Binary Interactions of the SNARE Proteins Syntaxin-4, SNAP23, and VAMP-2 and Their Regulation by Phosphorylation†

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ABSTRACT: The SNARE hypothesis proposes that synaptic vesicles dock at presynaptic membranes via interactions among the vesicular, integral membrane proteins VAMP (vesicle-associated membrane protein) and synaptotagmin and the target membrane proteins SNAP25 (synaptosome-associated protein with an M_r of 25 kDa) and syntaxin-1. Non-neuronal cells express isoforms of these proteins, believed to mediate secretory vesicle docking and/or fusion. Secretion in neuronal and non-neuronal systems differs in time course, Ca^{2+} dependence, and regulatory input. It is not known whether the non-neuronal protein isoforms form complexes akin to those of their neuronal counterparts. In this study, we defined the binding characteristics of three SNARE proteins: SNAP23, VAMP-2, and syntaxin-4. Binary, saturable interactions among all three partners (VAMP-2−syntaxin-4, VAMP-2−SNAP23, and SNAP23−syntaxin-4) were measured in vitro. Unlike its neuronal counterpart, SNAP23 did not potentiate VAMP-2 binding to its putative t-SNARE partner, syntaxin-4. The susceptibility of SNARE proteins to phosphorylation by exogenous kinases and their impact on binary interactions were explored. Syntaxin-4 was efficiently phosphorylated by casein kinase II (CKII) and cAMP-dependent protein kinase (PKA) (incorporating 0.8 and 3.9 mol of phosphate/mol of syntaxin-4, respectively), while syntaxin-1 was only strongly phosphorylated by CKII. Each of the syntaxin isoforms was weakly phosphorylated by protein kinase C (PKC) (<0.05 mol of phosphate/mol of syntaxin-4). Importantly, PKA but not casein kinase II phosphorylation of syntaxin-4 disrupted its binding to SNAP23. We hypothesize that PKA may modulate syntaxin-4-dependent SNARE complex formation to regulate exocytosis in non-neuronal cells.

The t-SNAREs[1] SNAP25 and syntaxin-1 as well as the v-SNARE VAMP-2 play a central role in synaptic vesicle exocytosis (*1*, *2*). Functional perturbation of any one of these three proteins by a variety of means abolishes or significantly decreases neurotransmitter release (*3*−*6*). Several laboratories have isolated a tripartite complex of VAMP-2, SNAP25, and syntaxin-1, and it has been suggested that formation of this complex is an essential step in the process of neurotransmitter release (*2*, *7*).

Recombinant forms of VAMP-2, SNAP25, and syntaxin-1 have been used to study the binding of these proteins in vitro. The interactions SNAP25−syntaxin-1 and VAMP-2−SNAP25 exhibit high affinity, in contrast to the VAMP-2−syntaxin-1 interaction which is comparatively weaker. However, VAMP-2 binds tightly to syntaxin-1 in the presence of SNAP25, leading to the conclusion that SNAP25 potentiates the binding of VAMP to syntaxin-1 (*8*, *9*). Together with the in vivo studies, these results have led to the suggestion that the t-SNAREs may form a high-affinity target for the v-SNARE which may thereby promote synaptic vesicle docking and subsequent fusion.

Isoforms of the three SNARE proteins described above have been shown to exist in other tissues. A SNAP25 isoform, SNAP23, was originally cloned from human B lymphocytes and is widely expressed outside of neuronal and neuroendocrine cells (*10*, *11*). Its mouse homologue was named syndet (*12*). SNAP23 is thought to be a t-SNARE because it can bind to recombinant VAMP-2 in vitro (*10*, *13*). The syntaxin-1 isoform, syntaxin-4, has also been found in a variety of tissues, including brain, lung, spleen, kidney, liver, skeletal muscle, and fat (*14*, *15*). Though other well-characterized syntaxin isoforms (syntaxins-2, -3, and -5) exist, only syntaxins-1 and -4 bind VAMP-2 (*8*). Thus, SNAP23 and syntaxin-4 have been considered putative t-SNARE partners for VAMP-2 in non-neuronal cells. Three well-characterized isoforms of the v-SNARE VAMP have been described to date, with VAMP-2 and

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[1] Abbreviations: NSF, *N*-ethylmaleimide-sensitive fusion factor; GST, glutathione *S*-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNAP, soluble NSF attachment protein; SNAP25, synaptosome-associated protein of 25 kDa; SNAP23, SNAP25-like protein of 23 kDa; VAMP, vesicle-associated membrane protein; SNARE, SNAP receptor; v-SNARE, vesicle SNARE; t-SNARE, target SNARE; cAMP, cyclic adenosine monophosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; CKII, casein kinase II; GLUT4, glucose transporter isoform 4; ECL, enhanced chemiluminescence.

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VAMP-3/cellubrevin having the widest distribution of expression. Both VAMP-2 and VAMP-3−cellubrevin have been detected in fat and muscle tissues (*16*−*18*) and in a variety of secretory cells (*19*−*21*).

Although VAMP-2, SNAP23, and syntaxin-4 have all been shown to bind to each other in binary combinations (*9*, *10*, *22*), the binding constants for these interactions have not been determined. Furthermore, the cellular mechanisms regulating these interactions are not understood. In this study, we have quantitated the interactions between VAMP-2, SNAP23, and syntaxin-4 in binary as well as ternary combinations and examined whether they can form complexes resistant to SDS. Further, we have screened the susceptibility of SNAP25, SNAP23, and syntaxins-1−4 to becoming phosphorylated by exogenous kinases and examined the consequence of phosphorylation on the binding parameters. The results described below suggest that interactions occurring between individual sets of SNARE isoforms have distinct properties and may be uniquely regulated.

