of both full-length (FL) and C-terminal half (CT) but not N-terminal half (NT) of syntaxin-1A (stx1A) to the DAPK C-terminal truncated mutant (DAPK-CT). Immobilized GST or GST fusion proteins (~1 μg) were incubated with His-tagged DAPK-CT, and binding was analyzed by SDS-PAGE followed by immunoblot with anti-His7-tag (lower panel) and anti-GST (upper panel) antibodies. D, HEK293T cells were co-transfected with DAPK-FL and stx1A-FL constructs. The expression of both proteins was confirmed by immunoblotting of co-transfected or untransfected cell extracts with antibodies as indicated. E, DAPK-stx1A complex was coimmunoprecipitated from co-transfected cell extracts by anti-stx1 antibody, and then detected by immunoblot with anti-DAPK antibody. Non-immune mouse IgG (Ms-IgG) was used as a control. 10% input of 293 cell lysates for coimmunoprecipitation was loaded as an indicator of relative amounts of proteins used. F, DAPK-stx1 complex was immunoprecipitated from Triton X-100-solubilized rat brain homogenates by anti-stx1 antibody. Homogenates were incubated with anti-stx1 antibody or control Ms-IgG, and the isolated immunoprecipitates probed with anti-DAPK. 10% input of brain homogenates for coimmunoprecipitation was loaded as an indicator of relative amounts of proteins used. G, stoichiometric binding between DAPK and stx1A. Immobilized GST-DAPK-CT (~250 ng) was incubated with increasing amounts of His-stx1A as indicated. Bound proteins were visualized by Coomassie Blue staining. The degradation band of GST-DAPK-CT is marked with an asterisk.

Fig. 1. DAP kinase (DAPK) structure and its specific interaction with syntaxin-1A. A. Domain structure of DAPK and its mutants. Human DAPK is a 1431-residue protein that contains an N-terminal kinase domain followed by a CaM binding sequence and a C-terminal syntaxin-binding domain. The K42A and C-terminal truncated (1157–1431) mutants are illustrated schematically. B, Ca2+-independent binding of GST-syntaxin-1A to DAPK in vitro. GST or GST fusion proteins (~1 μg each) were immobilized on glutathione-Sepharose beads and then incubated with HEK293 cell lysates transfected with a construct encoding HA-tagged DAPK full-length construct in the presence of either Ca2+ (500 μM)/CaM (1 μM) or EGTA (2 mM) as indicated. Bound protein complexes were eluted from the matrix, separated by SDS-PAGE, and immunoblotted with anti-DAPK antibody (lower panel). Membranes were then stripped and reprobed with anti-GST antibody (upper panel). C, binding of both full-length (FL) and C-terminal half (CT) but not N-terminal half (NT) of syntaxin-1A (stx1A) to the DAPK C-terminal truncated mutant (DAPK-CT). Immobilized GST or GST fusion proteins (~1 μg) were incubated with His-tagged DAPK-CT, and binding was analyzed by SDS-PAGE followed by immunoblot with anti-His7-tag (lower panel) and anti-GST (upper panel) antibodies. D, HEK293T cells were co-transfected with DAPK-FL and stx1A-FL constructs. The expression of both proteins was confirmed by immunoblotting of co-transfected or untransfected cell extracts with antibodies as indicated. E, DAPK-stx1A complex was coimmunoprecipitated from co-transfected cell extracts by anti-stx1 antibody, and then detected by immunoblot with anti-DAPK antibody. Non-immune mouse IgG (Ms-IgG) was used as a control. 10% input of 293 cell lysates for coimmunoprecipitation was loaded as an indicator of relative amounts of proteins used. F, DAPK-stx1 complex was immunoprecipitated from Triton X-100-solubilized rat brain homogenates by anti-stx1 antibody. Homogenates were incubated with anti-stx1 antibody or control Ms-IgG, and the isolated immunoprecipitates probed with anti-DAPK. 10% input of brain homogenates for coimmunoprecipitation was loaded as an indicator of relative amounts of proteins used. G, stoichiometric binding between DAPK and stx1A. Immobilized GST-DAPK-CT (~250 ng) was incubated with increasing amounts of His-stx1A as indicated. Bound proteins were visualized by Coomassie Blue staining. The degradation band of GST-DAPK-CT is marked with an asterisk.
human brain cDNA library via the yeast two-hybrid selection technique. Screening of $\sim 2 \times 10^6$ colonies led to the isolation of six classes of cDNAs encoding α-SNAP (10 clones), β-SNAP (13 clones), SNAP-25 (3 clones), syntaphilin (2 clones), syntaxin-1A (5 clones), SNAP-29 (2 clones), and one cDNA encoding the C-terminal domain (1386–1431) of DAP kinase (Fig. 1A) (47).

