Lipid-dependent Recruitment of Neuronal Src to Lipid Rafts in the Brain*

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Although most Src family tyrosine kinases are modified by palmitoylation as well as myristoylation, Src itself is only myristoylated. Dual acylation is important for attachment to liquid-ordered microdomains or lipid rafts. Accordingly, Src is excluded from lipid rafts in fibroblasts. Evidence of partial genetic redundancy between Src and Fyn for brain-specific targets suggests that these two kinases may occupy overlapping subcellular locations. Neuronal Src (NSrc), an alternative isoform of Src with a 6-amino acid insert in the Src homology 3 domain, is highly expressed in neurons. We investigated whether this structural difference in NSrc allows it to associate with lipid rafts. We found that perinatal mouse brains express predominantly NSrc, which is partly (10–20%) in a lipid raft fraction from brain but not fibroblasts. The association of Src with brain lipid rafts does not depend on the NSrc insert but depends on the amino-terminal myristoylation signal. A crude lipid fraction from brain promotes NSrc entry into rafts in vitro. Moreover, lipid raft-localized NSrc is more catalytically active than NSrc from the soluble fraction, possibly because raft localization alters access to other tyrosine kinases and phosphatases. These findings suggest that NSrc may be involved in signaling from lipid rafts in mouse brain.

The Src family of nonreceptor tyrosine kinases (SFKs)1 is broadly expressed and involved in many cell surface receptor-mediated signaling cascades (for review, see Refs. 1 and 2). Elucidation of normal SFK function has been difficult because most stimuli that activate SFKs also activate non-SFK tyrosine kinases. Moreover, redundancy within the SFK family has confounded attributing a specific signaling process exclusively to one kinase. Gene disruption studies have sometimes revealed exclusive functions for specific SFKs in certain cell types. Src in osteoclasts (3) and Lck in T cells (4) for instance, but, for the most part, deficiency of one SFK is compensated by others (5).

The subcellular localizations of SFKs have provided valuable clues toward understanding their functions. Src in focal adhesions plays a key role in integrin-dependent signaling events that affect cellular adhesion and motility (6–8), and Lck is recruited to endosomes in CD2-activated T cells where it is involved in CD2 receptor internalization (9). Localization of SFKs to various subcellular locations can be affected by protein-protein interactions involving their SH3 or SH2 domains (7, 10). However, lipid-lipid interactions involving amino-terminal acyl groups on SFKs are the primary mechanism for membrane localization of SFKs (11–14), particularly localization to membrane microdomains or lipid rafts.

