SUPPLEMENTARY MATERIALS AND METHODS

DNA constructs

Full-length rat PSD-95 cDNA in pGW1 vector was generously provided by M Sheng (Harvard University, Boston, MA, USA) and has been described elsewhere (Kim et al, 1995). PSD-95/pME18S deletion constructs ΔPDZ1/2, ΔPDZ3 and ΔSH3/GuK were gifts from H Umemori and T Yamamoto (University of Tokyo, Tokyo, Japan) and have been previously described (Tezuka et al, 1999). PSD-95 deletion construct Δ(1-54)/SH3/GuK was generated by PCR and subcloned into the pME18S vector. Mutant Δ(14-54) PSD-95 construct was generated by PCR and ligated into the pCR-Blunt II-TOPO vector (Invitrogen) before subsequent subcloning into the pGW1 vector. Mouse neuronal Src/pRcCMV mutant constructs (gifts from S Hanks, Vanderbilt University, Nashville, TN, USA) have been described elsewhere (Polte & Hanks, 1997) and included R175K Src, constitutively active Y527F Src and catalytically inactive K295R Src. Wild-type mouse neuronal Src was generated using the QuikChange XL site-directed mutagenesis kit (Stratagene) with Y527F Src/pRcCMV as the template. Rat NR1-1a/pRK5, rat NR2B/pRK5 and rat NR2A/pcDNA3 constructs were gifts from J MacDonald (University of Toronto, Toronto, ON, Canada). The following GST fusion constructs were generated by PCR and subcloned into the GST fusion vector pGEX-2T or pGEX-6P-1 (Amersham): Src unique domain (aa 4-86), Src SH3 domain (aa 88-146 of mouse neuronal Src), wild-type Src SH2 domain (aa 148-247), R175K Src SH2 domain, PSD-95(1-54) (aa 1-54), PSD-95 PDZ1 domain (aa 64-151) and PSD-95 PDZ2 domain (aa 160-246). According to convention, all of the amino acid residue numbers of Src correspond to chicken c-Src except where indicated. All DNA constructs were sequence verified.
**GST fusion proteins**

GST fusion proteins were expressed in *Escherichia coli* BL21 and were affinity purified using glutathione Sepharose beads (Amersham) according to the manufacturer’s instructions. For pull down assays, GST fusion proteins were not eluted from the beads. For ELISAs, GST fusion proteins were eluted and then dialyzed extensively against PBS. PSD-95(1-54) peptide was expressed as a GST fusion protein and then cleaved from GST with PreScission Protease (Amersham) according to the manufacturer’s protocol. Purity of all recombinant proteins was assessed by SDS-PAGE and Coomassie staining.

**Immunoblotting**

The following antibodies were used for immunoblotting: anti-PSD-95 mouse monoclonal (Affinity BioReagents, Oncogene, Transduction Labs), anti-Src mouse monoclonal (a gift from J Bolen, DNAX, Palo Alto, CA, USA), anti-pY416 Src mouse monoclonal (a gift from A Laudano, University of New Hampshire, Durham, NH, USA), anti-NR2A/B rabbit polyclonal (Chemicon), anti-NR2B rabbit polyclonal (Novus), anti-phosphotyrosine mouse monoclonal (clone 4G10, Upstate) and anti-GAPDH mouse monoclonal (Cell Signaling). Anti-PSD-95 rabbit polyclonal antibody (Synaptic Systems) was raised against aa 18-32 of rat PSD-95. Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were purchased from Amersham. Signals were detected with ECL (Amersham) and developed on X-ray film. For densitometric quantification, immunoblots were digitized on a flatbed scanner and digital images were quantified using Scion Image software (Scion Corp). A 5-point dilution series of rat forebrain proteins was included on each immunoblot to be quantified. A standard curve was generated from the dilution series and used to determine the linear range of detection for each antibody. Only values falling within the linear range were incorporated into the final analysis.
Direct binding between GST-Src SH2 and the PSD-95 peptides was assessed with a modified ELISA using previously described methods (Gilmer et al, 1994). Briefly, 96-well Immunolon-4 HBX microtiter plates (Thermo Labsystems) were coated with PSD-95(1-54) (20ng/µl in PBS) or with PSD-95(6-20), PSD-95(19-33), PSD-95(32-46) or PSD-95(43-57) (250ng/µl in PBS) overnight at 4°C and then were washed with PBS. Next, the plates were blocked with 1% (w/v) BSA in PBS overnight at 4°C and then washed with PBS. Increasing amounts of GST alone or GST-Src SH2 were diluted in PBS with 0.05% (v/v) Tween 20 (PBS-T) as indicated and added to the coated wells. Following incubation, wells were washed with PBS-T and then anti-GST HRP conjugated antibody (Santa Cruz) diluted in PBS-T was added for 1 h at room temperature. After a final wash step, colorimetric HRP substrate ABTS (Rockland) was added to the wells and incubated at room temperature for ~30 min. Each experiment was performed in duplicate. Optical density (OD) was measured at A405 on a VMAX plate reader (Molecular Devices). Background binding of GST alone was subtracted. OD values were normalized to maximum A405 reading for each experiment. Prior to the experiments, the linear range of the plate reader was determined for OD measurements at A405 using a dilution series. Experiments in which measurements fell outside of the linear range were excluded from the final analysis.