Because the yeast two-hybrid system may identify low affinity interactions that may not normally occur either in vitro or in vivo, we conducted four lines of experiments to confirm the selective and direct interaction between DAP kinase and syntaxin-1A. First, we performed in vitro binding assays with recombinant proteins. While HA-tagged full-length DAP kinase selectively bound to GST-syntaxin-1A in a Ca$^{2+}$/calmodulin-independent manner, no binding was detectable to GST-VAMP2 or GST alone (Fig. 1B). Next, while DAP-kinase-CT (1157–1431, Fig. 1A) bound to both GST-syntaxin-1A full-length and GST-syntaxin-1A-CT (181–288), no binding was found to GST-syntaxin-1A-NT (2–190), GST-VAMP2 or GST alone (Fig. 1C), consistent with a specific association of DAP kinase with syntaxin-1A via a direct interaction of their C-terminal domains. Furthermore, we sought to confirm the DAP kinase-syntaxin-1A interaction in a mammalian expression system. A cDNA encoding His-tagged full-length DAP kinase was co-transfected into HEK293T cells with the cDNA encoding syntaxin-1A. The association of endogenous DAP kinase with syntaxin-1A was then confirmed by immunoprecipitation with anti-syntaxin-1 antibody (Fig. 1, D and E). DAP kinase was coimmunoprecipitated with syntaxin-1A from rat brain homogenate by anti-syntaxin-1 antibody but not by non-immune mouse IgG (Fig. 1F).

Finally, we performed a Coomassie-based estimation of the stoichiometry of the syntaxin-DAP kinase-CT interaction using purified recombinant proteins. Our studies show that, under our experimental conditions, the interaction of His-tagged syntaxin-1A with GST-DAPK-CT occurs at relative concentrations approaching a 1:1 molar ratio, as determined by semi-quantification using purified recombinant proteins as standards (Fig. 1G). The results from our yeast two-hybrid selection, in vitro binding assays, and immunoprecipitation studies indicate that DAP kinase directly interacts with syntaxin-1A.

**Distribution and Subcellular Localization of DAP Kinase**—DAP kinase mRNA has been found to be present in brain tissue, and particularly in the hippocampus of adult rats (49–50). To determine the expression of DAP kinase protein in brain, we performed immunoblot using a monoclonal anti-DAP kinase antibody on various homogenates of anatomically and functionally distinct areas of adult rat brain including cortex, hippocampus, olfactory bulb, mesencephalon, midbrain, cerebellum, and spinal cord. Only cortex, hippocampus, and olfactory bulb showed prominent expression of DAP kinase in the adult rat brain (Fig. 2A). To examine the subcellular distribution of DAP kinase in neurons, we prepared a subcellular fractionation assay from crude synaptosomal preparations. By sucrose density gradient centrifugation, rat cerebral synaptosomes were fractionated into cytosol (synaptosol), synaptic vesicle, and synaptic plasma membrane fractions and then analyzed by sequential immunoblotting with various antibodies as indicated (Fig. 2B). The relative purity of these subcellular
fractions was confirmed by immunoreactivity corresponding to markers of synaptic vesicles (synaptophysin), plasma membrane (Na+/K+-ATPase), and cytosol (LDH). DAP kinase was present predominantly in the cytosolic fraction and, to a lesser extent, in the plasma membrane fraction, and was absent from the synaptic vesicle fraction, consistent with structural predictions given its lack of a hydrophobic transmembrane segment. The presence of DAP kinase in the plasma membrane fraction is significant given our description of its interaction with the plasma membrane protein syntaxin.