EXPERIMENTAL PROCEDURES

Recombinant Fusion Proteins. GST fusion proteins containing the cytoplasmic domains of syntaxins-1a and -2−4 as well as full-length SNAP23 and SNAP25 were prepared as described previously (*9*, *11*, *23*). The full cytoplasmic domain of VAMP-2 and full-length SNAP25 were expressed as N-terminal six-His fusion proteins using the pQE30 expression vector in BSJ72-competent *Escherichia coli*. Both six-His proteins were purified as described (*24*), but without cation exchange chromatography. Syntaxin-1 and syntaxin-4 were cleaved from GST by incubating 3 μg of thrombin/mg of GST−syntaxin for 150 min at 25 °C. SNAP23 was cleaved from GST by incubating 13 μg of thrombin/mg of GST−SNAP23 for 24 h at 4 °C. The concentration of recombinant proteins bound to glutathione−agarose beads was determined by boiling the beads to remove protein, for analysis by SDS−PAGE, and comparing the Coomassie blue staining of the supernatant protein with that of known amounts of bovine serum albumin.

In Vitro Binding Assays. Binding assays were performed following the procedure of Pevsner et al. (*8*) for neuronal SNAREs. Briefly, recombinant GST−SNARE fusion proteins (termed the "fixed" SNARE) was bound to glutathione−agarose beads and mixed with soluble recombinant SNARE(s) cleaved from GST as described above. The proteins were incubated in 200 μL of binding buffer [5 mM HEPES (pH 7.4), 70 mM KCl, 1 mM $MgCl_2$, and 0.25% Triton X-100] for 2 h at 4 °C with end-over-end rotation. The beads were collected with a low-speed centrifugation (10 s at ~1000*g*) and washed twice with 1 mL of binding buffer. Twenty microliters of 2× SDS sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.01% bromophenol blue] was added, and each tube was boiled for 2−3 min for separation by SDS−PAGE on 14% polyacrylamide gels (*25*).

SDS-Resistant Complexes. Rat brain homogenate solubilized in Triton X-100 was prepared as described (*26*). 3T3-L1 mouse adipocyte crude membranes were prepared as described (*11*). The SDS resistance of endogenous SNARE complexes was assayed using 20 μg of Triton X-100-solubilized rat brain protein or 90 μg of Triton X-100-solubilized 3T3-L1 mouse adipocyte crude membrane protein incubated at 4 °C for 2 h. The SDS resistance of recombinant protein complexes was tested using equimolar amounts of the recombinant forms of VAMP-2, SNAP25, SNAP23, syntaxin-1, and syntaxin-4, all cleaved of GST. The proteins were incubated for 2 h at 4 °C in binding buffer. SDS sample buffer (2×) was added, and some of the samples were boiled for 3 min.

Coimmunoprecipitation. Whole cell lysates of 3T3-L1 mouse adipocytes were prepared as described (*22*) and precleared by incubation for 2 h at 4 °C with 40 μL of protein A−Sepharose. Antibodies to SNAP23 and syntaxin-4 (see antibody description below, 2 μg of IgG each) were precoupled to protein A−Sepharose by incubation for 2 h at 4 °C in immunoprecipitation buffer [20 mM HEPES (pH 7.4), 100 mM KCl, 2% Triton X-100, and 2 mM EDTA]. The precoupled beads were pelleted, washed twice with immunoprecipitation buffer, and added to the supernatant from the preclearing step. Lysate and antibody were incubated for 2 h at 4 °C with constant rotation, then the Sepharose was pelleted and washed three times with immunoprecipitation buffer. Fifty microliters of SDS sample buffer was added; the samples were analyzed by SDS−PAGE in 14% polyacrylamide gels, and proteins were transferred to nitrocellulose and immunoblotted as described below.

Protein Detection and Antibody Description. For SDS resistance assays and in vitro binding assays, proteins were separated by SDS−PAGE, transferred to nitrocellulose, and stained with 0.05% Ponceau S in 1% acetic acid for 3−4 min. The nitrocellulose membranes were cut horizontally to separate various SNAREs and immunoblotted with the following antibodies: αSN23.C12, a rabbit polyclonal antibody raised against a hemocyanine-linked peptide comprising the 12 C-terminal amino acids of SNAP23 (*11*); αVAMP-2, a rabbit polyclonal antibody raised against a GST fusion protein encoding the cytoplasmic domain of VAMP-2 (*16*); αSy4, an affinity-purified polyclonal antibody raised against a GST fusion protein encoding the cytoplasmic domain of syntaxin-4 (*14*); SP5, a monoclonal antibody against syntaxin-1 (Serotec, Hornby, ON); and C171.1, a monoclonal antibody against SNAP25 (generous gift from R. Jahn, Yale University, New Haven, CT). For the majority of immunoblots, goat-α-mouse−HRP (1:5000) or goat-α-rabbit−HRP (1:7500) conjugates were used to detect monoclonal or polyclonal primary antibodies, respectively. To confirm the accuracy of ECL, ^{125}I-conjugated protein A (1:10000) was also used to detect polyclonal primary antibodies. ECL was used to detect binding of horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch), while phosphorimaging was used to detect [^{125}I]protein A.

Quantitation of Immunoblots. Bands detected by ECL were scanned with a ScanMaker IIHR flatbed scanner (Microtek, Redondo Beach, CA) and quantitated using NIH Image 1.61 software (National Institutes of Health, Bethesda, MD). Care was taken during exposure of the X-ray film to ensure that all readings were in the linear range. Confirming the quantitative validity of the results, the half-maximal binding value measured with ECL (0.55 μM) was found to be comparable to the value measured with ^{125}I (0.57 μM) in one of the individual experiments measuring syntaxin-4−SNAP23 coupling. Titration curves were fitted, and apparent

half-maximal binding coefficient values were determined using Origin 4.1 (Microcal). Student's *t* tests were performed using Excel 5.0 (Microsoft, Redmond, WA).