Lipid rafts are “liquid-ordered” microdomains in cell membranes (15, 16) which have been shown to exist in live cells at 37 °C (17–19). Enriched in cholesterol, sphingolipids, and phosphoinositides, these membrane microdomains contain proteins involved in vesicular trafficking and signal transduction. Lipid rafts often contain structural proteins such as caveolins and flotillins, but some do not (20). SFKs (12), receptor tyrosine kinases (platelet-derived growth factor receptor, epidermal growth factor receptor) (21–24), and G proteins (25, 26) are found in lipid rafts. Lipid rafts are important for the inclusion of SFKs such as Fyn, Lyn, Lck, and Hck p59 into lipid rafts of fibroblasts and leukocytes (30, 31, 34). Introducing a palmitoylation signal into wild-type Src causes relocalization to rafts (31). The exclusion of Src from rafts implies that it may not encounter substrates that are localized in or traffic through lipid rafts, and hence Src may be performing distinct functions from raft-localized SFKs. This notion is supported by the finding that EphrinA5 tyrosine phosphorylation in brain lipid rafts is reduced when Fyn is absent, even though Src is present (35). On the other hand, some other brain proteins are phosphorylated by either Src or Fyn. For example, Src and Fyn are redundant for tyrosine phosphorylation of p190 RhoGAP in the brain (36). And although Reelin-induced tyrosine phosphorylation of Dab1 in neurons appears to be primarily mediated by Fyn, Src can phosphorylate Dab1 when Fyn is absent (37). This implies that both Fyn and Src can access p190 RhoGAP and Dab1.
It is known that an alternative form of Src, neuronal Src (NSrc), is expressed during neuronal differentiation (38). NSrc differs from the normal splice form (called here cSrc) by 6 amino acids (RKVDVR) that are inserted into the SH3 domain because of an alternative splicing event (39, 40). The activity of NSrc is 2–4-fold higher than that of cSrc (41, 42). It is thought that these 6 amino acids destabilize the inactive conformation of Src, thereby leading to enhanced activity (43). It is also possible that the inclusion of these amino acids alters the localization of Src in cells and perhaps redistributes Src into lipid rafts. Consistent with this hypothesis, the NSrc insert is on a surface of the SH3 domain which is thought to be close to lipid rafts. When NSrc is expressed in fibroblasts, it does not enter rafts. Localization to lipid rafts in brain requires only the amino-terminal myristoylation signal and not the SH3 region containing the NSrc insert and depends on brain lipids. Finally, to begin to understand the function of NSrc in rafts, we explored the kinase activity of NSrc in mouse brain and found that total NSrc kinase activity is dependent not only on the isoform but also on raft association.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture, and Transfection—SYF1 cells, derived from src–/-; yes–/-; fyn–/- mouse embryos using T antigen, were described previously (49). To generate SYF1 cells reexpressing either mouse cSrc or mouse NSrc a standard retroviral infection protocol was used (50). The retroviral vector pLXSH containing Ssrc cDNA was used to generate viruses in 293T cells, followed by exposure of target SYF1 to these viruses at a low multiplicity of infection. The day after viral infection, SYF cells were selected with 0.2 mg/ml hygromycin B (Calbiochem). Src expression was verified by Western blotting. After the initial selection, stable cell lines (selected as pools, not clones) were maintained in the absence of hygromycin, and all cell lines were used at low passage number for all experiments. Fyn3 cells, SYF1 cells reexpressing Fyn cDNA, were provided by Dr. L. A. Cary. SYF1 cells, Src- or Fyn-reexpressing SYF cells, and human embryonic kidney 293T cell lines were all grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). 293T cells were transfected with DNA:calcium phosphate coprecipitates using the HEPES-buffered calcium phosphate method. Transfection medium was replaced by fresh culture medium 24 h after transfection, and cells were lysed 48 h after transfection. For retrovirus production 4 ml of fresh medium was added after 48 h, and virus was collected between 60 to 72 h post-transfection. SYF cells were transfected using LipofectAMINE Plus (Invitrogen) in Opti-MEM (Invitrogen) using methods described by the manufacturer. Transfection medium was replaced with fresh culture medium 12 h post-transfection, and cells were lysed 48 h post-transfection.

Animals—all mice used in this study were hybrid C57BL6J/129Sv. Genotyping typing was performed by PCR on tail DNA.

Expression Plasmids—Construction of mouse Src cDNA expression plasmids pSGT-cSrc, pSGT-NSrc, pLXSH-cSrc, and pLXSH-NSrc are described in Ref. 37. Hemagglutinin epitope (HA)-tagged cSrc and NSrc cDNAs were constructed by replacing the stop codon of Src cDNA in pSGT-Src with an XhoI site and subcloning the sequenced BamHI-XhoI fragment into pRES-hrGFP-2a.1 (Stratagene). Use of plasmids expressing GST-NSrcSH3 and GST-FynSH3 fusion proteins are described in Ref. 51. Src NH2-terminal 7 amino acids were fused to GFP (pCMX-
Ssrc7aa-GFP) by first replacing the start codon of GFP in pCMX-GFP with a HindIII-ATG-KpnI cassette (pCMX-Δ ATG-GFP). A HindIII-KpnI adaptor encoding the first 7 amino acids of Src, MGSNKK, was prepared by boiling the oligonucleotides 5′-AGCTTCTAGGGGCAAGCA-ACAAAGCAAGGGTAAC-3′ and 5′-CCCTCGCTTGTGCTGCGCCATG-GA-3′ and annealing them at room temperature. The double-stranded adaptor was purified from a 15% acrylamide-TBE gel after ethidium bromide staining, eluting DNA from the gel piece by overnight incubation in water. Double-stranded adaptors encoding the G2A mutant, MASNSKK, were purified similarly. These adaptors were cloned into HindIII- and KpnI-digested pCMX-Δ ATG-GFP. All plasmids were sequenced to confirm the appropriate changes.

