Interaction of the Tyrosine Kinase Pyk2 with the N-Methyl-D-aspartate Receptor Complex via the Src Homology 3 Domains of PSD-95 and SAP102*

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The protein-tyrosine kinase Pyk2/CAKβ/CADTK is a key activator of Src in many cells. At hippocampal synapses, induction of long term potentiation requires the Pyk2/Src signaling pathway, which up-regulates the activity of N-methyl-D-aspartate-type glutamate receptors. Because localization of protein kinases close to their substrates is crucial for effective phosphorylation, we investigated how Pyk2 might be recruited to the N-methyl-D-aspartate receptor complex. This interaction is mediated by PSD-95 and its homolog SAP102. Both proteins colocalize with Pyk2 at postsynaptic dendritic spines in the cerebral cortex. The proline-rich regions in the C-terminal half of Pyk2 bind to the SH3 domain of PSD-95 and SAP102. The SH3 and guanylate kinase homology (GK) domain of PSD-95 and SAP102 interact intramolecularly, but the physiological significance of this interaction has been unclear. We show that Pyk2 effectively binds to the Src homology 3 (SH3) domain of SAP102 only when the GK domain is removed from the SH3 domain. Characterization of PSD-95 and SAP102 as adaptor proteins for Pyk2 fills a critical gap in the understanding of the spatial organization of the Pyk2-Src signaling pathway at the postsynaptic site and reveals a physiological function of the intramolecular SH3-GK domain interaction in SAP102.

At low stimulus frequency, synaptic transmission depends largely upon α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)[1]-type glutamate receptors (1, 2). High frequency stimulation promotes Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptors, thereby inducing long term potentiation (LTP) (1, 3, 4). LTP is a lasting increase in synaptic transmission that may underlie learning and memory (5, 6). NMDA receptors likely consist of one or two NR1 and two or three NR2 subunits; each subunit has an extracellular N terminus and an intracellular C terminus (7–10). The very C termini of NR2 subunits interact with the first two PDZ domains of PSD-95/SAP90 and the related proteins SAP102 and PSD-93/chapsyn110. PSD-95 and its homologs (SAP102, PSD-93, and SAP97) are scaffolding proteins consisting of three PDZ domains, one SH3 domain, and one GK domain. PSD-95, PSD-93, and SAP102 are thought to be involved in clustering glutamate receptors together with other proteins at postsynaptic sites (11–15). SAP97 interacts with the AMPA receptor subunit GluR1 (16–18). SAP97 colocalizes with GluR1, but not GluR2/3, at postsynaptic sites (17). This interaction may already occur early in the secretory pathway and may be involved in trafficking or targeting of AMPA receptors (19).

SH3 and GK domains of PSD-95 and its homologs form intramolecular and, to some degree, intermolecular interactions (20–24). A point mutation in the *Drosophila* protein Dlg (a homolog of PSD-95) results in the substitution of a conserved leucine in the SH3 domain to a proline; this mutation leads to the loss of septate junction formation and overproliferation of the imaginal disc (25). Replacing the homologous leucine (Leu-460) with a proline in the SH3 domain of PSD-95 inhibits the interaction of the mutant SH3 domain with the GK domain (20, 22). Collectively these findings indicate the physiological importance of the SH3-GK domain interaction, but a molecular function of this interaction remains to be established.

Tyrosine phosphorylation of NR2B (26, 27) is augmented on LTP induction (28, 29), and several observations suggest that up-regulation of NMDA receptor activity by Src-mediated tyrosine phosphorylation is an important step in the induction of LTP. 1) Src increases NMDA receptor activity (30–32) by elevating its peak current (33), 2) the activity of Src is up-regulated during LTP (34), 3) inhibition of Src can prevent LTP (30), and 4) an increase in synaptic transmission on Src activation occludes subsequent LTP induction (34).

It is well established that Src can be activated by another tyrosine kinase, Pyk2/CAKβ/CADTK (35–38). Pyk2 is stimulated upon Ca^{2+} influx, activation of PKC by phorbol esters, or stimulation of G_q-linked receptors such as the muscarinic acetylcholine receptor M1 or the metabotropic glutamate receptor mGluR1 (35, 37, 39–48). Neither Ca^{2+} nor PKC appear to directly regulate Pyk2 *in vitro* (39); how Ca^{2+} or PKC activates Pyk2 *in vivo* is unknown. When stimulated, Pyk2 autophosphorylates itself on tyrosine 402. The SH2 domain of Src binds to this phosphorylated tyrosine of Pyk2, resulting in activation of Src (35, 36). Recent evidence indicates that similar to Src, Pyk2 is necessary for the induction of LTP, likely because it increases the activity of Src (49).

Precise localization of protein kinases is a critical requirement for fast and specific signaling via phosphorylation of a

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[1] The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CRNK, CADTK, CAKβ-related non-kinase; FAK: focal adhesion kinase; FITC, fluorescein isothiocyanate; GK domain, guanylate kinase homology domain; GST, glutathione-S-transferase; LTP; long-term potentiation; NMDA, N-methyl-D-aspartate; PKC, protein kinase C; TSA, tyramide signal amplification; SH, Src homology.

variety of substrates (50–52). For example, efficient regulation of AMPA receptors requires that PKA be localized in close proximity to protein kinase A anchor proteins (53). Similarly, Ca^{2+}-calmodulin-dependent protein kinase II is recruited to the postsynaptic site via multiple, highly regulated interactions with the NMDA receptor (54–58). We now show that Pyk2 binds to PSD-95 and SAP102 in the NMDA receptor complex, which also contains Src. More specifically, the proline-rich region in the C-terminal half of Pyk2 directly interacts with the SH3 domains of PSD-95 and SAP102. This binding is inhibited by the intramolecular interaction of the GK domain with the SH3 domain in SAP102 by regulating Pyk2 binding to the SH3 domain.

EXPERIMENTAL PROCEDURES

Materials—Glutathione-Sepharose was purchased from Amersham Biosciences and protein A-Sepharose from Sigma. Antibodies against Pyk2 and pcDNA3 plasmids for the expression of hemagglutinin-tagged full-length rat Pyk2 and FLAG-tagged human CRNK (residues 692–1009 of Pyk2) (59, 60) in HEK293 cells were generously provided by Drs. L. M. Graves and H. S. Earp (University of North Carolina, Chapel Hill). We also purchased monoclonal antibodies against the C terminus of Pyk2 from BD Transduction Laboratories. Mammalian pCMV expression vectors of Myc-tagged full-length rat PSD-95 and rat SAP102 and bacterial expression vectors for the production of GST fusion proteins containing the PDZ1-3 (residues 57–405), PDZ2 (residues 156–248), and GK (residues 534–723) domains of PSD-95 and the SH3-GK domains (residues 537–647) of SAP102 (13, 61, 62) were graciously supplied by Dr. C. C. Garner (Stanford University, Palo Alto, CA), and plasmids for the production of GST fusion proteins of the PDZ3 (residues 302–402; originally in pGAD; subcloned into the *Eco*RI site of pGEX4T-1) and SH3 (residues 431–500) domains of PSD-95 were graciously supplied by Dr. M. Sheng (Howard Hughes Medical Institute, Massachusetts Institute of Technology, Boston) (63, 64). Expression vectors for GST-CRNK (residues 671–1009 in pGEX2TK) and GST-ΔN-CRNK (residues 876–1009) were generously made available by Drs. T. Sasaki (Sapporo Medical University, Sapporo, Japan), and M. D. Schaller (University of North Carolina, Chapel Hill). Other reagents were of standard biochemical quality and from established providers.

Immunoprecipitation and Immunoblotting—Crude membrane fractions were prepared from rat forebrain as described (65). NMDA receptor complexes were extracted either directly from hippocampal tissue or from membrane fractions with deoxycholate (1%) at 0–4 °C (18, 55, 65). In some experiments membrane fractions were solubilized with 1% SDS for 30 min at 60 °C. This SDS extraction protocol dissociates the NMDA receptor complex and subsequently allows the specific immunoprecipitation of individual subunits after neutralization of SDS by excess Triton X-100 (18, 55, 65). Immunoprecipitation and immunoblotting were performed as described (18, 65) with antibodies against NR1-, NR2A-, NR2B-, and NR2A/B (18, 65), PSD-95 (residues 494–510 (66)), SAP102 (residues 21–119 (66)), Src (monoclonal antibody 327 (67)), and portions of the N- and the C-terminal half of Pyk2 (the antibodies Pyk2-N and Pyk2-C were produced by immunizing rabbits with GST fusion proteins encompassing residues 1–80 and 680–860, respectively, as described (68)). The specificities of the antibodies have been carefully characterized (17, 18, 65–68). Chromatographically purified nonspecific mouse or rabbit IgGs (Zymed Laboratories Inc.) were used for control immunoprecipitations to test for nonspecific interactions of proteins with antibodies.