In Vitro Phosphorylation. Human recombinant CKII, rat brain PKC [a mixture of α (type III), βI, βII (type II), and γ (type I)], and the bovine heart catalytic subunit of PKA were obtained from Boehringer Mannheim (Laval, PQ) and used according to the manufacturer's instructions. To measure phosphorylation by PKA, 20 pmol of the indicated SNARE was incubated for 5 min at 30 °C in 50 μL of 50 mM MES (pH 6.9), 10 mM $MgCl_2$, 0.5 mM EDTA, 150 μM ATP (0.4 μCi/nmol [γ-^{32}P]ATP), and 1 mM dithiothreitol, with or without 4 milliunits/mL PKA. To measure phosphorylation by CKII, 20 pmol of the indicated SNARE was incubated for 5 min at 37 °C in 70 μL of 20 mM MES (pH 6.9), 130 mM KCl, 50 μM ATP (0.86 μCi/nmol [γ-^{32}P]-ATP), 10 mM $MgCl_2$, and 4.8 mM dithiothreitol, with or without 0.71 milliunits/mL CKII. To measure phosphorylation by PKC, 20 pmol of the indicated SNARE was incubated for 5 min at 30 °C in 200 μL of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM ATP (6 μCi/nmol [γ-^{32}P]-ATP), and 0.5 mM dithiothreitol, with or without 0.25 milliunits/mL PKC, and with or without the activators 500 μM $CaCl_2$, 100 μg/mL phosphatidylserine, and 20 μg/mL 1,2-dioleoyl-*sn*-glycerol (Boehringer Mannheim). Where indicated, to maximize phosphate incorporation by PKC, 8 pmol of SNAP25 or SNAP23 was incubated for 30 min at 20 °C in 5 μL of 50 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 50 mM imidazole, 0.06% CHAPS, 100 μM $CaCl_2$, 50 μM EGTA, 1 mM dithiothreitol, and 40 μM ATP (50 μCi/nmol [γ-^{32}P]ATP), with or without 4 milliunits/mL PKC (Calbiochem, La Jolla, CA).

For preparation of large amounts of phosphorylated syntaxin-4, 100 pmol of agarose-coupled syntaxin-4 was incubated under conditions similar to those described above with the following exceptions: no [γ-^{32}P]ATP was used, the kinase and ATP concentrations were doubled, and the reactions were allowed to proceed for 45 min. The agarose beads were then collected and washed twice with binding buffer to remove reaction components and then used in SNARE binding assays as described above.

RESULTS

In Vitro SNARE Binding. Binding assays were performed to quantitate interactions between the three possible binary combinations of SNAP23, VAMP-2, and syntaxin-4. For the two combinations involving syntaxin-4, a fixed concentration of the GST−syntaxin-4 fusion protein (0.3 μM) was titrated with increasing concentrations of SNAP23 or VAMP-2. To study the interaction of VAMP-2 with SNAP23, 0.3 μM GST−VAMP-2 was titrated with increasing concentrations of SNAP23. Specific and saturable binding of the soluble SNAREs to the fixed SNAREs was observed for all three binary interactions (Figure 1). The syntaxin-4−SNAP23 interaction was stronger than that of the other two pairs (syntaxin-4−VAMP-2 or SNAP23−VAMP-2), with an apparent half-maximal binding coefficient of 0.76 ± 0.15 μM (Figure 1a).

Saturation of syntaxin-4 with VAMP-2 was eventually reached at approximately 10 μM VAMP-2, and the resulting apparent half-maximal binding coefficient was 4.4 ± 1.5 μM

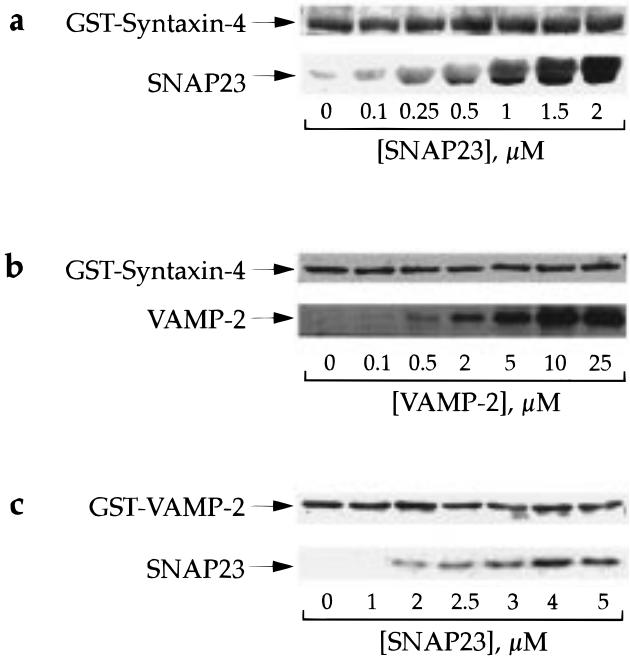

FIGURE 1: Binary binding of SNAP23, syntaxin-4, and VAMP-2. Increasing concentrations of SNAP23 (a and c) or VAMP-2 (b), as indicated, were used to titrate fixed 0.3 μM GST−syntaxin-4 (a and b) or fixed 0.3 μM GST−VAMP-2 (c) to saturation. Glutathione−agarose was used to sediment proteins bound to syntaxin-4. The pellets were analyzed by 14% SDS−PAGE and transferred to nitrocellulose. Ponceau S was used to visualize the fixed GST fusion protein constructs (i.e., GST−syntaxin-4 and GST−VAMP-2), while soluble SNAREs were immunoblotted with appropriate antibodies (αSN23.C12 at 1:2000 and αVAMP-2 at 1:1000). Shown is one experiment representative of three experiments.

(Figure 1b). SNAP23 bound to VAMP-2 with an apparent half-maximal binding coefficient of 2.8 ± 0.1 μM (Figure 1c). Nonspecific binding of VAMP-2 and SNAP23 to GST alone was found to account for less than 10% of the total soluble SNARE pulled down by GST−syntaxin-4 (results not shown). Since all of the recombinant proteins may not be in a fully native conformation, these values represent the minimum binding affinities of the individual SNARE proteins.