**Antibodies**—Anti-Src monoclonal antibody (MAb) LA074 was produced from the LP01B mouse hybridoma, maintained at the Biosafety Laboratories, Camden, NJ, and diluted from an unpurified cell supernatant. This MAb was raised against amino acids 2–17 of v-Src. The anti-Src MAb 327 was a kind gift from Dr. J. S. Brugge (Harvard University). An anti-pan-Src antibody that recognizes Src, Yes, and Fyn, SRC2, and anti-Fyn antibody, FYN3, were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology. Anti-phospho-Src Tyr-418 (referred to as such for anti-phospho autophosphorylated tyrosine) and anti-phospho-Src Tyr-529 (referred to as such for anti-phospho carboxyl-terminal tyrosine) antibody were from Biosource. Anti-HA antibody HA.11 was from Covance, anti-caveolin antibody from Transduction Laboratories, and anti-GFP antibody from Roche Applied Science. The anti-GST antibody 38.3 was described earlier (52). Horseradish peroxidase-conjugated secondary antibodies were purchased from Bio-Rad.

**Preparation of Lipid Rafts**—Lipid rafts were isolated from cells and brain tissue primarily as described earlier (53). Briefly, frozen brain tissue was weighed and homogenized in an ice-cold Triton X-100 lysis buffer containing 1% Triton X-100 and 5% glycerol in buffer A (50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 0.15 mM NaCl, 20 mM NaF, 1 mM Na3VO4, 5 mM β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) at a ratio of 8:1 (v/w) by 5 strokes with a loose pestle followed by 10 strokes with a tight pestle of an all glass homogenizer, while keeping the entire apparatus chilled on ice. The brain lysate mixture was filtered (through Whatman filter paper No. 1) to remove tissue debris. The supernatant was collected and used for preparation of lipid rafts. Cultured adherent cells, in culture dishes, were washed with cold phosphate-buffered saline (PBS) and scraped and collected in chilled PBS. The cells were centrifuged gently in a clinical centrifuge, and PBS was aspirated. The packed cell volume was estimated, and the resulting cell pellet was gently resuspended in ice-cold Triton X-100 lysis buffer at a ratio of 8:1 (v/w). After mixing on ice for 20 min, the cell lysates were centrifuged at 500 × g for 5 min in a cooled tabletop microcentrifuge to sediment nuclei and tissue debris. The supernatant was collected and used for preparation of lipid rafts. The Triton X-100 lysates collected as described above were mixed on ice for at least 1 h. Then, to 1 ml of the lysate, 1 ml of an 80% sucrose solution in buffer A was gradually added with continuous mixing on ice resulting in a solution containing 40% sucrose. 1.9 ml of this mixture was placed at the bottom of a 5-ml ultracentrifuge tube (Ultraclear, Beckman) and PBS was aspirated. The packed cell volume was estimated, and the resulting cell suspension was mixed on ice for at least 2 h prior to mixing with 20% sucrose solution in buffer B. In some experiments, a Triton X-100-soluble fraction of a cell lysate was isolated by ultracentrifugation of a 1% Triton X-100 lysate in an air-driven ultracentrifuge (Beckman) at maximum air pressure and at 4 °C, thus precipitating insoluble material.

**Immunoprecipitation, Gel Electrophoresis, and Western Blot Analysis**—Total protein levels in lysates were measured using the Bio-Rad protein assay dye, and equivalent amounts of protein were used for immunoprecipitation from Triton X-100 fractions. Because the total protein in lipid raft fractions was limitingly low for measurement, some volume equivalents of raft fraction as soluble fractions were used when comparing abundance of proteins in each fraction. Additionally, for standardization purposes prior to immunoprecipitation kinase assays, or to compare phosphorylation status of proteins, a range of amounts of immunoprecipitates was analyzed.

1% Triton X-100 lysates, with or without added octyl β-D-glucopyranoside, were mixed with modified radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM Na3VO4, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) to obtain a total volume of 600 μl. Immunoprecipitations were performed by first incubating the indicated primary antibodies with the cell lysates for 3 h or overnight at 4 °C. Immune complexes formed with rabbit antibodies were precipitated by incubating the reactions with protein A-immobilized Sepharose CL-4B beads (Sigma) for an additional 1 h at 4 °C. Immune-com
plexes formed with mouse antibodies were immunoprecipitated by a similar incubation with either protein G-immobilized Sepharose 4 Fast Flow (Amersham Biosciences) or with rabbit anti-mouse immunoglobulin G and protein A-immobilized Sepharose CL-4B beads. Immunoprecipitates were washed three times with modified radioimmunoprecipitation assay buffer and were either re suspended in SDS-PAGE sample buffer for Western blotting or processed through additional washes in preparation for kinase assays.