Transient Expression of Pyk2, CRNK, PSD-95, and SAP102 in HEK293 Cells—HEK293 fibroblasts were plated at 20–30% and maintained at 50–90% confluency in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% heat-inactivated fetal bovine serum (NovaTech), 2 mM L-glutamine, and 1 mM sodium pyruvate. HEK293 cells were transiently transfected following a modified (69) calcium phosphate precipitation protocol (70) with pCMV-PSD-95 or -SAP102 and pcDNA3-Pyk2 or -CRNK expression constructs. To enhance transfection efficiency, an equimolar amount of pAdVantage (Promega) was present during each transfection (71). Cells were collected 24–48 h post-transfection and lysed with RIPA buffer (10% glycerol, 1% Nonidet P-40, 0.4% deoxycholate, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA in 50 mM Tris, pH 7.4) or 1% Triton X-100 in 25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA. Both lysis buffers contained pepstatin A (1 μg/ml), leupeptin (10 μg/ml), aprotinin (20 μg/ml), and phenyl-methanesulfonyl fluoride (200 nM). Lysates were cleared by ultracen-

trifugation and used for either immunoprecipitation or pull-down experiments with GST fusion proteins.

Expression Constructs for GST Fusion Proteins of the SH3 and GK Domains of SAP102—A vector for expressing GST-SH3 fusion protein carrying residues 537–647 of rat SAP102 was produced by removing the GK domain from the original SAP102 GST-SH3-GK vector (a modified pGEX2T vector (13)) with *Nde*I and *Eco*RI. Overhangs were filled in with Klenow fragment, followed by blunt-end ligation. Positive clones were identified by colony PCR. The vector for expressing the GST-GK fusion protein covering residues 658–837 of full-length SAP102 was obtained through amplification of the GK domain by PCR using primers containing *Eco*RI restriction sites (5′primer, 5′ CAA GAA GAA TTC TAT GCC AGG CCT GTG ATC ATC TTG G 3′; 3′primer, 5′ CCA AAT GAA TTC TCA AGA CTG GTC CTC AAT GAT TTG TTT G 3′). The PCR product was digested with *Eco*RI and inserted into *Eco*RI-digested pGEX4T-1 vector before transformation into *Escherichia coli*. Clones containing pGEX4T-1 vectors with the GK domain inserted in the correct orientation were identified by colony PCR. All new and old vectors used in this study were confirmed by DNA sequencing.

Interaction Assays with GST Fusion Proteins—GST and GST fusion proteins of the PDZ1-3, PDZ2, PDZ3, SH3, and GK domains of PSD-95 or SH3, GK, and SH3-GK domains of SAP102, along with CRNK and ΔN-CRNK, were expressed from pGEX vectors (13) in the *E. coli* strain BL21 (Novagen) as described (18). Equal amounts of the different GST fusion proteins or GST (~25 μg) were absorbed onto glutathione-Sepharose (~20 μl), and the residues were incubated for 2 h at 4 °C with lysates (prepared with RIPA buffer) from HEK293 cells transiently overexpressing Pyk2, CRNK, PSD-95, or SAP102. Typically, the aliquots of the HEK293 lysates contained 300 μg of total protein as determined with the BCA assay (Pierce). Resins were washed with 500 mM NaCl, 10 mM Tris-Cl, pH 7.4, with 0.1% Triton X-100 in TBS (150 mM NaCl, 10 mM Tris-Cl, pH 7.4) and with TBS alone and then analyzed by immunoblotting as described (18, 55). Lysate containing 5–10 μg of protein was loaded onto gels as a positive control. Pyk2 was detected in these experiments with an antibody against the C-terminal portion of the kinase (residues 833–997; purchased from BD Transduction Laboratories). The presence of equal amounts of the GST fusion proteins was routinely verified by Ponceau S staining (Sigma) or probing with an anti-GST antibody (18).

Immunofluorescence—Four male Sprague-Dawley rats (200–350 g; Charles River) were deeply anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally), and intracardially perfused with heparinized saline followed by freshly depolymerized 0.5% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Coronal 50-μm sections were cut on a Vibratome and collected in cold PB. For single immunofluorescence, sections were incubated overnight in antibody to Pyk2 (1:1,000). Immunoreactivity was then visualized by donkey anti-rabbit conjugated to fluorescein isothiocyanate (FITC; Jackson ImmunoResearch). Because Pyk2, SAP102, and NR2A/B antibodies were raised in rabbit, tyramide signal amplification (TSA) was used for one of them, and subsequent conventional fluorescent staining was used for one of the other antibodies for double-labeling (72–74). After preincubation in 10% normal donkey serum, sections were incubated with Pyk2 antiserum at a dilution not recognized by a conventional fluorochrome-conjugated secondary antibody (1:20,000) and then reacted with biotinylated secondary antibody. Biotin was localized by a FITC-TSA direct kit (PerkinElmer Life Sciences). After preincubation in 1% normal donkey serum, SAP102 antiserum (1:1,000; Chemicon) or NR2 antiserum (1:1,000; Chemicon) was applied overnight. Immunoreactivity was visualized with donkey anti-rabbit antibody, conjugated to CY-3 (Jackson ImmunoResearch). Sections were then processed for synaptophysin (1:1,000; Clone SVP-38; Sigma) or PSD-95 (1:1,000; Clone 16; Transduction Laboratories). After overnight incubation in primary antibody, immunoreactivity was visualized with donkey anti-mouse antibody conjugated to Cy-5 (Jackson ImmunoResearch). Control sections were processed as above, except that primary or secondary antibodies were omitted or replaced by nonspecific control antisera. To control for possible cross-reaction between the first primary antibody and the second secondary antibody, tissue was processed as above, except that the second primary antibody was omitted. Omission of primary or secondary antibodies eliminated specific labeling. Furthermore, consistent results were obtained after reversing the first and the second primary antibodies, using first SAP102 or NR2 (1:20,000) with FITC-TSA and then Pyk2 (1:1,000) with Cy-3-conjugated secondary antibody.
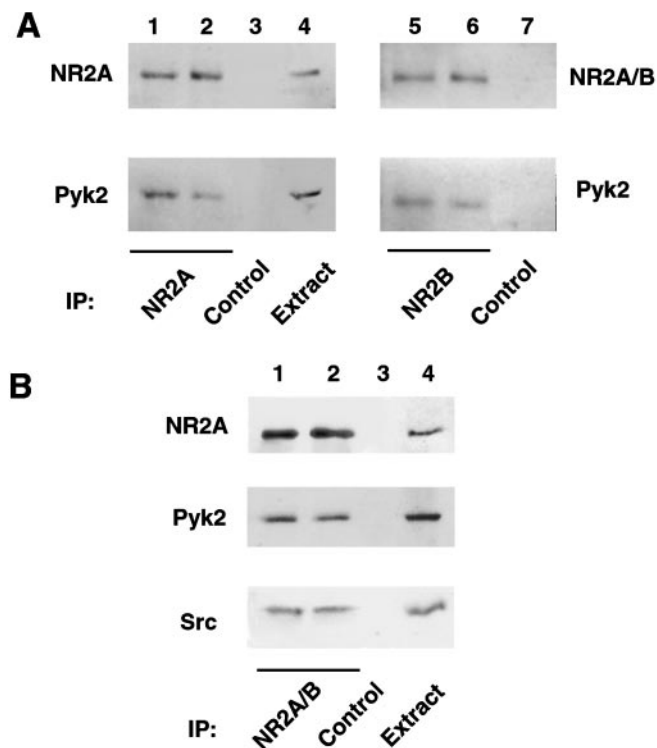
Pyk2 Interactions with the NMDA Receptor Complex

FIG. 1. **Pyk2 is associated with the NMDA receptor complex.** NMDA receptor complexes were extracted with 1% deoxycholate from rat hippocampal tissue, and immunoprecipitations (*IP*) were performed with antibodies against NR2A (*A, lanes 1* and *2*), NR2B (*A, lanes 5* and *6*), a mixture of antibodies against NR2A and -2B (*B, lanes 1* and *2*), or non-immune control antibodies (*A, lanes 3* and *7, B, lane 3*). Immunoblots of these samples were cut, and the portions comprising the range around 200 kDa were probed with antibodies against NR2A (*lanes 1–4* in *A* and *B*), or an antibody that recognizes NR2A and -2B (*A, lanes 5–7*). The portions surrounding the range around 120 and 60 kDa were incubated with the Pyk2-N antibody (*A* and *B*) and the antibody against Src (*B*), respectively (indicated on the *left* and *right side* of each blot). For comparison, 20 μl of extract was also directly applied to SDS-PAGE. Specific coprecipitations of Pyk2 and Src with the NMDA receptor precipitations are shown in duplicate to document reproducibility of the coprecipitations and were performed several times with similar results.