SNAP25 has been shown to enhance the binding of VAMP to syntaxin-1 in vitro (*8*, *9*). It is not known whether SNAP23, syntaxin-4, and VAMP-2 form a similar complex, although this has been suggested by making a simple analogy to their neuronal counterparts (*27*). Through a similar experimental approach like that used for the neuronal SNAREs, we titrated 0.3 μM syntaxin-4 with increasing concentrations of VAMP-2 in the presence of a saturating concentration (2 μM) of SNAP23. Whereas VAMP-2 binding to syntaxin-4 alone was saturable (Figure 1b), we were not able to saturate syntaxin-4 with VAMP-2 in the presence of SNAP23 (Figure 2). VAMP-2 association with syntaxin-4 was observed at high concentrations of VAMP-2, but this coincided with a decrease in the SNAP23 association with syntaxin-4.

Coimmunoprecipitation of SNARE Proteins. Given the binding affinities measured in vitro, we explored the possibility that endogenous SNAP23, syntaxin-4, and VAMP-2 interact in vivo. Previously, we have reported that immunoprecipitates of syntaxin-4 from 3T3-L1 adipocyte cell lysates contain VAMP-2 (*22*). In this study, syntaxin-4 and

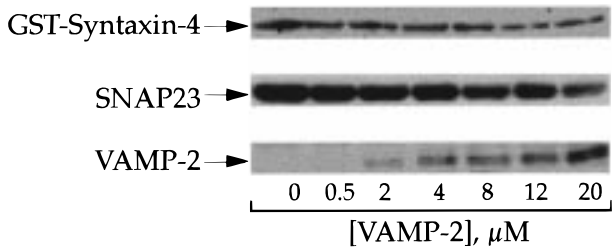

FIGURE 2: Ternary binding of SNAP23, syntaxin-4, and VAMP-2. GST−syntaxin-4 (0.3 μM) was incubated in the presence of a saturating concentration of SNAP23 (2 μM) and the mixture titrated with increasing concentrations of VAMP-2. Glutathione−agarose was used to sediment proteins bound to syntaxin-4. The pellets were analyzed by 14% SDS−PAGE and transferred to nitrocellulose. GST−syntaxin-4 levels on the nitrocellulose were visualized with Ponceau S staining, while SNAP23 and VAMP-2 were detected by immunoblotting with appropriate antibodies (αSN23.C12 at 1:2000 and αVAMP-2 at 1:1000). Shown is one experiment representative of three experiments.

FIGURE 3: Coimmunoprecipitation of SNARE proteins from 3T3-L1 adipocytes. SNAP23 or syntaxin-4 was immunoprecipitated (IP) from detergent-solubilized whole cell extracts of 3T3-L1 adipocytes as indicated. Samples were analyzed by 14% SDS−PAGE, transferred to nitrocellulose, and immunoblotted (IB) with the αVAMP-2, αSN23.C12, or αSy-4 antibody as described in Experimental Procedures. The positions of VAMP-2, syntaxin-4, and SNAP23 are indicated.

SNAP23 were immunoprecipitated sequentially from 3T3-L1 whole cell lysates as described in Experimental Procedures. After SDS−PAGE and transfer to nitrocellulose, the SNAP23 immunoprecipitate was probed for syntaxin-4 and VAMP-2 while the syntaxin-4 immunoprecipitate was probed for SNAP23. Syntaxin-4 and VAMP-2 both coimmunoprecipitated with SNAP23, but interestingly, SNAP23 did not coimmunoprecipitate with syntaxin-4 (Figure 3).

SDS-Resistant SNARE Complexes. A property of the neuronal SNARE complex of SNAP25, syntaxin-1, and VAMP-2 is its ability to withstand denaturation by SDS (*7*). It is not known whether non-neuronal SNAREs can form such a complex or whether any one of them can substitute for their cognate neuronal SNARE in the complex. We therefore examined the ability of SNAP23 and syntaxin-4 to participate in SDS-resistant complexes. We determined the ability of both of the proteins to replace or be replaced by their neuronal counterparts as well as any possible role that GST may play in the formation of complexes by GST−syntaxin proteins.

The results depicted in Figure 4 confirm previous observations that syntaxin-1 and SNAP25 form a SDS-resistant complex with VAMP-2. These complexes could be detected in homogenized brain tissue or in mixtures of recombinant proteins. In contrast, however, substitution of either syntaxin-1 or SNAP25 with their respective non-neuronal counterparts did not allow formation of a SDS-resistant complex (Figure 4). In addition, SNAP23 and syntaxin-4

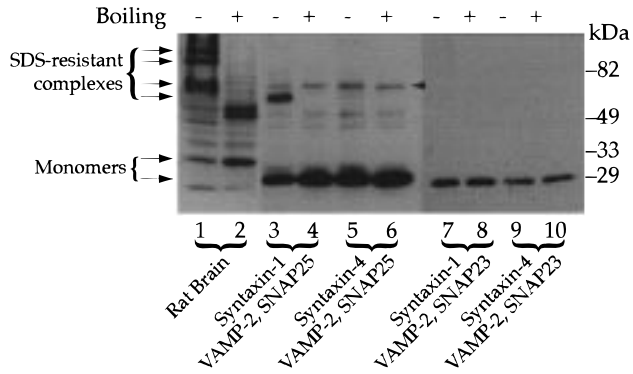

FIGURE 4: Only neuronal SNAREs form SDS-resistant complexes in vitro. Triton X-100-solubilized rat brain microsomes (lanes 1 and 2) were run as a positive control for SDS-resistant complexes. Equimolar concentrations (3.3 μM) of VAMP-2 (lanes 3−10), SNAP25 (lanes 3−6), SNAP23 (lanes 7−10), syntaxin-1a (lanes 3, 4, 7, and 8), and syntaxin-4 (lanes 5, 6, 9, and 10) were incubated for 2 h at 4 °C to allow complexes to form. SDS sample buffer similar in composition to SDS−PAGE buffer (see Experimental Procedures) was added, and samples were either boiled (lanes 2, 4, 6, 8, and 10) or not boiled (lanes 1, 3, 5, 7, and 9). Samples were analyzed by 12% SDS−PAGE, transferred to nitrocellulose, and immunoblotted with the syntaxin-1 antibody (lanes 1 and 2), SNAP25 antibody (lanes 3−6), or SNAP23 antibody (lanes 7−10) as described in Experimental Procedures. Bands of 60 kDa, indicated in lanes 3−6 by an arrowhead, are nonspecific immunoreactive contaminants. Shown is one experiment representative of six experiments.