SDS-PAGE and Western blotting were performed as described previously (8); however, different acrylamide concentration in gels and different acrylamide:bisacrylamide ratios were used to obtain optimal separation of proteins. The different gel properties are described in the legends of each experiment. Most notable are the 8 or 9% acrylamide gels with a 20:1 acrylamide:bisacrylamide ratio which were used to separate Src or Fyn from the immunoglobulin G heavy chain in immunoprecipitates. The proteins were transferred to pure nitrocellulose filters (0.22 μm pore size), and the were filters stained with Ponceau S to confirm proper transfer and subsequently blocked with 2% bovine serum albumin in Tris-buffered saline (TBS) containing 0.05% Tween 20.

FIG. 3. Recruitment of Src to lipid rafts in vitro. A, SYF cells (1 mg of protein) expressing HA epitope-tagged NSrc (NSrcHA) were separated into soluble and raft fractions, without or with mixing with postnatal day 1 (P1) brain lysate (1.5 mg of protein) and processed as described (Fig. 1). Western protein blots (WB) were probed for Src (La074), HA epitope, Fyn (FYN3), or cavolin (Cav) antibody. B, SYF cells (1 mg of protein) expressing cSrcHA or NSrcHA were fractionated into soluble and raft fractions. The soluble fraction was separated with postnatal day 1 brain lysate (1.5 mg of protein) and fractionated again. WBs were probed for Src (La074). C, 1.5 mg of Triton X-100 lysate of SYF cells expressing NSrc was mixed with a suspension of brain lipids and fractionated as above. D, bacterially expressed NSrc SH3 or Fyn SH3 GST fusion proteins were mixed with postnatal day 1 brain lysate and fractionated. GST fusion proteins were detected with anti-GST antibody. E, Triton X-100 soluble fraction of 293T cells expressing either GFP or an N terminally Src-GFP fusion were fractionated after mixing with 2 mg of Triton X-100 lysate of postnatal day 1 brain.
Electrophoresis was started and continued until the dye front was 5 mm from the resolving gel allowing in-gel protease digestion to occur. After 30 min, electrophoresis was resumed and continued until the dye front reached the bottom of the gel. The gel was then dried and exposed to a PhosphorImager screen.

**GST Fusion Protein Expression**—GST fusion proteins were expressed and purified as follows. Briefly, 1 ml of an overnight culture from a single colony was diluted into 200 ml of fresh LB containing ampicillin and cultured at 37°C until the A600 reached 0.5. The cultures were induced with 100 μM isopropyl-1-thio-D-galactopyranoside and cultured at room temperature for 3 h, and cells were collected by centrifugation. The bacterial pellet was washed once with PBS and then sonicated on ice in 8 ml of 2 PBS containing 10% glycerol, 1% Triton X-100, and 1 mM PMSF. The lysate was centrifuged at 9,000 g for 10 min at 4°C, and the supernatant was incubated with 250 μl of a 70% slurry of glutathione-Sepharose 4B beads (Amersham Biosciences), with mixing, at 4°C for 1 h. The beads were washed once with 10 ml of 2 PBS containing 10% glycerol and resuspended in 4 ml of 2 PBS containing 10% glycerol and 1 mM PMSF. An aliquot of this slurry was centrifuged, supernatant removed, and GST fusion proteins were eluted by incubating the beads with 10 mM reduced glutathione in 50 mM Tris, pH 8, for 10 min at room temperature. The mixture was centrifuged, and the supernatant was collected and dialyzed overnight against PBS containing 1 mM PMSF. Eluted GST fusion proteins were separated with SDS-PAGE and detected by Western blotting.