RESULTS

Coimmunoprecipitation of Pyk2 and Src with NMDA Receptors—Because kinases are often localized in close proximity to their targets, we hypothesized that kinases involved in tyrosine phosphorylation of the NMDA receptor may be associated with this receptor. In fact, an earlier report (31) suggests that Src, which can up-regulate NMDA receptor activity, binds to the NMDA receptor complex. To test whether Pyk2, an activator of Src, associates with the NMDA receptor complex, rat forebrain samples were solubilized with deoxycholate and used for immunoprecipitations with antibodies against NR2A and -2B. Previously, we showed (18, 55, 65) that the interactions between the NMDA receptor and its associated proteins including PSD-95, SAP102, and Ca^{2+}-calmodulin-dependent protein kinase II are well preserved under these conditions. Subsequent immunoblotting revealed that Pyk2 specifically and reproducibly coprecipitated with the NMDA receptor complex (Fig. 1*A*). We also observed copurification of Src with the Pyk2-NMDA receptor complex, especially if a mixture of NR2A and 2B antibodies was used for immunoprecipitation of the NMDA receptor (Fig. 1*B*). Coprecipitation of Pyk2 and Src with the NMDA receptor was specific because precipitations with non-immune control antibodies did not result in isolation of Pyk2 or Src.

Coimmunoprecipitation of Pyk2 with PSD-95 and SAP102—PSD-95 and its homologs SAP102 and PSD-93 mediate the association of the NMDA receptor with various signaling proteins including neuronal nitric-oxide synthase and Ras-activating SynGAP (75–77). Therefore, we tested whether Pyk2 might be associated with PSD-95 or SAP102. Immunoprecipitation with antibodies against SAP102 resulted in coprecipitation of Pyk2 as detected with the antibody against the N-terminal 80 residues of Pyk2 (Pyk2-N; Fig. 2*A, lanes 6* and *7*). Probing of total membrane extract with the Pyk2-N antibody demonstrated the specificity of this antibody for Pyk2, which possesses a molecular mass of about 115 kDa (*lane 1*). Immunoprecipitation with both Pyk2-N and Pyk2-C also yielded the 115-kDa polypeptide that is immunoreactive for Pyk2-N (*lanes 3* and *4*, respectively). Pyk2 copurified with PSD-95 (*lane 11*), as well. To test whether Pyk2 might bind to SAP102 or PSD-95 after extraction with detergents rather than being associated with those two proteins *in vivo*, we solubilized brain mem-

FIG. 2. **Coimmunoprecipitation of Pyk2 with PSD-95 and SAP102 from rat brain.** Rat forebrain membrane fractions were extracted with 1% deoxycholate on ice or, when indicated, with SDS at 60 °C (*dissociated*; *A, lanes 8, 9,* and *13*) and used for immunoprecipitations (antibodies are indicated at the *bottom* of each blot) and subsequent immunoblotting. *NR2A/B* and *Control* indicate immunoprecipitations (*IP*) with a mixture of antibodies against NR2A and -2B and with nonspecific control IgG, respectively. 20 μl of extract were also directly applied to SDS-PAGE when indicated (*Extract*). Blots were probed with antibodies against Pyk2-N (68) (*A*) or SAP102 (*B*). Similar results were obtained in several other experiments.
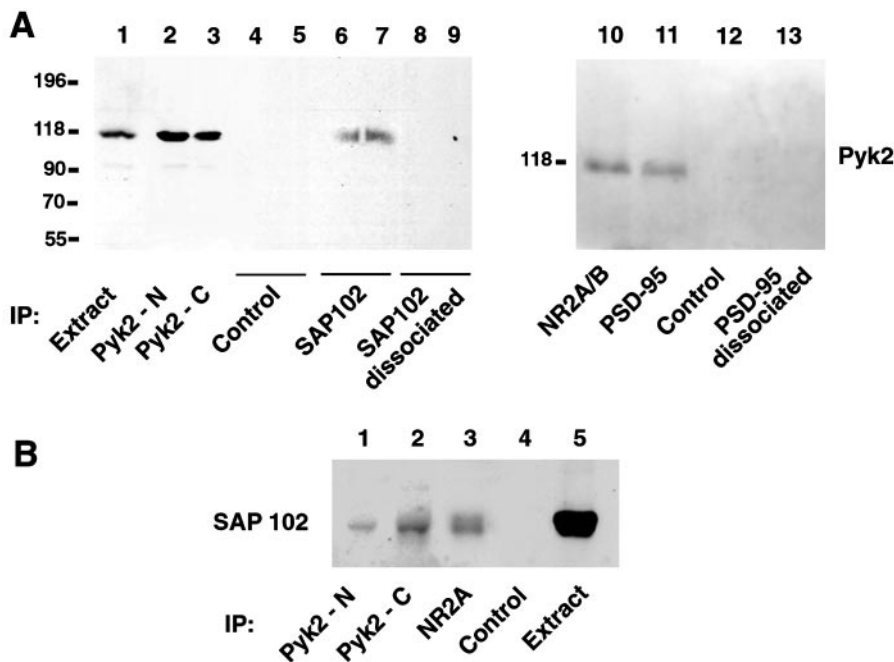
branes with 1% SDS at 60 °C and subsequently added an excess of Triton X-100, which forms mixed micelles with SDS, thereby neutralizing it. This treatment dissociates the different
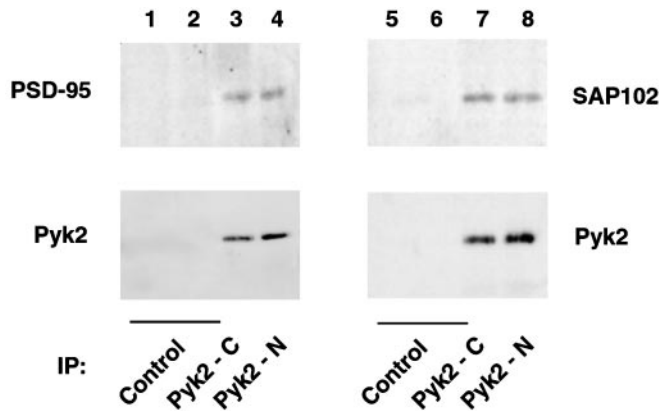

FIG. 3. **Pyk2 interacts with PSD-95 and SAP102 in HEK293 cells.** Pyk2 was transiently expressed in HEK293 cells together with either PSD-95 or SAP102. HEK293 cells were solubilized with RIPA buffer. Immunoprecipitations (*IP*) were performed with the Pyk2-N and the Pyk2-C antibody (68) and with non-immune rabbit IgG (*Control*). PSD-95 and SAP102 were detected by immunoblotting with the corresponding antibodies (*upper panels*). Blots were stripped and reprobed with Pyk2-N to confirm the presence of Pyk2 and the specificity of the immunoprecipitations (*lower panels*). Two other experiments yielded similar results.

components of the NMDA receptor complex including PSD-95 and SAP102 (9, 55, 62, 65). Under these conditions Pyk2 did not coprecipitate with either SAP102 (*lanes 8* and *9*) or PSD-95 (*lane 13*). These observations indicate that the interactions between Pyk2 and PSD-95 or SAP102 do not form during the extraction procedure and are likely to occur *in vivo*. These interactions were further confirmed by immunoprecipitation of Pyk2 with either Pyk2-N or Pyk2-C and subsequent detection of SAP102 (Fig. 2*B*) and PSD-95 (data not shown) in immunocomplexes by immunoblotting.

Pyk2 Interacts with PSD-95 and SAP102 in HEK293 Cells in the Absence of the NMDA Receptor—To evaluate whether the association between Pyk2 and PSD-95 or SAP102 depends on the presence of the NMDA receptor or is due to a direct interaction, we expressed Pyk2 with either PSD-95 or SAP102 in the heterologous HEK293 cell line. Immunoprecipitation of Pyk2 with either Pyk2-N or Pyk2-C from cell lysates resulted in coprecipitation of PSD-95 and SAP102 (Fig. 3, *upper panels*). The specificities of these coprecipitations were demonstrated by the lack of PSD-95 and SAP102 immunoreactivity in immunoprecipitations with control IgG. Because HEK293 cells are non-neuronal and do not express NMDA receptors, these results suggest that Pyk2 interaction with PSD-95 and SAP102 does not require the NMDA receptor and might be direct.