In vitro kinase assay

Full-length recombinant Src or Fyn (Upstate, 3.0 U) was pre-incubated with PSD-95(43-57), EPQ(pY)EEIPA or EPQYEEIPA in kinase reaction buffer (100 mM Tris-HCl pH 7.2, 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 250 µM sodium orthovanadate, 2 mM DTT) without
ATP for 5 hr at 4°C prior to performing the kinase assays. Peptides were used at a concentration of 1 mM as this concentration is known to be required for activation of Src by EPQ(pY)EEPIA, by preventing auto-inhibition through the SH2 domain (Liu et al, 1993). Although EPQ(pY)EEPIA has a high affinity for the SH2 domain, it must compete with the intramolecular binding of phosphorylated tyrosine in the tail of the kinase, which keeps Src in an inactive conformation. Following pre-incubation with the peptides, Src assay kit (Upstate) was used according to the manufacturer’s instructions to measure kinase activity in vitro. Src or Fyn was combined with the substrate peptide (150 µM) diluted in kinase reaction buffer. The sequence of the substrate peptide corresponded with aa 6-20 of p34cdc2 (KVEKIGEGTYGVVYK). The kinase reaction was initiated with the addition of [γ-32P]ATP (Amersham) and the reaction proceeded for 10 min at 30°C. Tyrosine kinase activity was evaluated by measuring transfer of [γ-32P]ATP by the kinase to the substrate peptide. Amount of phosphorylated peptide was quantified with a scintillation counter.

**Miniature EPSC recordings**

Methods for whole cell recordings are described in Pelkey et al (2002). Briefly, cultures were bathed in extracellular solution containing (in mM): 140 NaCl, 5.4 KCl, 25 HEPES, 1.3 CaCl₂, 33 glucose, pH 7.35 (310-320 mOsm) supplemented with TTX (0.5 µM), strychnine (10 µM), bicuculline methiodide (10 µM), glycine (1 µM). Patch electrodes (4-7 MΩ) were filled with intracellular solution (ICS) containing (in mM): 140 CsCl, 10 HEPES, 10 BAPTA, 2 Mg-ATP, pH 7.25 (300-310 mOsm). ICS was supplemented with GST (5 µg/ml) or GST-PSD-95(1-54) (5 µg/ml) as indicated, and osmolarity was re-adjusted accordingly. Membrane potential was held constant at −60 mV and whole cell currents were recorded using an Axopatch 200B
amplifier (Axon Instruments). All recordings were performed at 22-24°C. mEPSCs were recorded immediately upon formation of the whole cell configuration and continuously monitored for 20-30 min thereafter. mEPSCs were low-pass filtered at 2 kHz and acquired on line with a personal computer using Strathclyde software (courtesy of J Dempster) with the detection level set to ~3× higher than the baseline noise. False events and traces with >1 event/200 ms recording period were eliminated by subsequent inspection of the raw data. Averaged mEPSCs were created using a minimum of 50 traces aligned by their rising edges. NMDAR-mediated currents (I_NMDA) were constructed by subtracting, from the averaged mEPSC, a current decaying at a single exponential rate equal to the fast component. Peak mEPSC amplitude was measured to determine the AMPAR component of averaged mEPSCs and integrated current during I_NMDA (charge) was measured to determine the NMDAR component of averaged mEPSCs.