**Lipid Extraction**—Brain lipids were extracted essentially as described previously (56). Briefly, perinatal day 1 mice brains were sonicated, in 15-s spurts, in ice-cold Folch Extraction Buffer (2:1 chloroform:methanol mixture) while keeping the sample chilled in an ice water bath. Tissue debris was discarded by centrifugation, and the supernatant was washed once with 0.2 volume of water and once with 0.2 volume of Folch Upper Phase (3:48:47 chloroform:methanol:water containing 0.1 M KCl), and the resulting brain lipid extract was used for further studies. An aliquot of the lipid extract was dried under a stream of argon, and the residue was resuspended into ice-cold 1% Triton X-100 buffer by sonication, again in a manner described above. This emulsion was used in mixing experiment.

**RESULTS**

**Mouse Brains Contain NSrc in Lipid Rafts**—We first tested whether Src is present in Triton X-100-insoluble lipid raft preparations isolated by floatation from mouse brain. Using La074, an antibody raised to the unique amino-terminal 17 residues of Src,Src was found in both soluble and lipid raft fractions from perinatal mouse brain (Fig. 1A; raft fractions were also enriched for caveolin). In comparing samples prepared from embryonic, perinatal, and weanling mouse brains, the proportion of Src that was present in lipid raft was maximal in adult brain and less in embryonic or perinatal brain (Fig. 1B). In various preparations, between 5 and 20% of Src was present in the raft fraction. In contrast, when a Fyn-specific antibody, FYN3, was used to study the same samples, a much larger proportion of Fyn (greater than 90%) was in the lipid raft fractions (Fig. 1B), as expected (31, 57).

The low abundance of Src in lipid rafts from brain could be the result of competition with Fyn or Yes for binding sites. We tested this by comparing lipid rafts from wild-type and fyn/−/− mice.

**Activated NSrc in Lipid Rafts from Brain**

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**Fig. 4. NSrc is more active than cSrc.** A, cSrc and NSrc were immunoprecipitated from SYF cell lysates (100 μg of protein) using La074 antibody. Immunoprecipitated Src was eluted in 60 μl of 1× SDS-PAGE sample buffer, and different volumes were separated on a 8% (20:1 acrylamide:bisacrylamide) gel. Blots (WB) were probed for Src (La074), phosphotyrosine 418 (P-Y418), and phosphotyrosine 529 (P-Y529). B, quantification of the ratio of phosphorylation of cSrc and NSrc at tyrosine 418 and 529. C–E, equivalent amounts of Src immunoprecipitated by the indicated antibodies were used in an in vitro kinase reaction using enolase as substrate. Protein samples were separated on a 9% (20:1 acrylamide:bisacrylamide) gel, and the protein blot was probed for Src (La074) (C), exposed to a PhosphorImager detection screen (D), and incorporation of radiolabeled into enolase was quantified (E).
perinatal brain samples (Fig. 1C). In both samples, ~10% of Src was in the raft fraction. The same results were obtained using \( \text{fyn}^{-/-} \), \( \text{yes}^{-/-} \), and \( \text{yes}^{-/-} \) brains (data not shown). Therefore, the quantity of Src in rafts is not limited by competition from Fyn and/or Yes.

Consistent with the possibility that Fyn is the prevalent SFK in brain lipid rafts, \( \text{fyn}^{-/-} \) brain lipid rafts contained much less tyrosine-phosphorylated protein in the molecular size range of Src and Fyn (Fig. 1D) and less material that reacted with antibodies to the phosphorylation sites conserved across the Src family, phosphotyrosine 418 and 529 (Fig. 1C). Nevertheless, other proteins contained in lipid rafts from \( \text{fyn}^{-/-} \) brains were tyrosine-phosphorylated at levels similar to those in lipid rafts from wild-type controls (Fig. 1D). This implies either that Fyn is responsible for little of the tyrosine phosphorylation of raft proteins or that Src or other tyrosine kinases can replace Fyn when Fyn is absent, even though Src distribution does not change.

The major form of Src present in brain has been reported to be NSrc (38, 39). However, distinguishing NSrc from cSrc by SDS-PAGE is difficult (e.g., see below, Fig. 4C) because they are very similar in size (541 and 535 residues, respectively). To test which form of Src was present in brain lipid raft and soluble fractions, we made use of a modified \( S. \text{ aureus} \) V8 protease digestion (55) protocol. The modifications included dephosphorylating the proteins to prevent possible phosphorylation-dependent mobility changes and then labeling with radioactive phosphate at Ser-17, using protein kinase A in the presence of a specific Src inhibitor (54). For markers, we used NSrc and cSrc that were expressed in SYF fibroblasts, which contain no endogenous Src (49). As seen in Fig. 2, cSrc and NSrc immunoprecipitated from fibroblasts differ only slightly in apparent size before digestion, but after V8 protease digestion, the sizes of the V3 and V4 NH\(_2\)-terminal fragments (Fig. 2A) are significantly different (Fig. 2B). Based on these markers, the "Src" proteins immunoprecipitated from both the lipid raft and non-raft fractions of mouse brain are predominantly NSrc. Therefore, NSrc is partitioned partly into the lipid raft fraction in mouse brain.