Colocalization of Pyk2 with SAP102 and PSD-95—Multiple immunofluorescence staining was performed to compare the cellular and subcellular distribution of Pyk2 with that of
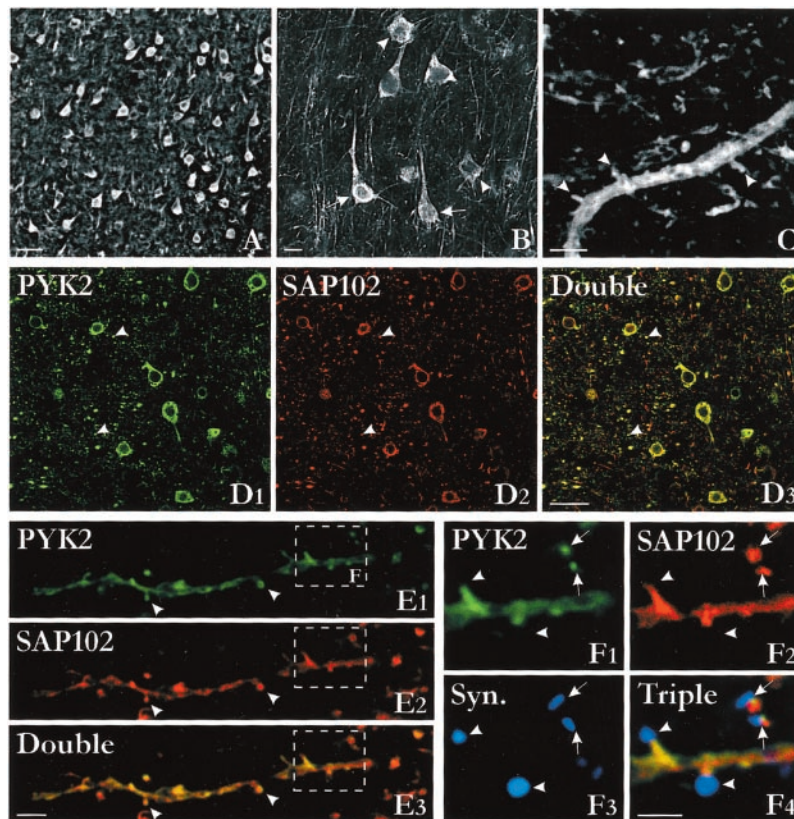

FIG. 4. **Pyk2 colocalizes with SAP102 at subcellular sites including synapses and postsynaptic spines in rat forebrain.** *A–C*, confocal immunofluorescence images of immunostaining with the Pyk2-N antibody in the cerebral cortex. *A*, low magnification view showing numerous stained neurons in cortical layer III. *B*, higher magnification view from layer V. Both pyramidal (*arrows*) and nonpyramidal neurons (*arrowheads*) were immunopositive. Staining was prominent in somata and proximal dendrites, especially in apical dendritic shafts of pyramidal neurons. *C*, Pyk2 immunoreactivity was also observed in many spines (*arrowheads*) and puncta in nearby neuropil. *D–F*, confocal images showing colocalization of Pyk2 (FITC, *green*) with SAP102 (Cy-3, *red*). *D*, low magnification view of cortical layer III. Nearly all cells immunoreactive for Pyk2 were also immunoreactive for SAP102. Likewise, in the neuropil, colocalization of Pyk2 and SAP102 was frequently observed (*arrowheads*). *E*, high magnification view of a spiny dendrite immunoreactive for both Pyk2 and SAP102. Many spines positive for Pyk2 were also positive for SAP102 (*arrowheads*). *F*, enlargement of area *boxed* in *E*; *arrowheads* point to double-labeled spines and *arrows* to double-labeled puncta, likely to correspond to synapses, as defined by their association with synaptophysin (*Syn.*, Cy-5, *blue*). *Scale bars*: *A*, 50 μm; *B*, 20 μm; *C*, 5 μm; *D*, 50 μm; *E*, 5 μm; and *F*, 3 μm.

FIG. 5. **Subcellular and synaptic co-localization of Pyk2 with PSD-95 and NR2 subunits in the cerebral cortex.** Confocal immunofluorescence images showing colocalization of Pyk2 (Pyk2-N antibody; FITC, *green*) with NR2 (Cy-3, *red*), and PSD-95 (Cy-5, *blue*). *Arrows* point to two triple-labeled pyramidal neurons in layer V. In the neuropil, most Pyk2-positive puncta are also positive for NR2 and PSD-95. However, many PSD-95-positive and some NR2-positive puncta are negative for Pyk2. *Insets* show an enlargement of the *boxed* area. Pyk2-positive dots colocalize with NR2 and PSD-95 immunoreactivity (*arrows*); *arrowheads* point to spines immunopositive for all three proteins. *Scale bars*, 20 μm; *insets*, 3 μm.
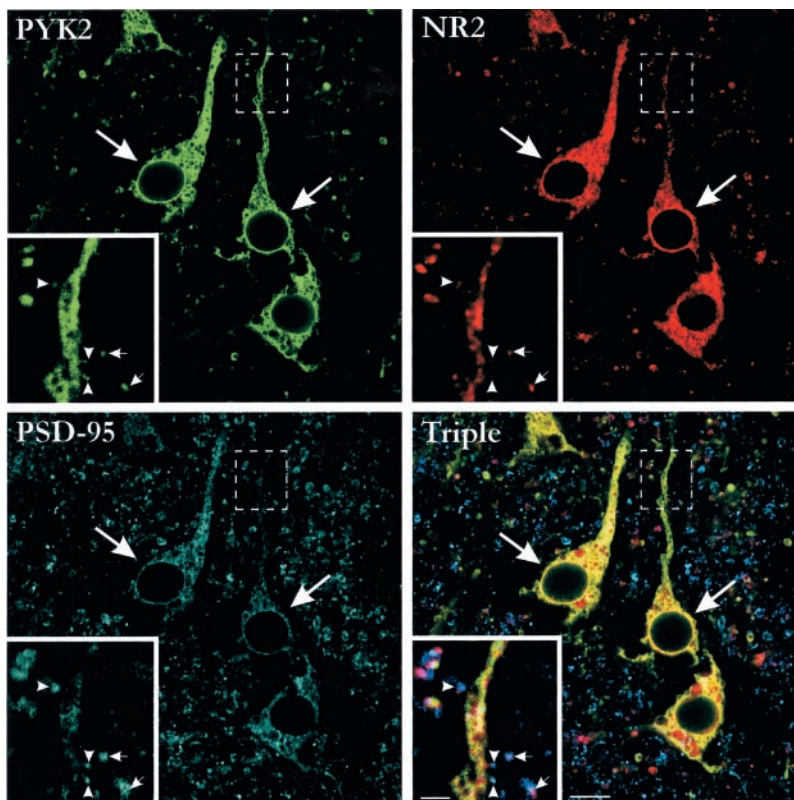
SAP102 and PSD-95. Pyk2 antibodies stained a population of neurons distributed throughout the cerebral cortex (Fig. 4). Some of these neurons can be identified as pyramidal neurons (Fig. 4*B, arrows*); others were non-pyramidal cells of various shapes and sizes (Fig. 4*B, arrowheads*). Staining was typically patchy within dendrites and somatic cytoplasm, excluding the nucleus. At higher magnification, Pyk2 immunoreactivity was obvious in many dendritic spines (Fig. 4*C*). Numerous Pyk2 immunoreactive puncta were observed throughout the neuropil (Fig. 4*D1, arrowheads*). Such a distribution pattern often reflects a synaptic distribution. Double labeling with the presynaptic marker synaptophysin confirmed that many of the Pyk2-positive puncta were at synaptic sites (*e.g.* see Fig. 4*F, 1, 3,* and *4, arrows*).

SAP102-immunopositive neurons were also widely distributed in the cerebral cortex in a pattern that matched Pyk2 staining (Fig. 4*D2*). Double labeling confirmed that almost all SAP102-positive neurons were also positive for Pyk2 and vice versa (Fig. 4*D3*). In the neuropil, punctate colocalization was prominent (Fig. 4*D3, arrowheads*). Many of these puncta are likely to be synapses as indicated by double labeling with synaptophysin (*e.g.* Fig. 4, *F2–4, arrows*). Furthermore, Pyk2 and SAP102 immunoreactivity codistributed along dendrites, concentrating at dendritic spines (Fig. 4*E1*) juxtaposed to presynaptic synaptophysin staining (Fig. 4*F*). These results indicate that Pyk2 and SAP102 are colocalized not only in neuronal somata and dendrites but also at postsynaptic sites, where they may be associated with NMDA receptors.

NR2 and PSD-95 antibodies stained a large population of pyramidal and non-pyramidal neurons throughout the cortex. Triple immunofluorescence labeling showed cellular colocalization of Pyk2 with NR2 and PSD-95 (Fig. 5). This distribution was patchy in somata and proximal dendrites; some of these patches exhibited all three antigens, but others were immunopositive only for NR2 (Fig. 5, *lower right*). In the neuropil, most Pyk2-positive puncta colocalized with NR2 and PSD-95 staining. Likewise, Pyk2-positive spines were also triple-la-

beled (Fig. 5, *arrowheads* in *insets*). However, many PSD-95 and some NR2-positive puncta were negative for Pyk2. These findings indicate that Pyk2 is colocalized with PSD-95 and NMDA receptors at many, although perhaps not all, postsynaptic spines where these proteins might be associated with each other.

The Proline-rich Region of Pyk2 Binds to the SH3 Domain of PSD-95—To define the interaction site of Pyk2 in PSD-95, we expressed GST fusion proteins of the PDZ1-3, PDZ2, PDZ3, SH3, and GK domains of PSD-95 in *E. coli*. Equal amounts of these fusion proteins were immobilized on glutathione-Sepharose, washed, and incubated with cell lysates of transfected HEK293 cells that ectopically expressed Pyk2, after lysates were pre-cleared with GST alone. Subsequent immunoblotting demonstrated that Pyk2 specifically bound to the SH3 domain of PSD-95 (Fig. 6*A*) and SAP102 (Fig. 6*B*). We obtained the same results when brain extracts were used as a source of native Pyk2 in similar pull-down experiments (data not shown). Immunoblots were stained with Ponceau S (Fig. 6, *lower panels*) and re-probed with anti-GST antibodies (not shown) to ensure that comparable amounts of each GST fusion protein were present in the various samples.