To confirm that DAP kinase is present at neuronal processes and to examine whether its localization in neurons would allow it to interact with syntaxin-1 at the plasma membrane, we performed double-labeled immunocytochemistry in cultured hippocampal cells using antibodies against DAP kinase and syntaxin-1. As shown in Fig. 2C, syntaxin-1 staining was detected in a punctate pattern along the plasma membrane surface of neuronal cell bodies and processes. While some staining for DAP kinase was found in glia, DAP kinase signal was seen predominantly in neurons. Consistent with our immunoblot findings in subcellular synaptosomal fractions, the majority of DAP kinase staining was detected in the cytoplasmic space, but extended throughout neuronal processes to the plasma membrane, where it was found to colocalize with syntaxin-1. These data demonstrate that DAP kinase partially colocalizes with syntaxin-1A at the plasma membrane of intact neurons, and suggest that our findings that DAP kinase and syntaxin-1A are binding partners could be physiological relevant in vivo. The restricted distribution of DAP kinase in adult brain to the hippocampus, cortex, and olfactory bulb, its presence in both the cytosolic and plasma membrane fractions of synaptosomes, and its partial colocalization with syntaxin-1 in hippocampal neurons suggest that DAP kinase-mediated signal transduction pathway may be involved in neuronal functions related to synaptic transmission or plasticity.

In Vitro Phosphorylation of Recombinant Syntaxin-1A by DAP Kinase—DAP Kinase was first reported as a calcium/calmodulin-dependent serine/threonine protein kinase that mediates γ-interferon-induced cell death (47); however, its potential roles in mature neurons is still unknown. Given our finding of a specific interaction between syntaxin-1A and DAP kinase, we wondered whether syntaxin-1A might be a substrate for DAP kinase phosphorylation. Due to its presence in synapses, we also speculated that DAP kinase activity might represent a signal transduction pathway coupled to synaptic activity, i.e., that syntaxin phosphorylation by DAP kinase might occur in a Ca\(^{2+}\)/CaM-dependent manner. First, we examined the ability of recombinant syntaxin-1A to serve as a substrate of DAP kinase. We incubated 50 pmol of purified recombinant syntaxin-1A-ΔTM cleaved from the GST tag with a truncated DAP kinase (KD-CaM-(1-320)) containing both the catalytic domain and CaM-binding regulatory domain, and [γ-\(^{32}\)P]ATP in buffer with 0, 0.1, 1, 10, or 100 μM free Ca\(^{2+}\). As shown in Fig. 3A, phosphorylation of syntaxin-1A is hardly observed in the absence of Ca\(^{2+}\), is very weak at 0.1 and 1 μM [Ca\(^{2+}\)], and increases sharply between 1 and 10 μM free [Ca\(^{2+}\)]. As synaptic vesicle exocytosis requires elevated intracellular free [Ca\(^{2+}\)] that occur with opening of voltage-dependent calcium channels at the synapses could lead to the activation of DAP kinase localized at or near active zones and consequently, the phosphorylation of syntaxin-1A. Interestingly,