**Lipid Raft Localization of Src Is Dependent on Brain Lipids**—It was possible that inclusion of Src in brain lipid rafts was caused by the presence of the NSrc insert. To test whether NSrc has an intrinsically higher propensity to enter rafts, we compared the localizations of NSrc and cSrc in fibroblasts. As shown in Fig. 1A, lipid rafts from SYF cells expressing cSrc or NSrc contain caveolin, but not cSrc or NSrc. Therefore, NSrc does not enter lipid rafts in fibroblasts but does in brain. In addition, lipid rafts from brain contained a larger proportion of caveolin molecules than those from fibroblasts (Fig. 1A), implying that lipid rafts have different properties depending on the cell type. This raises the possibility that lipid rafts from brain may have a higher affinity for NSrc and caveolin than those from fibroblasts.

We postulated that NSrc is recruited to lipid rafts from brain by protein or lipid interactions that are peculiar to the brain rafts. To test this, NSrc was expressed in fibroblasts and mixed with brain lysate before fractionation. The NSrc was tagged with HA epitope at the COOH terminus so that it could be distinguished from the endogenous brain NSrc. Both NSrc-HA and cSrc-HA, expressed in SYF1 fibroblasts, entered lipid rafts when mixed with brain extracts (Fig. 3A and data not shown). This implies that the presence of NSrc in lipid rafts prepared from brain is caused by a distinct property of the lipid rafts from this tissue.

Because only 5–20% of NSrc in brain is in the lipid raft fraction, we wondered whether this population of NSrc is different from the remainder or whether the quantity in the rafts is limited for some other reason, such as affinity. To test this, NSrc-HA or cSrc-HA, isolated from the nonraft fraction of fibroblasts (Fig. 3A, first lane, and not shown), was mixed with additional brain lysate, and detergent-insoluble lipid rafts were again prepared. A portion of the previously soluble Src redistributed into the lipid raft fraction (Fig. 3B, second and fourth lanes). This implies that the amount of NSrc or cSrc in the brain lipid raft fraction is limited by the protein or lipid composition of the rafts. To distinguish these possibilities, NSrc from SYF cells was mixed with crude brain lipids before fractionation. A small fraction of the NSrc appeared in the raft...
fraction (Fig. 3C). This shows that the specific lipids present in brain extracts contribute to the presence of NSrc in lipid rafts from brain.

The primary difference between cSrc and NSrc is in the SH3 domain and because both Src molecules show similar affinity for lipid rafts (Fig. 3D) it is unlikely that raft localization of Src in brain is the result of molecular interactions involving the SH3 domain. Indeed, the cSrc and Fyn SH3 domains, expressed as GST fusion proteins, did not enter lipid rafts if they were mixed with brain lysates before floatation (Fig. 3D). In contrast, when the first 7 amino acids of Src, containing the myristoylation signal (58), was fused to a heterologous protein (GFP), some of this chimeric molecule was recruited to lipid raft preparations, whereas GFP alone was not (Fig. 3E). Conversely, a G2A mutant of Src-GFP, which cannot be myristoylated, was not recruited to lipid rafts (data not shown). These results suggest that the NH2-terminal 7 residues of Src, including the myristoylation signal, are sufficient to recruit a small fraction of Src to lipid rafts from brain and further imply that myristoylation may account for the presence of endogenous NSrc in lipid rafts in brain.