(or GST−syntaxin-4) together with VAMP-2 do not form a complex resistant to SDS (Figure 4). No SDS-resistant complexes were seen in solubilized crude membrane fractions from 3T3-L1 adipocytes (results not shown), possibly due to the low abundance of SNARE proteins in these cells relative to that in neuronal cells. Experiments identical to these were also probed with primary antibodies to each of the different components of the complexes, to eliminate the possibility of epitope masking by the complex. The results confirmed the formation of SDS-resistant complexes among the neuronal SNAREs but not when syntaxin-4 or SNAP23 was used.

In Vitro Phosphorylation of SNAREs by Exogenous Kinases. Protein phosphorylation can regulate certain protein−protein interactions in vivo. As the first step in a search for possible phosphorylation events regulating SNARE proteins, we examined the susceptibility of recombinant SNAP25 and syntaxin-1 and their isoforms to phosphorylation by exogenous PKA, CKII, and PKC. GST alone as well as GST fusion proteins containing full-length SNAP25, full-length SNAP23, or cytoplasmic domains of syntaxins-1−4 was exposed to [γ-^{32}P]ATP in the presence or absence of each of the three kinases. Autoradiography was used to detect the phosphorylated species after SDS−PAGE. Analysis of the amino acid sequences of SNARE proteins by the PROSITE database revealed multiple possible sites for action of CKII, PKA, and PKC sites in SNAP25, SNAP23, and syntaxins-1−4. Using the experimental conditions described in Experimental Procedures, CKII phosphorylated syntaxin-1 and -4 (Figure 5a), and PKA phosphorylated syntaxin-4 only (Figure 5b). PKC weakly incorporated phosphate into all four syntaxin isoforms, albeit to different extents (Figure 5c), but long exposure times were required to detect this phosphorylation. None of the kinases could phosphorylate SNAP25 or SNAP23 under the conditions recommended by

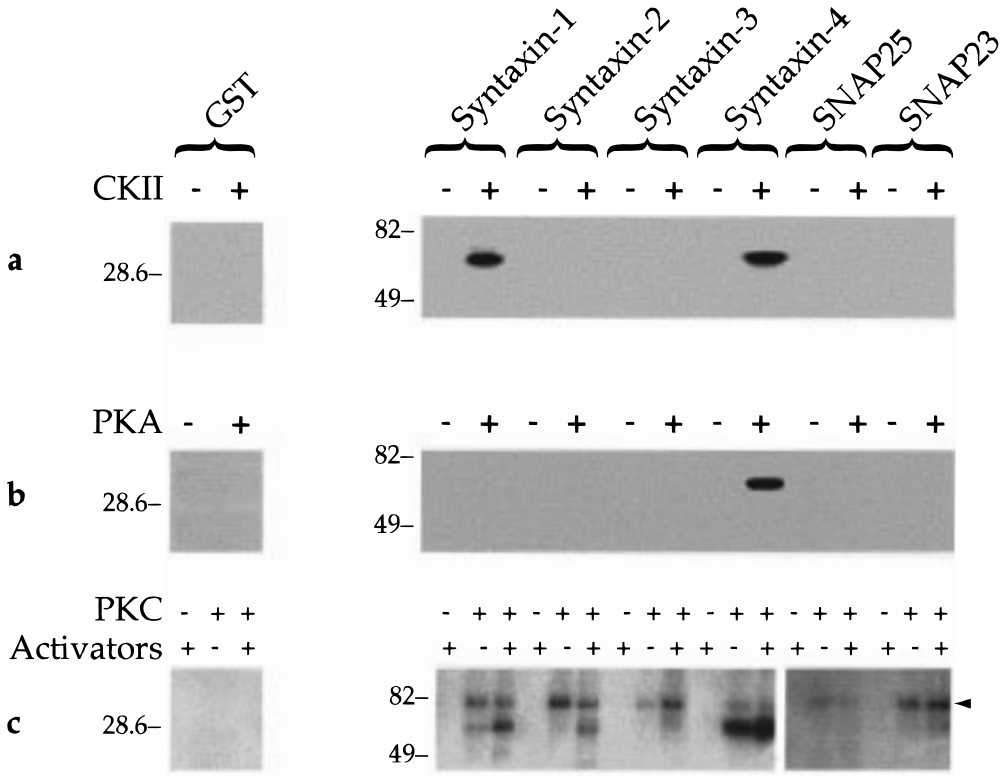

FIGURE 5: Syntaxin-4 is phosphorylated by CKII, PKA, and PKC. GST−SNARE constructs (20 pmol) were incubated with [^{32}P]ATP in the presence (+) or absence (−) of (a) CKII, (b) PKA, or (c) PKC and in the presence (+) or absence (−) of activators (Ca^{2+}, diacylglycerol, and phosphatidylcholine). Phosphorylation reaction products were separated by 10% SDS−PAGE, and phosphorylated bands were detected by autoradiography. Films in panels a and b were exposed for 24 h, and that in panel c was exposed for 170 h. The arrowhead in panel c denotes autophosphorylated PKC enzyme. Shown is one experiment representative of three experiments.

the kinase manufacturer. When conditions were altered to maximize phosphate incorporation by increasing enzyme, substrate, and ATP concentrations and specific activity (see Experimental Procedures), PKC was able to phosphorylate SNAP25 and to a lesser extent SNAP23 (results not shown). Importantly, none of the kinases tested were able to phosphorylate GST.