**Fig. 3. Ca\(^{2+}\)/CaM-dependent phosphorylation of Syntaxin-1A by DAPK in vitro.** A, phosphorylation of stx-1A by DAPK is Ca\(^{2+}\)/CaM-dependent. 50 pmol of purified stx1A-ΔTM was incubated with [γ-\(^{32}\)P]ATP and purified DAPK KD-CaM in a 25-μl reaction in 0, 0.1, 1, 10, or 100 μM free Ca\(^{2+}\) buffer. Phosphorylation reactions were terminated by boiling in SDS sample buffer, the products separated by SDS-PAGE, and the gels stained with Coomassie blue (lower panel), and dried and exposed to x-ray film for autoradiography (upper panel). B, serine 188 is a prominent DAPK-phosphorylation site in stx1A. His-tagged stx1A-ΔTM or its S188A mutant (50 pmol each) were incubated with purified DAPK catalytic domain (3 μg/ml) under conditions described under “Experimental Procedures,” and the products separated by 10–20% SDS-PAGE; gels were then stained with Coomassie Blue (left panel), dried, and exposed to x-ray film for autoradiography (right panel). (*, degradation product of stx1A-ΔTM). C and D, stoichiometry of syntaxin phosphorylation by DAPK. stx1A-ΔTM (3.8 pmol) or MLC (10 pmol) was incubated with the DAPK catalytic domain (KD) (3 μg/ml) for phosphorylation under conditions described under “Experimental Procedures” for the indicated time periods. Additional DAPK-KD was added every 60 min. Reactions were terminated by the addition of SDS sample buffer and boiling, the products separated by SDS-PAGE, and the gel exposed to film for 16 or 5 h for stx1A-ΔTM or MLC, respectively. Gel slices containing labeled stx1A or MLC were excised, scintillation levels were counted, and the moles of P\(_{i}\) incorporated per mole of stx1A or MLC were calculated and plotted as a function of time.
DAP kinase (KD-CaM) was also found to be autophosphorylated in a Ca^{2+}-regulated manner (Fig. 3A). While increasing Ca^{2+} levels activate the phosphorylation of syntaxin 1A by DAP kinase, DAP kinase autophosphorylation was stronger in the absence than in the presence of free [Ca^{2+}]. In addition, DAP kinase autophosphorylation inhibited its phosphorylation of syntaxin-1, an observation consistent with previous findings (61) suggesting a mechanism for Ca^{2+}-mediated regulation of DAP kinase activity.

To map potential DAP kinase phosphorylation sites in syntaxin-1A, we examined the capacity of syntaxin deletion mutants to serve as DAP kinase substrates. We incubated 50 pmol of purified His-tagged syntaxin-1A full-length, syntaxin-1A-NT (2–190), or syntaxin-1A-CT (151–264) with immobilized full-length DAP kinase and found that syntaxin-1A-CT fragment was efficiently phosphorylated in vitro by DAP kinase in a Ca^{2+}/CaM-dependent manner (data not shown). As the consensus sequence for DAP kinase phosphorylation has not yet been illustrated, we used site-directed mutagenesis to generate seven syntaxin-1A mutants in which the serine, at positions 188, 200, 208, 249, or 259, or the threonine, at positions 197 or 251, was substituted with alanine. While T197A, S200A, S208A, S249A, T251A, and S259A mutants served as efficient substrates for the purified DAP kinase KD (data not shown), the S188A mutation effectively eliminated 32P incorporation (251), was substituted with alanine. While T197A, S200A, S208A, S249A, T251A, and S259A mutants served as efficient substrates for the purified DAP kinase KD (data not shown), the S188A mutation effectively eliminated 32P incorporation following incubation with DAP kinase KD (Fig. 3B), indicating that serine 188 of syntaxin-1A is the primary phosphorylation site for DAP kinase. The DAP kinase-mediated phosphorylation sequence of syntaxin-1A, X(S/T)X/K/R/QAL, is conserved among the syntaxin-1–4 isoforms, but is unique from the consensensus phosphorylation sequences of CaMKII, PKC, PKA, and casein kinases I and II.