**Lipid Raft-associated NSrc Is Activated**—NSrc is reportedly more catalytically active than cSrc (41, 42). Activity is known to be regulated by dephosphorylation of the COOH-terminal regulatory tyrosine, Tyr-529 (Tyr-535 in NSrc), and phosphorylation on the activation loop tyrosine (Tyr-418; Tyr-424 in NSrc) (1, 43). Phosphospecific antibodies were used to compare phosphorylation of cSrc and NSrc, both made in SYF cells and immunoprecipitated in equivalent quantities (Fig. 4A). Although both proteins were phosphorylated to a similar extent on Tyr-529, phosphorylation on Tyr-418 was ~2.5-fold higher for NSrc than cSrc (Fig. 4, A and B). To assay kinase activity directly, equal amounts of cSrc and NSrc, bound to equal amounts of either of two different monoclonal antibodies (Fig. 4C), were incubated with radioactive ATP and enolase as an exogenous substrate (59). NSrc was about 2.5 times more active than cSrc when bound to either antibody (Fig. 4, D and E).

Protein-tyrosine phosphatase inhibitors inhibit dephosphorylation of proteins phosphorylated by Src (and other tyrosine kinase) and also can activate SFKs by increasing phosphorylation at Tyr-418 (60–62). Serum-starved SYF1 cells reexpressing cSrc, NSrc, or Fyn were incubated in the absence or presence of orthovanadate for 3 h (Fig. 5). As shown in Fig. 5A, phosphorylation of total cell proteins on tyrosine increased in all cell types but most strongly in cells expressing NSrc. Correspondingly, phosphorylation on Tyr-418 was increased, modestly on cSrc and disproportionately on NSrc (Fig. 5B). This implies that phosphate turnover on the activation loop of NSrc is more rapid than on cSrc, consistent with increased activity of NSrc.

To examine whether raft localization further activates NSrc, we compared phosphorylation and kinase activity of NSrc prepared from the raft and soluble fractions of mouse brain (Fig. 6). NSrc in lipid rafts is more active than NSrc in soluble fraction of mouse brain lysates. A, Src was immunoprecipitated (IP) with La074 from either Triton X-100-soluble fraction (Sol) or lipid raft (Raft) fraction from postnatal day 1 mouse brain. Immunoprecipitated Src was eluted in SDS-PAGE sample buffer and separated on a 9% (20:1 acrylamide:bisacrylamide) gel and probed for Src or phosphotyrosine 418. The results were quantified and expressed as the ratio of phosphotyrosine 418 to total Src. The experiment was repeated twice with similar results. B, as in A, but immunoprecipitation was with anti-Src 327 antibody. Based on a preliminary quantification of Src in the samples (upper panels), equal amounts of Src from soluble or lipid raft fractions were used in an in vitro kinase reaction. Reactions were incubated for 5 (5') or 10 (10') min before electrophoresis and autoradiography. Incorporation of radiolabel into enolase was assayed by a scintillation counter. The experiment was repeated twice with similar results.
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6. When equal amounts of NSrc were compared, the phosphotyrosine 418 content is about 2-fold higher in NSrc from lipid rafts than in NSrc from the soluble fraction (Fig. 6A). NSrc immunoprecipitated from rafts was also more active in phosphorylating an exogenous substrate than NSrc immunoprecipitated from the soluble fraction (Fig. 6B). This implies that NSrc, already partially activated because of its altered SH3 domain, is further activated by localization to lipid rafts.

DISCUSSION

Numerous lines of evidence suggest that there is functional redundancy within the Src family of nonreceptor tyrosine kinases. The general protein interaction and catalytic domain structures of these molecules are very similar; hence, it may not be surprising that under extreme challenges, such as in knockout mice, one kinase largely fulfills the function of another. However, there is evidence that SFKs can be differentially localized, especially when activated (63), so not all SFKs have access to the same substrates. In this regard, the absence of a palmitoylation signal in Src means that it does not enter lipid rafts in fibroblasts (31) (Fig. 1A), whereas Fyn and most other SFKs are concentrated in lipid rafts. Hence it is curious that in brain Src can compensate for the absence of Fyn for phosphorylation of some substrates (36, 37) and that some investigators have detected Src in rafts (44–48).