Pyk2 contains two segments that are proline-rich and constitute potential interaction sites for SH3 domains. These segments are toward the C terminus of Pyk2 (residues 713–720 and 855–862). We used the C-terminal Pyk2 fragment spanning residues 671–1009 for interaction assays. This fragment included the two proline-rich regions together with flanking sequences on both ends. It lacked the N-terminal region of Pyk2, which contains a homologous band 4.1 domain and the central catalytic domain. This catalytically inactive fragment, called CRNK (CADTK or CAKβ-related non-kinase) or Pyk2-related non-kinase, is expressed as a truncated version of Pyk2 in brain and other tissues (59, 78, 79). Analogous to a similar C-terminal fragment of the related focal adhesion kinase FAK, which is also expressed *in vivo* and inhibits certain functions of FAK (80–82), CRNK is thought to interfere with those signal-
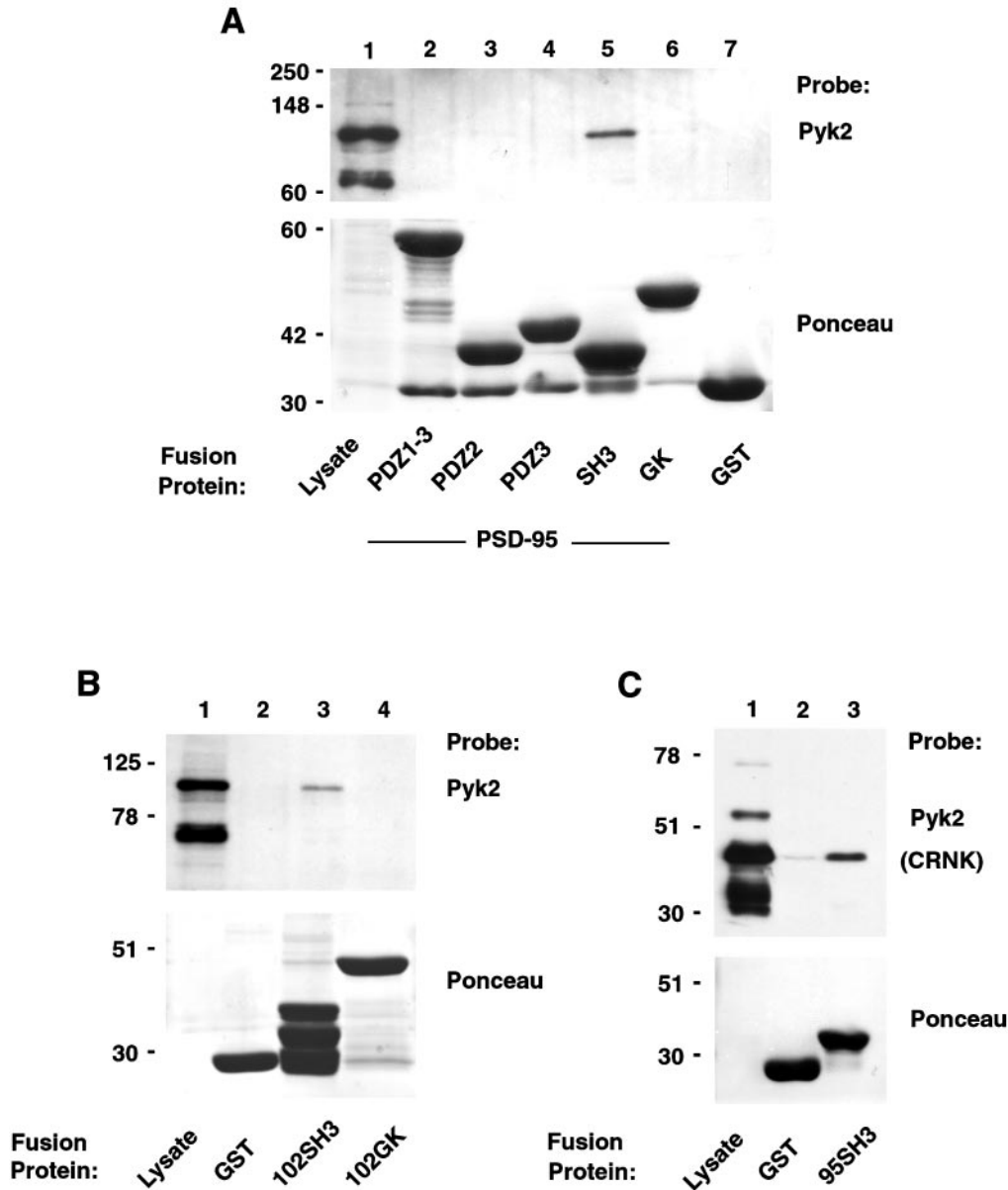
FIG. 6. **Interaction of the SH3 domain of PSD-95 and SAP102 with the C-terminal region of Pyk2.** GST and the GST fusion proteins given at the *bottom* of each panel were expressed in *E. coli*, adsorbed onto glutathione-Sepharose, and incubated with RIPA buffer lysates from HEK293 cells transiently overexpressing Pyk2 (*A* and *B*) or its truncated version, CRNK (*C*). After washing, samples were analyzed by immunoblotting. Cell extract (*Lysate*) containing 5 μg of protein was used as positive control. Probing blots with an antibody against the C-terminal portion of Pyk2 (*upper panels*) demonstrated that Pyk2 specifically bound to the SH3 domain of PSD-95 (*A*) and SAP102 (*B*). This Pyk2 antibody was from Transduction Laboratories and recognizes full-length Pyk2 (*A* and *B*) as well as CRNK (*C*) but does not cross-react with the GST moiety of our fusion proteins (in contrast to the Pyk2-N and Pyk2-C antibodies, which had been produced against GST fusion proteins of the corresponding Pyk2 regions (68)). Binding to PSD-95 was mediated by the C-terminal region of Pyk2 (CRNK; *C*). Blots were stained with Ponceau S (*lower panels*) before blocking and probing to show that comparable amounts of GST fusion proteins were present on each resin sample. Similar results were obtained in several other experiments.

ing mechanisms of Pyk2 that depend on the interaction of the C-terminal domain of Pyk2 with corresponding binding partners (59, 78, 79). It is, therefore, not only of biochemical but also of physiological interest to determine whether the SH3 domain of PSD-95 binds to CRNK. Like Pyk2, we expressed CRNK in HEK293 cells and used lysates of these cells for pull-down experiments with the PSD-95 derived GST-SH3 fusion protein. CRNK bound to this fusion protein but not to GST alone (Fig. 6B) indicating a specific interaction with the SH3 domain of PSD-95.

To test whether the proline-rich segments of Pyk2 are responsible for PSD-95 and SAP102 binding, we expressed GST fusion proteins of CRNK (residues 671–1009) and ΔN-CRNK (residues 876–1009, lacking the two proline-rich segments) as

GST fusion proteins. Lysates of HEK293 cells ectopically expressing full-length PSD-95 or SAP102 were used for pull-down experiments with GST fusion proteins of CRNK, ΔN-CRNK, and the SH3 and GK domains of PSD-95 or SAP102. CRNK but not ΔN-CRNK specifically interacted with full-length PSD-95 and SAP102 in these *in vitro* binding assays (Fig. 7). These results indicate that the proline-rich segments mediate the Pyk2-PSD-95 interaction. We consistently observed much weaker binding of SAP102 to CRNK than PSD-95 and therefore analyzed the availability of the SH3 domains in full-length PSD-95 and SAP102 for binding. Biochemical and crystallographic studies demonstrated that the SH3 and GK domains of PSD-95 and its homologs form intramolecular interactions (20–24). Intermolecular binding of the SH3 domain of one
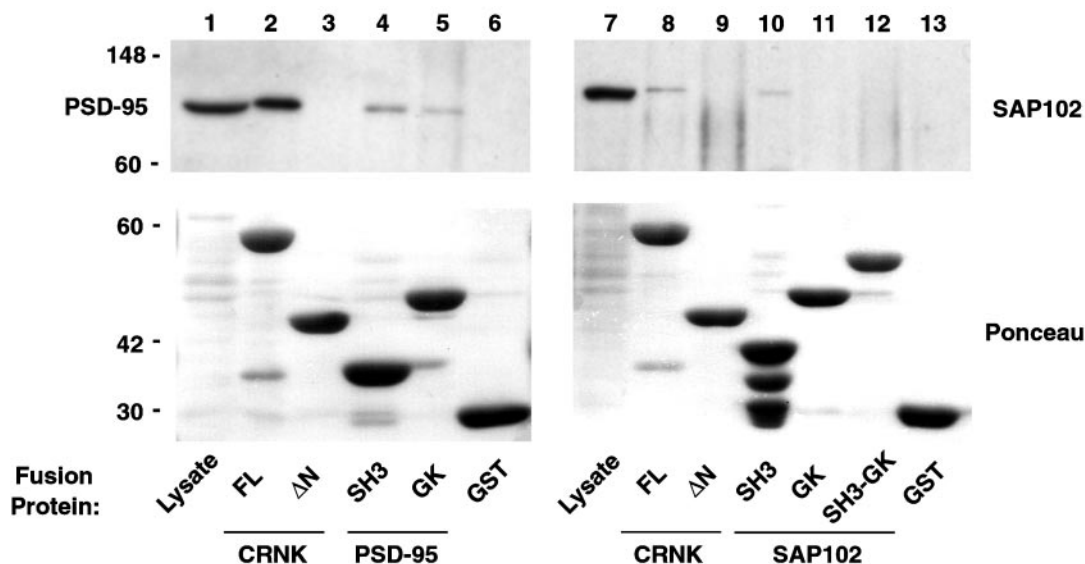
FIG. 7. **Requirement of the proline-rich region of Pyk2 for binding to PSD-95 and SAP102.** GST and the GST fusion proteins outlined at the *bottom* of the panels (*CRNK*, residues 671–1009; ΔN-CRNK, residues 876–1009 of Pyk2) were adsorbed onto glutathione-Sepharose, incubated with 1% Triton X-100 lysates of HEK293 cells transiently overexpressing Myc-tagged PSD-95 (*left*) or SAP102 (*right*), and analyzed by immunoblotting with anti-Myc antibody (*upper panels*). Cell extract (*Lysate,* 10 μg of protein) was loaded as positive control. CRNK specifically interacted with PSD-95 and SAP102 only when the proline-rich region was present (*FL-CRNK*). Consistently less SAP102 than PSD-95 was pulled down by either CRNK or the respective SAP102 SH3 domain. Blots were stained with Ponceau S (*lower panels*) before probing to confirm that equal amounts of GST fusion proteins were present. Results were replicated in several additional experiments.