To confirm that serine 188 is a dominant phosphorylation site of syntaxin and argue against trace levels of syntaxin phosphorylation, we performed stoichiometric analysis of phosphorylation of purified syntaxin-1A by DAP kinase in vitro. The reactions included equal amounts (3.8 pmol) of syntaxin-1A or myosin light chain (MLC) (10 pmol) and excess DAP kinase and [γ-32P]ATP, and were terminated at various time points by the addition of SDS sample buffer and boiling. The products were separated by SDS-PAGE, stained with Coomassie Blue to verify equal amounts of protein loading, the gel dried, and finally submitted to autoradiography. To quantify total P incorporation, gel slices corresponding to phosphorylated syntaxin bands were excised and scintillation counted. Stoichiometry values were expressed as the ratio of moles of phosphate (Pi) incorporated per mole of syntaxin-1A or MLC and plotted against reaction time. The maximal stoichiometric ratio of ~0.4 under our phosphorylation conditions approximates that of MLC (Fig. 3B, C, and D), a classic DAP kinase substrate with one dominant phosphorylation site (62); this is consistent with the results of our mutagenetic studies, which identified one phosphorylation site in syntaxin for DAPK. The relatively low stoichiometry for phosphorylation of both syntaxin and MLC (~0.4) is probably a result of our phosphorylation assay, which does not replicate the optimal conditions for DAP kinase activity or lacks a cofactor for maximal activation of the recombinant DAP kinase in vitro.

In Vivo Phosphorylation of Syntaxin-1A by DAP Kinase—While our biochemical experiments showed that DAP kinase incorporates 32P into syntaxin-1A in a Ca^{2+}-dependent manner, the conditions used for in vitro phosphorylation may not reflect conditions found in the native cellular environment. In addition, treatment of proteins with detergent for solubilization may expose sites that are normally not available for phosphorylation in vivo. Therefore, to investigate in vivo phosphorylation, we performed back-phosphorylation assays (40) in HEK293T cells co-transfected with cDNAs of syntaxin-1A and DAP kinase. In this procedure, endogenous phosphate is incorporated in vivo into syntaxin-1A after stimulation with ionomycin, a Ca^{2+} ionophore that induces Ca^{2+} channel-independent Ca^{2+} influx. The cell lysates from stimulated and non-stimulated HEK293T cells are then processed for in vitro phosphorylation (back-phosphorylation) with immunoprecipitated full-length DAP kinase to incorporate [γ-32P]ATP into syntaxin-1A that was left unphosphorylated in vivo after stimulation. In this protocol, a decrease in back-phosphorylation in vitro reflects in vivo phosphorylation of syntaxin-1A by DAP kinase in transfected HEK293T cells in response to Ca^{2+} influx.

It has been reported that the DAP kinase K42A mutant, in which the conserved lysine 42 in the kinase subdomain II is substituted with alanine, is catalytically inactive to phosphorylate MLC (48). We therefore generated a DAP kinase K42A mutant to use as a negative control in our in vivo phosphorylation studies. To confirm that K42A DAP kinase is catalytically inactive, both HA-tagged DAP kinase wild type and K42A mutant expressed in HEK293T cells were immunoprecipitated with anti-HA antibody and then incubated with recombinant syntaxin-1A-CT in the presence of Ca^{2+}, calmodulin, and [γ-32P]ATP. Syntaxin-1A-CT was phosphorylated by DAP kinase wild type but not by the K42A mutant (Fig. 4A), consistent with previous findings that mutation of this site abolishes kinase activity (48). The K42A mutation does not affect binding of DAP kinase to syntaxin since equal amounts of DAP kinase was coimmunoprecipitated by anti-syntaxin-1 antibody from the lysates of the co-transfected HEK293T cells (Fig. 4, B and C).