Stoichiometry of Phosphorylation. To examine the degree of phosphorylation obtained, we determined the stoichiometry of phosphate incorporation into syntaxin-4 by each of the kinases. Under the conditions used in these experiments, PKA incorporated 3.9 ± 0.4 mol of P/mol of syntaxin-4. CKII incorporated 0.81 ± 0.09 mol of P/mol of syntaxin-4. PKC incorporated <0.05 mol of P/mol of syntaxin-4. Very little phosphorylation was also observed for SNAP25. Only 0.01 mol of P/mol of SNAP25 could be phosphorylated using the high-efficiency reaction conditions. The level of phosphorylation of SNAP23 was even lower. Therefore, the following binding experiments were performed only with syntaxin-4 phosphorylated by PKA and CKII.

Effects of Syntaxin-4 Phosphorylation on SNARE Binding in Vitro. To determine the effect of phosphorylation of syntaxin-4 by CKII and PKA on its ability to bind soluble SNAP23 and/or VAMP-2, assays similar to those used in the titrations described in Figure 1 were performed with phosphorylated protein. In these binary interaction assays, a SNAP23 concentration of either 0.5 or 1.0 μM was used, while the VAMP-2 concentrations used were 2 and 8 μM. The concentrations were chosen to span the half-maximal binding values for their interactions with unphosphorylated syntaxin-4. In the ternary binding assay, 1.0 μM SNAP23 and 8 μM VAMP-2 were used with 0.3 μM (phospho)GST−syntaxin-4.

CKII phosphorylation did not affect the ability of syntaxin-4 to bind SNAP23 or VAMP-2 in either binary or ternary combinations (results not shown, $p > 0.05$). On the other hand, PKA-phosphorylated syntaxin-4 consistently bound 30% less SNAP23 than nonphosphorylated syntaxin-4, in both binary (Figure 6) and ternary (Figure 7) assays. In contrast, the binding of VAMP-2 did not appear to be affected by PKA phosphorylation in either the binary assay (results not shown, $p > 0.05$) or the ternary assay (Figure 7).

DISCUSSION

It is well established that SNARE proteins are required for the docking and fusion of vesicles with their target membrane in a variety of organisms and tissues (for reviews, see refs *1* and *27*). The observation that neuronal isoforms of SNARE proteins can assemble into a number of multimeric complexes has led to the suggestion that the stepwise progression of SNARE proteins from binary to ternary to 7S and finally to 20S complexes precedes the fusion of the vesicle with the target membrane (*2*, *28*). The natural extension of such a hypothesis is that individual SNARE isoforms would have cognate partners which would be able to form analogous complexes leading to fusion of specific vesicle classes with their appropriate target membranes. Many lines of evidence support the notion that SNAP23 and syntaxin-4 are the t-SNARE partners for the broadly expressed v-SNARE VAMP-2 (*10*, *22*, *23*, *29*). Furthermore, we and others have shown that these proteins are capable of

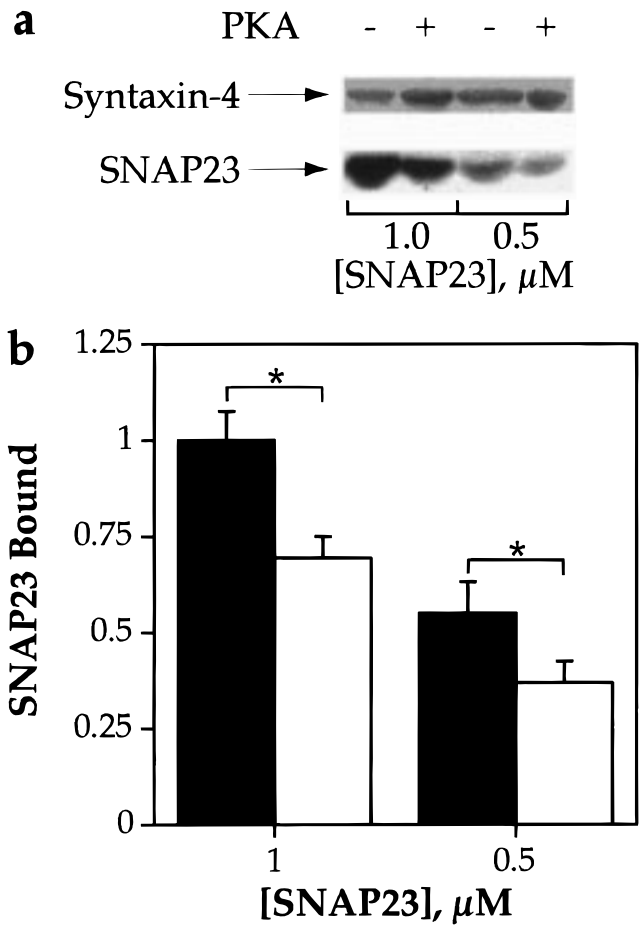

FIGURE 6: PKA phosphorylation of syntaxin-4 inhibits SNAP23 binding. (a) GST−syntaxin-4 was phosphorylated by PKA as in Figure 4. Samples at 0.3 μM were incubated with 0.5 or 1.0 μM SNAP23. Glutathione−agarose was used to sediment proteins bound to syntaxin-4. The pellets were analyzed by 14% SDS−PAGE and transferred to nitrocellulose. GST−syntaxin-4 levels on the nitrocellulose were visualized with Ponceau S staining, while SNAP23 was detected by immunoblotting (1:2000). Shown is one replicate of one representative experiment. (b) Quantitation of three experiments of three replicates each. Dark bars represent SNAP23 bound to nonphosphorylated GST−syntaxin-4, and open bars represent SNAP23 bound to PKA-phosphorylated GST−syntaxin-4 (an asterisk denotes a Student's t test, $p < 0.05$, relative to nonphosphorylated control).