PSD-95 molecule with the GK domain of another PSD-95 molecule can occur, but the intramolecular interaction is favored and occludes exogenous SH3 from binding to the GK domain and vice versa. The GST-SH3 and GST-GK fusion proteins specifically bound full-length PSD-95 on a weak basis, suggesting that, under our conditions, there is some access for the SH3 domain to its binding site on the GK domain, and vice versa, in full-length PSD-95. The interaction of GST-SH3 with SAP102 was weaker, and binding of GST-GK to SAP102 was not detectable. These results raise the possibility that the intermolecular interaction between the SH3 and GK domains of SAP102 may limit the interaction between Pyk2 and the SH3 domain.

Intramolecular GK Domain Association with the SH3 Domain Inhibits Pyk2 Binding—To test whether the presence of the GK domain within the same polypeptide influences the interaction of the SH3 domain of SAP102 with Pyk2, we performed pull-down experiments with GST fusion proteins carrying the SH3 or GK domain separately or in tandem. Whereas the GST fusion protein carrying only the SH3 domain of SAP102 pulled down Pyk2 from HEK293 cell lysates, a similar amount of a GST fusion protein with the combined SH3-GK domain did not (Fig. 8). The GK domain by itself did not mediate precipitation of Pyk2 in these pull-down experiments. These experiments indicate that SAP102 binds via its SH3 domain to Pyk2 and that the GK domain can inhibit this interaction.

DISCUSSION

Our results indicate that Pyk2, like Src, is associated with the NMDA receptor complex (Fig. 1) via the SH3 domains of PSD-95 and SAP102. The Pyk2 interaction with PSD-95 and SAP102 appears to be rather stable, as it can be detected after immunoprecipitation from brain (Fig. 2), a procedure that takes several hours, and also from HEK293 cells ectopically expressing these proteins (Fig. 3). Re-association does not appear to play a role during these immunoprecipitations; after dissociation of the Pyk2-PSD-95 and Pyk2-SAP102 complexes with SDS and neutralization of SDS with Triton X-100, Pyk2
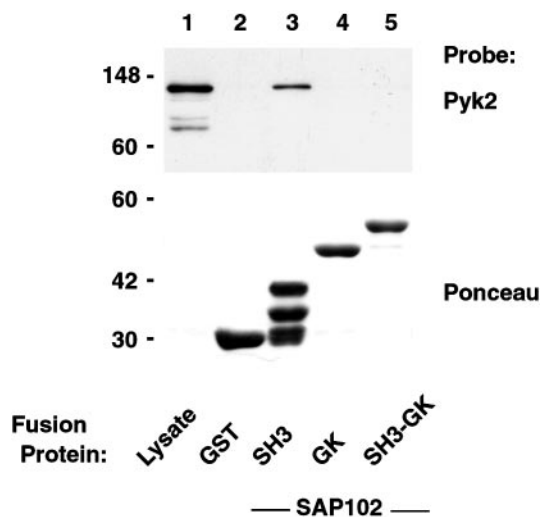

FIG. 8. **Inhibition of Pyk2 binding to the SH3 domain of SAP102 by an intramolecular SH3-GK domain interaction.** GST and the GST fusion proteins indicated at the *bottom* of the panels were adsorbed onto glutathione-Sepharose, incubated with RIPA buffer lysates of HEK293 cells transiently overexpressing Pyk2, and analyzed by immunoblotting with an antibody against the C-terminal portion of Pyk2 (Transduction Laboratories; *upper panel*). Cell extract (*Lysate*, 5 μg of protein) was used as positive control. Pyk2 only bound to the isolated SH3 domain of SAP102 but not to the GST fusion protein carrying the combined SH3-GK domains. Ponceau S staining (*lower panels*) detected similar amounts of GST fusion proteins in each resin sample. Similar results were obtained in several other experiments.

does not rebind to PSD-95 or SAP102 (Fig. 2). An *in vivo* interaction of Pyk2 with PSD-95 and SAP102 is further supported by our immunofluorescence microscopy studies. Pyk2 colocalizes with SAP102 and PSD-95 at synapses, which were identified by triple labeling with the presynaptic marker protein synaptophysin (Fig. 4) or with antibodies against the NR2A and -2B subunits of the NMDA receptor complex (Fig. 5).

Pyk2 did not interact with the different PDZ domains of PSD-95 in pull-down experiments (Fig. 6), consistent with the

fact that the C terminus of Pyk2 (Pro-Pro-Ala-Glu) does not conform to a classic PDZ domain binding motif. Rather, it specifically bound to the SH3 domains of PSD-95 and SAP102. An ~300-residue-long C-terminal fragment of full-length Pyk2 containing two proline-rich regions (713–720 and 855–862) was sufficient for binding to the SH3 domain. This portion of Pyk2, known as CRNK, is expressed in parallel with full-length Pyk2 in several tissues including brain (59, 78, 79), similar to the homologous fragment FAK-related non-kinase of the related focal adhesion kinase FAK (80–82). Signaling pathways that depend on the interaction of the C-terminal domain of Pyk2 with corresponding binding partners can be inhibited by CRNK (59, 78, 79). Therefore, CRNK may regulate the Pyk2 interaction with PSD-95 and SAP102 and thereby the efficiency of NMDA receptor regulation by Pyk2. In support of this speculation, the dominant negative Pyk2 mutant K457A (35, 39) prevents up-regulation of NMDA receptor peak currents by endogenous Pyk2 (49), suggesting the mutant blocks access of the endogenous wild-type Pyk2 to the SH3 domains of PSD-95 and SAP102 in the NMDA receptor complex.

Only a few binding partners for the SH3 domain of PSD-95 and its homologs have been identified, including the KA2 subunit of the kainate-type glutamate receptor (83), A kinase anchor protein 150 (84), and huntingtin (85). In addition to these interactions, the SH3 and GK domains of PSD-95 and its homologs can undergo intramolecular and, to some extent, intermolecular interactions (20–24). Mutations that disrupt this intramolecular association in the PSD-95 homolog Dlg result in overproliferation of the imaginal disc in *Drosophila* (25). These observations suggest that the regulation of protein-protein interactions between the SH3 and GK domains has a critical function *in vivo*. Recent experiments indicate that disruption of the SH3-GK domain interaction interferes with the channel clustering activity of PSD-95 (22); however, the precise mechanism underlying this phenomenon is unclear.

The linker domain between the SH3 and GK domain has been implicated as a potential binding site for ligands that might induce conformational changes of the SH3-GK module (21, 86, 87). This region is also involved in targeting Dlg to septate junctions in epithelial cells (88) and to synapses at the neuromuscular junction (89) of *Drosophila*. Our present results indicate that the SH3 domain of SAP102, and perhaps also PSD-95, primarily binds Pyk2 when the SH3 domain is not engaged in an intramolecular interaction with the GK domain. We hypothesize that specific regulatory mechanisms, such as association of the GK or linker domain with a binding partner or a post-translational modification, like phosphorylation of SAP102 and PSD-95, control the SH3-GK interaction and, thereby, Pyk2 binding to the SH3 domain.