As expected, the amount of 32P incorporation during in vitro back-phosphorylation was significantly reduced after stimulation of DAP kinase/syntaxin-1A co-transfected cells with ionomycin. However, stimulation of cells co-transfected with DAP kinase K42A/syntaxin-1A caused no significant reduction in [γ-32P]ATP incorporation in vitro when compared with the non-stimulation control (Fig. 4D). Coomassie Blue staining corroborated the identity of the phosphorylated band (35 kDa) as syntaxin-1A and was used to normalize back-phosphorylation to the amount of syntaxin-1A immunoprecipitated by the anti-syntaxin-1 antibody. Quantitative analysis of the results of seven independent experiments showed that ionomycin stimulation significantly reduced back-phosphorylation of syntaxin-1A from the cells transfected with the cDNAs of DAP kinase/syntaxin-1A to 82.1 ± 5.9% of the non-stimulated control value (mean ± S.E., n = 7, p < 0.05; Fig. 4E). In contrast, no significant decrease in back-phosphorylation of syntaxin-1A (93.5 ± 5.3%, mean ± S.E., n = 7, p > 0.05) from the cells transfected with the cDNAs of DAP kinase K42A/syntaxin-1A was observed. These data suggest that syntaxin-1A is a substrate for DAP kinase-mediated phosphorylation in vivo through a Ca^{2+}-dependent pathway.

Biochemical Effects of DAP Kinase Phosphorylation of Syntaxin-1A—Since SNAP-25 (1) and Munc18-1, a regulatory protein involved in synaptic vesicle docking and fusion (53 and 63–64), are two of the most important binding partners of syntaxin-1A in neurotransmitter release, we first tested whether syntaxin-1A phosphorylation by DAP kinase affected the mutually exclusive interactions of syntaxin 1A with these two proteins. We incubated immobilized DAPK-phosphorylated or unphosphorylated GST-syntaxin-1A(TM1–264), a mutant lacking the C-terminal transmembrane segment or GST as a control with His-tagged Munc18-1 or SNAP-25. As shown in Fig. 5, while phosphorylated syntaxin-1A did not affect its
binding to SNAP-25, its binding to Munc18-1 decreased dramatically to 54.80 ± 7.98% (n = 8, p < 0.01) of Munc18-1 binding by unphosphorylated syntaxin-1A.

Our mutagenesis studies demonstrated syntaxin-1A serine 188 to be the primary site for DAP kinase phosphorylation. Ser-188 is located in the linker region between N-terminal Habc and C-terminal H3 coil domains, adjacent to the binding site for Munc18-1. To test whether introduction of a negatively charged residue at this site has any effect on binding of syntaxin-1A to Munc18-1, we attempted to mimic complete phosphorylation of serine 188 by mutating it to a negatively charged residue at this site. To test whether introduction of a negatively charged residue at this site has any effect on binding of syntaxin-1A to Munc18-1, we attempted to mimic complete phosphorylation of serine 188 by mutating it to a negatively charged residue at this site. To test whether introduction of a negatively charged residue at this site has any effect on binding of syntaxin-1A to Munc18-1, we attempted to mimic complete phosphorylation of serine 188 by mutating it to a negatively charged residue at this site.
wild-type syntaxin-1A. Munc18-1, but did not affect binding to SNAP-25 relative to that of

binding to other SNAREs further supports the notion that only the C-terminal H3 coiled-coil domain, but not the syntaxin linker region, is involved in SNARE core complex formation (1, 51, 67).