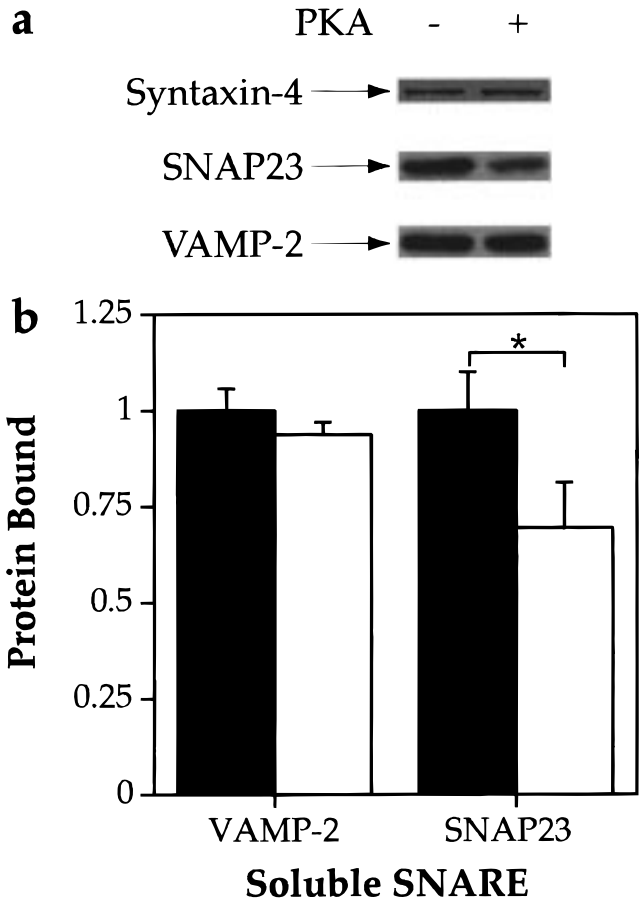

FIGURE 7: PKA phosphorylation of syntaxin-4 affects only SNAP23 binding. (a) GST−syntaxin-4 was phosphorylated by PKA as in Figure 4. Samples at 0.3 μM were incubated with 1.0 μM SNAP23 and 8 μM VAMP-2. Glutathione−agarose was used to sediment proteins bound to syntaxin-4. The pellets were analyzed by 14% SDS−PAGE and transferred to nitrocellulose. GST−syntaxin-4 levels on the nitrocellulose were visualized with Ponceau S staining, while SNAP23 and VAMP-2 were detected by immunoblotting (1:2000 and 1:1000, respectively). Shown is one replicate of one representative experiment. (b) Quantitation of three experiments of three replicates each. Dark bars represent soluble SNARE protein bound to nonphosphorylated GST−syntaxin-4, and open bars represent soluble SNARE protein bound to PKA-phosphorylated GST−syntaxin-4 (the asterisk denotes a Student's t test, $p < 0.05$, relative to nonphosphorylated control).

forming binary complexes (*8*−*10*, *22*). Here we show that they do so to the same rank order of affinity as the neuronal isoforms. The interaction between SNAP23 and syntaxin-4 was the strongest of the three pairs (apparent half-maximal binding coefficient of 0.76 ± 0.15 μM); the interaction between SNAP23 and VAMP-2 was found to be of intermediate affinity (apparent half-maximal binding coefficient of 2.8 ± 0.1 μM), and the binding of VAMP-2 to syntaxin-4 was the weakest. However, in contrast with previous results in which binding of VAMP to syntaxin-1 could not be saturated (*8*), we were able to saturate syntaxin-4 with VAMP-2 (apparent half-maximal binding coefficient of 4.4 ± 1.5 μM). Consistent with the strong binary interactions we observed in vitro, we also show that SNAP23 can coimmunoprecipitate both VAMP-2 and syntaxin-4. Although syntaxin-4 immunoprecipitates were shown to contain VAMP-2 (*22*), we found that these immunoprecipitates do not contain SNAP23 (Figure 3). This could potentially be due to a lower abundance of SNAP23 relative to other SNAREs in 3T3-L1 cells, or consistent with the in vitro results, it could be due to displacement of SNAP23 by VAMP-2 (see below). It is not likely to be due to the selectivity of the immunoprecipitating antibody for free syntaxin-4 since the antibody used to immunoprecipitate syntaxin-4 is a polyclonal one raised against the full cytoplasmic domain of syntaxin-4.

An unexpected finding of our studies is that we could not observe ternary complex formation among the non-neuronal combination of SNARE proteins, in either nondenaturing binding assays or SDS resistance assays. Not only was VAMP-2 unable to form a SDS-resistant complex with SNAP23 and syntaxin-4, but neither of these two proteins on their own was able to replace SNAP25 or syntaxin-1 in the neuronal SDS-resistant complex. Whereas the lack of SDS resistance does not directly prove the lack of ternary complex formation, it does suggest that if the complex exists the forces that keep the proteins together are much weaker than those involved in the tripartite complex of the neuronal

isoforms. Furthermore, when VAMP-2 at increasing concentrations was added to syntaxin-4 and SNAP23, VAMP-2 association with syntaxin-4 resulted in decreased SNAP23 association. These results suggest that SNAP23 and VAMP-2 may compete for a similar site on syntaxin-4 or, alternatively, that excess VAMP-2 removes unbound SNAP23 from its equilibrium with syntaxin-4. While SNAP23 immunoprecipitates contained both VAMP-2 and syntaxin-4, this does not constitute evidence of a ternary SNARE complex, since the stoichiometry of the three SNAREs in the immunoprecipitate is not known. It is conceivable that the immunoprecipitate contains binary complexes of SNAP23−VAMP-2 and SNAP23−syntaxin-4.