Our results indicate that 5–20% of Src in brain is present in a lipid raft fraction made using nonionic detergent and floatation on sucrose. This is one of several operational definitions of a lipid raft fraction (16), and rafts made in this way contain >90% of Fyn and a significant amount of caveolin (Fig. 1, A and B). The limited amount of Src in rafts probably results from a low affinity interaction because additional Src could be recruited from the nonraft fraction into rafts by mixing in vitro (Fig. 3A). A low affinity is consistent with the results from fibroblasts showing that dually acylated (myristoylated, palmitoylated) but nonmyristoylated (myristoylated) SFKs enter rafts with high affinity (30, 31). Nevertheless, the monoacylation of Src is important for its recruitment to rafts in brain: we found that appending the NH2-terminal 7 residues of Src (MG-NSKSK) to a heterologous protein (GFP) permitted that protein to associate with lipid rafts (Fig. 3E). After cleavage of the initiating methionine and myristoylation, this implies that myristoyl-NSKSK is sufficient for low affinity raft targeting. On the other hand, the peptide MASNKS, which would not be myristoylated, was unable to target GFP to rafts.

It was found previously that caveolin-2 tyrosine phosphorylation by endogenous tyrosine kinases present in purified caveolin-rich domains from lung is inhibited by a synthetic myristoylated MGNKSK, which is not by a nonmyristoylated Src peptide or by either myristoylated or nonmyristoylated peptides from Yes (45). This may indicate that the specific myristoylated NH2-terminus of Src (but not Yes) displaces the main caveolin-2 kinase (possibly endogenous Src) from these caveolae preparations. It is thus possible that not only a myristoyl group but also a specific NH2-terminal sequence is important for raft association. It is conceivable that the basic residues present in the NH2-terminus of Src may contribute to raft localization, for example by interacting with acidic phospholipid head groups, but, in the case of K-Ras, farne-sylation and basic residues constitute a targeting signal for nonraft (disordered) membrane (64).

We found that both the majority of Src expressed in perinatal mouse brain is the NSrc splice form, and this distributes into nonraft and raft fractions. This is in accordance with previous studies done on O1A1 cells (41) and cultured rat neurons (42) showing that upon differentiation, neurons switch from expressing cSrc to expressing NSrc. However, the NSrc-specific insert in the SH3 domain did not account for the ability of a subpopulation of Src to enter lipid rafts in brain but not fibroblasts. Indeed, either cSrc or NSrc could be recruited into lipid rafts by mixing with brain extracts, and the isolated SH3 domains had no propensity to associate with rafts. These results imply that the presence of Src in lipid raft fractions from brain is caused by the lipid or protein composition of those rafts relative to those from fibroblasts. Indeed, a crude lipid fraction from brain could recruit added Src into a raft fraction. It is possible that the particular composition of brain lipid rafts may provide an environment with a low affinity for myristoylated Src.

One consequence of lipid raft localization is increased proximity to lipid raft residents like Cbp (53, 65, 66). This protein, when tyrosine phosphorylated by SFKs, recruits the protein kinase Csk, which in turn inactivates the SFK. The 80% or more of NSrc that is not (or only weakly) associated with lipid rafts may thus be more resistant to inhibition by Cbp and Csk. However, this does not explain our observation that activity is actually increased in NSrc immunoprecipitated from raft versus nonraft fractions of brain (Fig. 6). We found that raft-derived NSrc is >2-fold more phosphorylated in its activation loop than nonraft-derived NSrc, and correspondingly is >1.8-fold more active when assayed on an exogenous substrate (Fig. 6). Altered phosphorylation at the inhibitory Csk site (Tyr 529) was not detected. It is known that SFKs with relatively high tyrosine 529 phosphorylation can still be catalytically active (43, 67, 68). Possibly, localization to lipid rafts may activate NSrc by increasing access to kinases, or decreasing access to phosphatases, which act on the activation loop tyrosine (69, 70). This activation is on top of the 2–3-fold higher activity of mouse NSrc relative to cSrc, which was determined for molecules from the nonraft fraction of fibroblasts (Fig. 4) and is similar to previous findings with chicken NSrc and chicken cSrc (41, 42). Stimulation of Src family kinase activity by raft localization was described very recently for Lck and attributed to reduced phosphatase activity at the activation loop phosphorylation site (71).

The weakly interacting SH3 domain in NSrc (72) suggests a reduced subset of SH3-interacting substrates for NSrc compared with cSrc and Fyn, and it is also possible that NSrc is relatively indifferent to polyproline ligand-dependent activation. This suggests a restriction of input signals as well as substrates for NSrc. To understand the role of NSrc in neuronal development, it may be informative to genetically replace the src locus with cSrc cDNA in a hy–/–→yes–/–→ mouse.

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