Several PKC phosphorylation sites have been identified on the NMDA receptor (65, 90–92) that influence receptor function. For example, phosphorylation of the C1 cassette of the NR1 C terminus may regulate the distribution of the NMDA receptor on the cell surface (91, 93). In addition, stimulation of PKC generally results in up-regulation of NMDA receptor activity (94–99). However, recent findings suggest that potentiation of NMDA receptors is not always due to direct phosphorylation of the receptor subunits by PKC (100). Elegant work by MacDonald and co-workers (33, 49) demonstrated that Pyk2 and Src act downstream of PKC to increase NMDA receptor activity. Earlier studies indicated that NR2A and -2B are phosphorylated on tyrosine residues *in vivo* (26, 27, 46, 101) and that NMDA receptor activity is up-regulated by Src (30–32,102). Furthermore, the increase in NMDA receptor activity by PKC was prevented by specific inhibitors of Src (33). These observations suggest that intermediary kinases, such as Pyk2

and Src, are involved in the PKC signaling cascade resulting in the phosphorylation of the NMDA receptor. This model is supported by coimmunoprecipitation of Src and the NMDA receptor (Fig. 1; see also Ref. 34) reflecting the formation of a Src-NMDA receptor complex for efficient and specific phosphorylation of the receptor. However, additional experiments are necessary to characterize further the interaction between Src and the NMDA receptor and the phosphorylation of NR2A and -2B by Src.

How does PKC control Src activity? Stimulation of PKC by phorbol esters or G_q-coupled receptors results in activation of Pyk2 (35, 38, 39, 46). Up-regulation of NMDA receptor activity by PKC can be inhibited by injection of a purified, dominant negative Pyk2 mutant into hippocampal pyramidal neurons (49). Furthermore, injection of purified wild-type Pyk2 increases NMDA receptor currents, and this effect is blocked by inhibition of Src. In contrast, the Src-mediated increase in NMDA receptor activity (31–33) is not antagonized by dominant negative Pyk2 (49). These results place PKC upstream of Pyk2 and Src downstream of Pyk2 in that signaling pathway (49). Because the induction of NMDA receptor-dependent LTP requires Src (34) and Pyk2 (49), we hypothesize that LTP may depend on the coassembly of Pyk2 and Src at the NMDA receptor. Because Ca^{2+} influx also leads to the activation of Pyk2 independent of PKC stimulation (39, 46), anchoring of the kinase at the NMDA receptor may place Pyk2 at a strategically ideal location where NMDA receptor-mediated Ca^{2+} influx into the cytosol may most effectively contribute to Pyk2 activation. As a result, Pyk2 autophosphorylation could lead to the association and activation of Src (35). However, it is yet unknown how Ca^{2+} influx or PKC activation induces Pyk2 activation *in vivo*.

As an integral component of the NMDA receptor complex, Pyk2 plays a critical role in the regulation of NMDA receptor activity and function (Fig. 1) (49). We demonstrated that Pyk2 binds to the SH3 domain of PSD-95 and SAP102 and that the interaction of Pyk2 with the SH3 domain is inhibited by the GK domain. Our biochemical studies indicate a functional role of the SH3-GK domain interaction in PSD-95 homologs, the regulation of Pyk2 binding. Our results will aid further studies concerning the precise regulation of the NMDA receptor by PKC, Pyk2, and Src signaling and the role of PSD-95 and its homologs in synaptic functions that depend on the targeting of these kinases.

REFERENCES

1. Collingridge, G. L., and Lester, R. A. (1989) *Pharmacol. Rev.* **41,** 143–210
2. Monaghan, D. T., Bridges, R. J., and Cotman, C. W. (1989) *Annu. Rev. Pharmacol. Toxicol.* **29,** 365–402
3. Bear, M. F., and Malenka, R. C. (1994) *Curr. Opin. Neurobiol.* **4,** 389–399
4. Malenka, R. C., and Nicoll, R. A. (1999) *Science* **285,** 1870–1874
5. Bliss, T. V., and Collingridge, G. L. (1993) *Nature* **361,** 31–39
6. Nicoll, R. A., and Malenka, R. C. (1995) *Nature* **377,** 115–118
7. Seeburg, P. H. (1993) *Trends Pharmacol. Sci.* **14,** 297–303
8. Hollmann, M., and Heinemann, S. (1994) *Annu. Rev. Neurosci.* **17,** 31–108
9. Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N., and Jan, L. Y. (1994) *Nature* **368,** 144–147
10. Blahos, J., II, and Wenthold, R. J. (1996) *J. Biol. Chem.* **271,** 15669–15674