**DISCUSSION**

Our current findings suggest that syntaxin-1 could serve as a substrate for DAP kinase, a Ca\(^{2+}\)/CaM-dependent protein kinase. Using the yeast two-hybrid selection approach with the C-terminal half of syntaxin-1A as bait, we have isolated the cDNA encoding DAP kinase. We then confirmed that syntaxin-1 and DAP kinase bind to each other directly via their C-terminal domains. We have found that DAP kinase is relatively enriched in hippocampus and cortex and present in synaptic preparations, and partially colocalizes with syntaxin-1 in the plasma membrane along the processes of cultured hippocampal neurons. Furthermore, we have demonstrated that syntaxin-1A is phosphorylated by DAP kinase both in vitro and in vivo in a Ca\(^{2+}\)-dependent manner, with maximal phosphorylation occurring when [Ca\(^{2+}\)] reaches 10 \(\mu M\). PCR-based site-directed mutagenesis pinpoints the syntaxin-1A phosphorylation site to serine 188. Finally, we show that phosphorylation of syntaxin-1A or its S188D mutant, which mimics a state of complete phosphorylation, significantly decreases the interaction of syntaxin-1A with Munc18-1, a protein that regulates the synaptic vesicle docking/priming process by forming a syntaxin-Munc18 complex (53, 63–64). DAP kinase was first reported to be a mediator of apoptosis (47); however, its downstream targets in apoptosis or otherwise have not yet been identified. Because of its restricted expression in adult rat hippocampus, cortex, and olfactory bulb, its presence in the cytosolic fraction of synaptosomes, its partial colocalization with syntaxin-1 in neuronal processes, its direct association with syntaxin-1A, and its Ca\(^{2+}\)-dependent phosphorylation of syntaxin-1A, we propose that DAP kinase plays a non-apoptotic role in modulating synaptic transmission.

The relatively low quantities of endogenous DAPK associated with syntaxin-1 in extracts derived from rat brain could be a consequence of the significant impact of detergents on the stability of a dynamic kinase-substrate complex. Furthermore, our immunocytochemical studies in cultured hippocampal neurons suggest a relative enrichment of DAPK in some restricted synapses; if this is the case, our brain homogenates, which are derived from a mixed population of neurons and glial cells, would not allow us to estimate the abundance of this complex in specific synapses. Finally, the conformation and thus the stability of the DAPK-syntaxin complex in solution with detergent may not reflect that of the complex in its in vivo membrane-bound environment. Thus, our comminoprecipitation data should not be considered as evidence of the relative enrichment of a native DAPK-syntaxin complex in intact synapses. Rather,
the in vivo data provide evidence showing that endogenous DAPK indeed associates with syntaxin, and strengthens the conclusions of our in vitro studies.

Most significantly, we have found that the phosphorylation of syntaxin-1A by DAP kinase is Ca^{2+}-dependent, and that this phosphorylation can exact a significant and selective effect on syntaxin binding with its regulatory protein Munc18-1, while maintaining its properties governing assembly and the binding stability of the SNARE complex. The sharp rise in intracellular calcium levels ([Ca^{2+}]_i) that accompanies the arrival of an action potential to the presynaptic terminal is critical not only as a trigger for synaptic vesicle fusion with the terminal plasma membrane, but also as a regulator of the multiple steps constituting the synaptic vesicle life cycle. Endocytosis is tightly regulated by [Ca^{2+}]_i levels during action potential firing at hippocampal synapses (68). Activity-dependent mobilization of synaptic vesicles is also elevated during stimulation (46, 69, 70). The priming stage of synaptic vesicles is also tightly regulated by [Ca^{2+}]_i, the supply of releasable vesicles is accelerated during high frequency stimulation in both chromaffin cells (71) and at different neuronal synapses (72–74) in a Ca^{2+}-dependent manner. One mechanism by which [Ca^{2+}]_i could affect multiple steps of vesicle trafficking is through protein phosphorylation/dephosphorylation by Ca^{2+}-dependent kinases/phosphatases.

Although our back-phosphorylation experiments detected a relatively low ratio (about 20%) of in vivo phosphorylation of syntaxin by DAPK in co-transfected 293 cells, multiple repeats (n = 7) of these experiments showed that this in vivo phosphorylation event is consistently and statistically significant when compared with syntaxin phosphorylation by the control K42A DAPK mutant. Several factors could contribute to the low ratio of in vivo syntaxin phosphorylation seen in 293 cells. First, the level of syntaxin expression in co-transfected 293 cells was much higher than that of DAPK; thus, only a relatively small percentage of total syntaxin could be phosphorylated by limited amounts of DAPK in vivo. Second, the targeting mechanisms responsible for syntaxin co-localization with DAPK at a subset of synapses are not present in 293 cells. Third, DAPK-mediated phosphorylation requires free Ca^{2+} levels above 10 μM, which would highly restrict syntaxin phosphorylation to some regions within the cell following ionomycin treatment. Finally, DAPK function in vivo could be further modulated by cellular cofactors. We lack knowledge of any potential activator or inhibitor of this newly discovered kinase, which could be limiting our capability to fully activate this kinase in vivo except by elevating intracellular Ca^{2+}.