**Hippocampal slice recordings**

Hippocampal slices were prepared from 3-4 week old male Sprague-Dawley rats and were placed in a holding chamber for ≥1 hr prior to recording. A single slice (300 µm) was then transferred to a recording chamber and superfused at 2 ml/min with artificial cerebrospinal fluid (ACSF) composed of (in mM): 136 NaCl, 3 KCl, 1.25 NaH2PO4, 2 MgCl2, 11 D-glucose, 20 NaHCO3, 2 CaCl2, pH 7.40 (315-325 mOsm) saturated with 95% O2 (balance 5% CO2) at 28-32°C. ACSF was supplemented with bicuculline methiodide (5 µM). Synaptic responses were evoked with bipolar tungsten electrodes located ~50 µm from the cell body layer in CA1. Test stimuli were delivered at 0.1 Hz and stimulus intensity was set to 35% of that which produced maximum synaptic responses. Whole-cell EPSC recordings were done using the visualized method (Zeiss Axioskop 2FS microscope) with patch pipettes (3-4 MΩ) containing ICS.
composed of (in mM): 132.5 Cs-gluconate, 17.5 CsCl, 10.0 HEPES, 10.0 BABTA, 2.0 Mg-ATP, 0.3 GTP, 5.0 QX-314, pH 7.25 (290 mOsm) supplemented with PSD-95(43-57) or sPSD-95(43-57) as indicated and placed in the cell body layer. For recording NMDAR-mediated synaptic responses, ACSF was supplemented with DNQX (5 µM) and neurons were held at −60 mV. NMDAR-mediated EPSC peak amplitudes were normalized to the first 2 min of whole cell recording. Current-voltage relationships for NMDAR EPSCs were performed at the end of each recording. Peak amplitudes of EPSCs were obtained at holding potentials from −100 to +80 mV in steps of 20 mV. EPSC amplitude at each membrane potential was normalized on a cell-by-cell basis to that of the EPSC obtained at a holding potential of +40 mV. TBS consisted of 15 bursts of 4 pulses at 100 Hz, delivered at an interstimulus interval of 200 ms. Whole-cell EPSP recordings were done using the visualized patch method with patch pipettes (3-4 MΩ) containing ICS composed of (in mM): 132.5 K-gluconate, 17.5 KCl, 10 HEPES, 0.2 EGTA, 2.0 Mg-ATP, 0.3 GTP, 5.0 QX-314, pH 7.25 (290 mOsm) supplemented with PSD-95(43-57) or sPSD-95(43-57) as indicated and placed in the cell body layer. EPSP slope was calculated as the slope of the rising phase between 10% and 65% of the peak of the response. Raw data were amplified using a MultiClamp 700A amplifier and a Digidata 1322A acquisition system sampled at 10 kHz and analyzed with Clampfit 9.0 (Axon Instruments) and Sigmaplot 7. Cells were discarded if the series resistance changed by more than 15%. Extracellular fEPSPs were recorded using glass micropipettes filled with ACSF placed in the stratum radiatum 60-80 µm from the cell body layer. fEPSP slope was calculated as the slope of the rising phase between 10% and 60% of the peak of the response. EPSP and fEPSP slopes were normalized to the average level during the 10 min of recording before stimulation. Data are presented as mean (±sem). Two way ANOVA with the Student-Newman-Keuls test were used for statistical comparison.
References


Polte TR and Hanks SK (1997) Complexes of focal adhesion kinase (FAK) and Crk-associated substrate (p130(Cas)) are elevated in cytoskeleton-associated fractions following adhesion and Src transformation. Requirements for Src kinase activity and FAK proline-rich motifs. *J Biol Chem* **272**: 5501-5509