The lack of evidence for ternary complex formation in vitro among a non-neuronal combination of SNARE isoforms is puzzling and raises some interesting possibilities. The simplest explanation is that these three proteins do not form a ternary complex in vivo and that pairwise interactions are the only requirement for docking and fusion steps involving these SNAREs. Indeed, the failure to observe a saturable binding of VAMP to syntaxin-1 in previous studies (*8*) may indicate that a ternary complex is needed to strengthen the v-SNARE−t-SNARE complex between those isoforms, but the stronger binding of VAMP-2 to syntaxin-4 may allow us to bypass this requirement. A second possibility is that additional factors such as NSF or αSNAP are required for the formation of a stable complex. Indeed, when a membrane extract of rat adipocytes was incubated with *myc*-epitope-tagged NSF and αSNAP, the endogenous syntaxin-4 and VAMP-2 sedimented upon *myc*-epitope immunoprecipitation (*18*), and the immunoprecipitate was later shown to contain SNAP23 (*30*). These results, however, do not prove that the three SNAREs form a single complex. A third possibility is that the ternary complex forms but its dissociation rate is too rapid to be detected by our assay. One major difference between regulated fusion events in neurons and in non-neural cells is their time course; i.e., fusion of synaptic vesicles in neurons occurs within fractions of a millisecond, while exocytosis in many other cell types occurs much more slowly. The rapidity of synaptic vesicle exocytosis is thought to occur because vesicles are docked at the plasma membrane in a prefusion state requiring only a calcium influx to complete the fusion process. In contrast, in many of these non-neuronal cell types, regulated exocytosis may be controlled in part by rate-limiting steps prior to the docking and fusion process (*31*). For example, in the chromaffin cell, depolymerization of cortical actin appears to be necessary to facilitate exocytosis (*32*). In some cases, docking and fusion steps may occur constitutively after the regulatory signal, in which case the ternary complex would not have to wait for a fusion "trigger" as the neuronal complex does, and may, therefore, not have to be particularly stable.

In such a case, cellular regulation of the assembly of the binary complexes may be important in the control of exocytosis. Protein phosphorylation has been established as a universal method of regulating protein−protein interactions in almost every field of biology. Some SNARE proteins have been identified as targets of various kinases both in vivo (*23*, *33*, *34*) and in vitro (*35*). In this study, we demonstrate that syntaxin-4 is an efficient in vitro substrate for PKA and CKII. No other syntaxin tested was phosphorylated by PKA. Syntaxin-1 was phosphorylated by CKII, and SNAP25 was phosphorylated by PKC to low levels, consistent with previous observations (*33*, *35*). Hirling and Scheller (*35*) reported that PKA can also phosphorylate SNAP25, albeit to a very low extent, so it is possible that this level of phosphorylation was too low to be detected under the conditions we used. Our results further show that SNAP23 cannot be phosphorylated in vitro by either PKA or CKII, and only minimally by PKC.

Importantly, we have identified a functional consequence of syntaxin-4 phosphorylation by PKA. PKA-phosphorylated syntaxin-4 cannot bind the same amount of SNAP23 at equilibrium that nonphosphorylated syntaxin-4 can. SNAP23 binding by syntaxin-4 is consistently decreased by 30% upon phosphorylation by PKA. Such a shift in the equilibrium binding constant could significantly alter the ability of proteins to interact in vivo where protein concentrations may be limiting and kinases may be temporally and spatially regulated. Identifying tissues where PKA regulation could lead to changes in exocytosis will be of interest.

One form of regulated exocytosis which may be partly under the control of PKA is the delivery of the glucose transporter GLUT4 to the plasma membrane of fat and muscle cells. In these tissues, intracellular vesicles containing glucose transporters translocate to and fuse with the plasma membrane in response to insulin (*36*−*39*). VAMP-2 and VAMP-3/cellubrevin exist on GLUT4 vesicles and other microsomal vesicles (*16*, *17*, *40*, *41*). SNAP23 is found on plasma membranes of fat and muscle cells (*11*−*13*, *42*), and syntaxin-4 is found on both plasma membranes and the GLUT4 storage vesicles (*14*, *43*). The intracellular location of syntaxin-4 is not unprecedented as, by analogy, syntaxin-1 can also be found on synaptic vesicles (*44*). There are now several lines of evidence to support the hypothesis that these isoforms of v- and t-SNAREs are required for GLUT4 translocation in response to insulin (*14*, *22*, *29*, *45*, *46*). The mechanisms by which insulin triggers exocytosis in these cell types are not yet known, but regulation by insulin may occur at the step of GLUT4 vesicle formation or translocation to the membrane (*27*, *47*) rather than at the docking and fusion steps.

CKII is known to be activated by insulin (*48*), but our results suggest that this event is not involved in direct regulation of SNARE protein interactions. However, isoproterenol, an activator of stimulatory G-proteins and hence adenylate cyclase and PKA, can inhibit insulin-stimulated glucose transport in rat adipocytes (*49*−*51*). In the presence of isoproterenol, insulin appears to be able to mobilize GLUT4 proteins to the plasma membrane as determined by Western blotting of plasma membrane fractions and by membrane-invasive, glucose transporter-specific labeling (*49*−*51*). However, GLUT4 does not appear to translocate to the plasma membrane as determined by exofacial labeling (*52*). One possible explanation of this phenomenon is that insulin stimulates the delivery of the GLUT4 vesicles to the plasma membrane, but upon arrival there, they are unable to fuse to it. Isoproterenol may have caused PKA to phosphorylate syntaxin-4, resulting in blockage of a syntaxin-4−SNAP23 interaction that is required for vesicle fusion with the plasma membrane.

In conclusion, we have detected and quantitated binary interactions between SNAP23, syntaxin-4, and VAMP-2 but have found no evidence for a ternary complex of these three

proteins under conditions which promote formation of stable ternary complexes among the neuronal isoforms. We have also shown that syntaxin-4, when phosphorylated by PKA, is deficient in its ability to bind SNAP23. Together, these studies demonstrate that individual sets of SNARE proteins may assemble into different types of complexes whose assembly may be regulated in response to extrinsic stimuli.

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