As our data suggest that syntaxin-1A could be phosphorylated by DAP kinase when synaptic [Ca^{2+}]_i is elevated during action potential stimulation, it seems rational to ask whether phosphosyntaxin-1A is able to affect the formation of the SNARE complex and the syntaxin-Munc18-1 complex. We found that phosphorylation of syntaxin-1A or its S188D mutation reduces its association with Munc18-1 by 50%, while no significant effect is detected on its binding with SNAP-25 (72). Furthermore, syntaxin-1A S188D mutation affects neither the assembly nor stability of the SNARE complex in vitro (Fig. 7). The formation of the trans-SNARE complex is thought to bring lipid membranes in close apposition, perhaps even resulting in merging of the two bilayers (10). Unlike VAMP-2 and SNAP-25, in which most of the protein sequence participates in core complex formation, the C-terminal third of the cytoplasmic region of syntaxin is involved in formation of the SNARE core complex. The N-terminal half of syntaxin forms an independently folded domain (75) and is involved in binding to several SNARE regulatory proteins, Munc13 (76) and Munc18-1 (77). Particularly, Munc18-1 was reported to bind syntaxin with high affinity, and this binding is mutually exclusive with SNARE complex formation (51, 65–66). In the three-dimensional crystal structure of the neuronal-Sec1/Munc18-1-syntaxin-1A complex (78), the N-terminal half of Habc domain of syntaxin-1A is folded back onto the C-terminal H3 domain, representing a “closed” conformation, and the linker region (residues 145–188) between the N-terminal half and C-terminal coil domain is structured in the favorable environment provided by the lower part of domain 3 of Munc18-1. For this reason, the linker region of syntaxin-1A is critical both as a structural switch for syntaxin to transform from a closed conformation to an open one, and for its binding affinity to Munc18 and other SNARE proteins, which was confirmed by an earlier finding that residue mutations in the linker region of syntaxin-1A abolishes its binding to Munc18-1 and consequently inhibits secretion in PC12 cells (51). Likewise, phosphorylation in the linker region would be predicted to have biochemical import for syntaxin conformation and syntaxin interactions with other binding partners. We speculate that DAP kinase phosphorylation of syntaxin-1A at serine 188, which is located in the end of the linker region, would induce conformational changes in syntaxin-1A and thereby affect its binding to Munc18-1. Our in vitro binding studies, which demonstrate that the phosphorylation of syntaxin-1A or its S188D mutant decreases its binding to Munc18-1 by about 50% without affecting the assembly and stability of the SNARE complex, supports this hypothesis.

Munc18 has been proposed to play both activating (63, 64) and inhibitory (79) roles in synaptic vesicle exocytosis by acting at different steps in the release pathway. One of the most well characterized roles for Munc18-1 is to sequester syntaxin from binding to SNAP-25 and inhibit formation of the SNARE complex. However, recent work from knockout animals showed that Munc18 could also function upstream of SNARE complex formation and promote large dense-core vesicle (LDCV) docking in chromaffin cells (53). Thus, the existence of cellular signal pathways to regulate the switch between the assembly/disassembly of the Munc18-syntaxin complex in response to synaptic activity could be of physiological import. Our present study provides a novel signal transduction pathway by which the formation of the syntaxin-Munc18 complex could be regulated via syntaxin phosphorylation in response to intracellular [Ca^{2+}]_i and synaptic activity.

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Ca$^{2+}$-dependent Phosphorylation of Syntaxin-1A by the Death-associated Protein (DAP) Kinase Regulates Its Interaction with Munc18
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