11. Brenman, J. E., Christopherson, K. S., Craven, S. E., McGee, A. W., and Bredt, D. S. (1996) *J. Neurosci.* **16,** 7407–7415
12. Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) *Science* **269,** 1737–1740
13. Muller, B. M., Kistner, U., Kindler, S., Chung, W. J., Kuhlendahl, S., Fenster, S. D., Lau, L.-F., Veh, R. W., Huganir, R. L., Gundelfinger, E. D., and Garner, C. C. (1996) *Neuron* **17,** 255–265
14. Sheng, M., and Sala, C. (2001) *Annu. Rev. Neurosci.* **24,** 1–29
15. Tomita, S., Nicoll, R. A., and Bredt, D. S. (2001) *J. Cell Biol.* **153,** F19–F24
16. Cai, C., Coleman, S. K., Niemi, K., and Keinanen, K. (2002) *J. Biol. Chem.* **277,** 31484–31490
17. Valtschanoff, J. G., Burette, A., Davare, M. A., Leonard, A. S., Hell, J. W., and Weinberg, R. J. (2000) *Eur. J. Neurosci.* **12,** 3605–3614
18. Leonard, A. S., Davare, M. A., Horne, M. C., Garner, C. C., and Hell, J. W. (1998) *J. Biol. Chem.* **273,** 19518–19524
19. Sans, N., Racca, C., Petralia, R. S., Wang, Y. X., McCallum, J., and Wenthold, R. J. (2001) *J. Neurosci.* **21,** 7506–7516
20. McGee, A. W., and Bredt, D. S. (1999) *J. Biol. Chem.* **274,** 17431–17436
21. McGee, A. W., Dakoji, S. R., Olsen, O., Bredt, D. S., Lim, W. A., and Prehoda, K. E. (2001) *Mol. Cell* **8,** 1291–1301
22. Shin, H., Hsueh, Y. P., Yang, F. C., Kim, E., and Sheng, M. (2000) *J. Neurosci.* **20,** 3580–3587
23. Tavares, G. A., Panepucci, E. H., and Brunger, A. T. (2001) *Mol. Cell* **8,** 1313–1325
24. Nix, S. L., Chishti, A. H., Anderson, J. M., and Walther, Z. (2000) *J. Biol. Chem.* **275,** 41192–41200
25. Woods, D. F., Hough, C., Peel, D., Callaini, G., and Bryant, P. J. (1996) *J. Cell Biol.* **134,** 1469–1482
26. Moon, I. S., Apperson, M. L., and Kennedy, M. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3954–3958
27. Lau, L. F., and Huganir, R. L. (1995) *J. Biol. Chem.* **270,** 20036–20041
28. Rosenblum, K., Dudai, Y., and Richter-Levin, G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10457–10460
29. Rostas, J. A., Brent, V. A., Voss, K., Errington, M. L., Bliss, T. V., and Gurd, J. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10452–10456
30. Xiong, Z. G., Pelkey, K. A., Lu, W. Y., Lu, Y. M., Roder, J. C., MacDonald, J. F., and Salter, M. W. (1999) *J. Neurosci.* **19,** RC37
31. Yu, X.-M., Askalan, R., Keil, G. J., II, and Salter, M. W. (1997) *Science* **275,** 674–678
32. Zheng, F., Gingrich, M. B., Traynelis, S. F., and Conn, P. J. (1998) *Nat. Neurosci.* **1,** 185–191
33. Lu, W. Y., Xiong, Z. G., Lei, S., Orser, B. A., Dudek, E., Browning, M. D., and MacDonald, J. F. (1999) *Nat. Neurosci.* **2,** 331–338
34. Lu, Y. M., Roder, J. C., Davidow, J., and Salter, M. W. (1998) *Science* **279,** 1363–1367
35. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) *Nature* **383,** 547–549
36. Blaukat, A., Ivankovic-Dikic, I., Gronroos, E., Dolfi, F., Tokiwa, G., Vuori, K., and Dikic, I. (1999) *J. Biol. Chem.* **274,** 14893–14901
37. Girault, J. A., Costa, A., Derkinderen, P., Studler, J. M., and Toutant, M. (1999) *Trends Neurosci.* **22,** 257–263
38. Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 19125–19132
39. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) *Nature* **376,** 737–745
40. Earp, H. S., Huckle, W. R., Dawson, T. L., Li, X., Graves, L. M., and Dy, R. (1995) *J. Biol. Chem.* **270,** 28440–28447
41. Felsch, J. S., Cachero, T. G., and Peralta, E. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5051–5056
42. Raja, S., Avraham, S., and Avraham, H. (1997) *J. Biol. Chem.* **272,** 10941–10947
43. Tokiwa, G., Dikic, I., Lev, S., and Schlessinger, J. (1996) *Science* **273,** 792–794
44. Tapia, J. A., Ferris, H. A., Jensen, R. T., and Garcia, L. J. (1999) *J. Biol. Chem.* **274,** 31261–31271
45. Siciliano, J. C., Toutant, M., Derkinderen, P., Sasaki, T., and Girault, J.-A. (1996) *J. Biol. Chem.* **271,** 28942–28946
46. Heidinger, V., Manzerra, P., Wang, X. Q., Strasser, U., Yu, S. P., Choi, D. W., and Behrens, M. M. (2002) *J. Neurosci.* **22,** 5452–5461
47. Okazaki, H., Zhang, J., Hamawy, M. M., and Siraganian, R. P. (1997) *J. Biol. Chem.* **272,** 32443–32447
48. Derkinderen, P., Siciliano, J., Toutant, M., and Girault, J. A. (1998) *Eur. J. Neurosci.* **10,** 1667–1675
49. Huang, Y., Lu, W., Ali, D. W., Pelkey, K. A., Pitcher, G. M., Lu, Y. M., Aoto, H., Roder, J. C., Sasaki, T., Salter, M. W., and MacDonald, J. F. (2001) *Neuron* **29,** 485–496
50. Bauman, A. L., and Scott, J. D. (2002) *Nat. Cell Biol.* **4,** E203–E206
51. Mochly-Rosen, D. (1995) *Science* **268,** 247–250
52. Pawson, T., and Scott, J. D. (1997) *Science* **278,** 2075–2080
53. Rosenmund, C., Carr, D. W., Bergeson, S. E., Nilaver, G., Scott, J. D., and Westbrook, G. L. (1994) *Nature* **368,** 853–856
54. Bayer, K. U., De Koninck, P., Leonard, A. S., Hell, J. W., and Schulman, H. (2001) *Nature* **411,** 801–805
55. Leonard, A. S., Lim, I. A., Hemsworth, D. E., Horne, M. C., and Hell, J. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3239–3244
56. Strack, S., and Colbran, R. J. (1998) *J. Biol. Chem.* **273,** 20689–20692
57. Strack, S., McNeill, R. B., and Colbran, R. J. (2000) *J. Biol. Chem.* **275,** 23798–23806
58. Leonard, A. S., Bayer, K.-U., Merrill, M. A., Lim, I. A., Shea, M. A., Schulman, H., and Hell, J. W. (2002) *J. Biol. Chem.* **277,** 48441–48448
59. Li, X., Dy, R. C., Cance, W. G., Graves, L. M., and Earp, H. S. (1999) *J. Biol. Chem.* **274,** 8917–8924
60. Lyons, P. D., Dunty, J. M., Schaefer, E. M., and Schaller, M. D. (2001) *J. Biol. Chem.* **276,** 24422–24431
61. Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D., and Garner, C. C. (1993) *J. Biol. Chem.* **268,** 4580–4583
62. Lau, L.-F., Mammen, A., Ehlers, M. D., Kindler, S., Chung, W. J., Garner, C. C., and Huganir, R. L. (1996) *J. Biol. Chem.* **271,** 21622–21628
63. Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N., and Sheng, M. (1995) *Nature* **378,** 85–88
64. Kim, E., Naisbitt, S., Hsueh, Y.-P., Rao, A., Rothschield, A., Craig, A. M., and Sheng, M. (1997) *J. Cell Biol.* **136,** 669–678
65. Leonard, A. S., and Hell, J. W. (1997) *J. Biol. Chem.* **272,** 12107–12115
66. Sans, N., Petralia, R. S., Wang, Y. X., Blahos, J., II, Hell, J. W., and Wenthold, R. J. (2000) *J. Neurosci.* **20,** 1260–1271
67. Lipsich, L. A., Lewis, A. J., and Brugge, J. S. (1983) *J. Virol.* **48,** 352–360
68. Yu, H., Li, X., Marchetto, G. S., Dy, R., Hunter, D., Calvo, B., Dawson, T. L., Wilm, M., Anderegg, R. J., Graves, L. M., and Earp, H. S. (1996) *J. Biol. Chem.* **271,** 29993–29998
69. Jordan, M., Schallhorn, A., and Wurm, F. M. (1996) *Nucleic Acids Res.* **24,** 596–601
70. Kingston, R. E. (1997) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., More, D. D., Seidenman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 9.1.4.–9.1.6., John Wiley & Sons, Inc., New York
71. O'Malley, R. P., Mariano, T. M., Siekierka, J., and Mathews, M. B. (1986) *Cell* **44,** 391–400
72. Shindler, K. S., and Roth, K. A. (1996) *J. Histochem. Cytochem.* **44,** 1331–1335
73. Hunyady, B., Krempels, K., Harta, G., and Mezey, E. (1996) *J. Histochem. Cytochem.* **44,** 1353–1362
74. Burette, A., Wyszynski, M., Valtschanoff, J. G., Sheng, M., and Weinberg, R. J. (1999) *J. Comp. Neurol.* **411,** 601–612
75. Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996) *Cell* **84,** 757–767
76. Kim, J. H., Liao, D., Lau, L.-F., and Huganir, R. L. (1998) *Neuron* **20,** 683–691
77. Chen, H.-J., Rojas-Soto, M., Oguni, A., and Kennedy, M. B. (1998) *Neuron* **20,** 895–904
78. Schaller, M. D., and Sasaki, T. (1997) *J. Biol. Chem.* **272,** 25319–25325
79. Xiong, W. C., Macklem, M., and Parsons, J. T. (1998) *J. Cell Sci.* **111,** 1981–1991
80. Gilmore, A. P., and Romer, L. H. (1996) *Mol. Biol. Cell* **7,** 1209–1224
81. Richardson, A., and Parsons, T. (1996) *Nature* **380,** 538–540
82. Schaller, M. D., Borgman, C. A., and Parsons, J. T. (1993) *Mol. Cell. Biol.* **13,** 785–791
83. Garcia, E. P., Mehta, S., Blair, L. A., Wells, D. G., Shang, J., Fukushima, T., Fallon, J. R., Garner, C. C., and Marshall, J. (1998) *Neuron* **21,** 727–739
84. Colledge, M., Dean, R. A., Scott, G. K., Langeberg, L. K., Huganir, R. L., and Scott, J. D. (2000) *Neuron* **27,** 107–119
85. Sun, Y., Savanenin, A., Reddy, P. H., and Liu, Y. F. (2001) *J. Biol. Chem.* **276,** 24713–24718
86. Masuko, N., Makino, K., Kuwahara, H., Fukunaga, K., Sudo, T., Araki, N., Yamamoto, H., Yamada, Y., Miyamoto, E., and Saya, H. (1999) *J. Biol. Chem.* **274,** 5782–5790
87. Paarmann, I., Spangenberg, O., Lavie, A., and Konrad, M. (2002) *J. Biol. Chem.* **277,** 40832–40838
88. Hough, C. D., Woods, D. F., Park, S., and Bryant, P. J. (1997) *Genes Dev.* **11,** 3242–3253
89. Thomas, U., Ebitsch, S., Gorczyca, M., Koh, Y. H., Hough, C. D., Woods, D., Gundelfinger, E. D., and Budnik, V. (2000) *Curr. Biol.* **10,** 1108–1117
90. Tingley, W. G., Roche, K. W., Thompson, A. K., and Huganir, R. L. (1993) *Nature* **364,** 70–73
91. Tingley, W. G., Ehlers, M. D., Kameyama, K., Doherty, C., Ptak, J. B., Riley, C. T., and Huganir, R. L. (1997) *J. Biol. Chem.* **272,** 5157–5166
92. Hall, R. A., and Soderling, T. R. (1997) *J. Biol. Chem.* **272,** 4135–4140
93. Ehlers, M. D., Tingley, W. G., and Huganir, R. L. (1995) *Science* **269,** 1734–1737
94. Chen, L., and Huang, L. Y. (1992) *Nature* **356,** 521–523
95. Kelso, S. R., Nelson, T. E., and Leonard, J. P. (1992) *J. Physiol.* (*Lond.*) **449,** 705–718
96. Xiong, Z. G., Raouf, R., Lu, W. Y., Wang, L. Y., Orser, B. A., Dudek, E. M., Browning, M. D., and MacDonald, J. F. (1998) *Mol. Pharmacol.* **54,** 1055–1063
97. Logan, S. M., Rivera, F. E., and Leonard, J. P. (1999) *J. Neurosci.* **19,** 974–986
98. Lan, J. Y., Skeberdis, V. A., Jover, T., Zheng, X., Bennett, M. V., and Zukin, R. S. (2001) *J. Neurosci.* **21,** 6058–6068
99. Lan, J. Y., Skeberdis, V. A., Jover, T., Grooms, S. Y., Lin, Y., Araneda, R. C., Zheng, X., Bennett, M. V., and Zukin, R. S. (2001) *Nat. Neurosci.* **4,** 382–390
100. Zheng, X., Zhang, L., Wang, A. P., Bennett, M. V., and Zukin, R. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 15262–15267
101. Cheung, H. H., and Gurd, J. W. (2001) *J. Neurochem.* **78,** 524–534
102. Wang, Y. T., and Salter, M. W. (1994) *Nature* **369,** 233–235