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AFAP-120

A VARIANT FORM OF THE Src SH2/SH3-BINDING PARTNER AFAP-110 IS DETECTED IN BRAIN AND CONTAINS A NOVEL INTERNAL SEQUENCE WHICH BINDS TO A 67-kDa PROTEIN*

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SH2 and SH3 domains have been characterized as functional domains that mediate protein-protein interactions in signal transduction. Recently, the cDNA sequence of a novel Src- and Fyn-binding protein called AFAP-110, for Actin-Filament Associated Protein-110 kDa, was reported. This protein was distinctive in that it is both an SH2 and SH3 binding partner for the nonreceptor tyrosine kinases Src and Fyn. Here, we report the characterization of an alternatively processed form of AFAP-110 that encodes an additional 258 base pair (bp) of open reading frame. Transient expression of this full-length clone reveals a molecular mass of 120 kDa. Western blot analysis indicate that a larger 120-kDa variant of AFAP-110 can be detected in brain and is not detectable in any other tissues examined. Northern blot analysis indicate that the novel 258-bp insert can be detected in brain RNA but not chick embryo fibroblast RNA. We propose the name AFAP-120, for Actin Filament-Associated Protein-120 kDa. Expression of the 258-bp novel insert (NINS) as a glutathione S-transferase-encoded fusion protein permits adsorption of a 67-kDa protein from tissue lysates. Deletion analysis of the NINS indicates that the interaction with p67 can be attributed to a proline-rich motif that resembles an SH3-binding motif. We hypothesize that AFAP-120 facilitates interactions in brain between SH2/SH3 signaling proteins and actin filaments and that a proline-rich motif in the NINS may exist to facilitate additional interactions between cellular proteins in brain and actin filaments.

SH2 and SH3 domains are recognized as conserved functional domains found in a variety of proteins known to play an important role in signal transduction, including the *src* family of nonreceptor tyrosine kinases, phospholipase C-γ, and phosphatidylinositol 3-kinase (1). The SH2 domain mediates protein-protein interactions by recognizing and binding to peptide motifs that contain a phosphotyrosine residue (1). The function of the SH3 domain is less clear (2). This domain, unlike the SH2 domain, is also found in proteins known to interact with the cytoskeleton and cell membrane (3, 4). Hence, the SH3 domain has been hypothesized to mediate protein-protein interactions with these cellular structures (1). Recent studies indicate that the SH3 domain may also serve to link proteins to *ras* signaling pathways (5–8). Evidence for this association was originally pointed out in the cloning of the *abl* SH3-binding protein, 3BP-1(9). This polypeptide contains sequences distinct from its SH3-binding motif which are homologous to the family of GTPase activating proteins that act upon the *rac*/*rho* family of small G-proteins. Additionally, recent evidence indicates that *rac* and *rho* control membrane ruffling and stress fiber organization, respectively (10, 11). Thus, these data indicate a link between SH3-containing proteins, *ras* signaling pathways, and cytoskeletal associations.

Recently, we described the predicted amino acid sequence of a novel Src-binding protein, called AFAP-110 (12). This novel cytoskeletal-associated protein was distinctive in that it could be independently adsorbed with bacterial encoded fusion proteins expressing either the SH2 or SH3 domains of $pp60^{src}$ or $pp59^{fyn}$ (12). Furthermore, AFAP-110 encodes predicted SH2- and SH3-binding motifs (12). These data indicate that AFAP-110 is both an SH2- and SH3-binding partner for Src and that it is capable of forming a stable interaction with more than one member of the Src family of nonreceptor tyrosine kinases. It was hypothesized that one possible function of AFAP-110 was to facilitate interactions between SH2/SH3-containing proteins and the cytoskeleton (12). These stable complexes may be significant as the binding of AFAP-110 to activated forms of Src correlate strongly with transformation (13, 14). Activated variants of Src that contain deletions in the SH2 or SH3 domains are both defective for transformation and unable to form a stable complex with AFAP-110 (13, 14). These data indicate that one criterion for transformation would require intact SH2 and SH3 domains to facilitate interactions with SH2 and SH3 cellular binding partners. Because AFAP-110 is associated with actin structures (12, 13, 15), and the concomitant disruption of actin cables is a hallmark of transformation by Src (16, 17), then AFAP-110 could play an important role in modulating the effects of Src on the cytoskeleton. In addition, AFAP-110 may represent an important component in the pathway of signal transduction through c-Src and c-Fyn.

In this report, we describe the isolation and characterization of a variant form of the Src SH2/SH3-binding protein AFAP-110. This larger variant, called AFAP-120, has a molecular mass of 120 kDa and encodes a novel domain containing 86 amino acids placed near the carboxyl terminus which do not disrupt the downstream reading frame homologous to AFAP-110. Northern and Western blot analysis demonstrate that this larger variant can be detected in brain. AFAP-120 retains predicted SH2- and SH3-binding motifs and can be efficiently

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This work is dedicated to the late Dr. Eric H. Humphries, whose input and critiques were instrumental toward the development of this project.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L20302.

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adsorbed by GST[1]-SH3 created from c-Src. The novel 86-amino-acid insert encodes a unique sequence that contain a proline-rich region resembling an SH3-binding motif. Expression and immobilization of the NINS as a GST-encoded fusion protein (GST-NINS) reveals that a 67-kDa protein from brain lysates will associate with GST-NINS, but not to GST alone. This association requires the integrity of a proline-rich motif in the NINS. We hypothesize that AFAP-120 may be an important binding partner for c-Src in brain and that the NINS may facilitate additional interactions between cellular proteins and actin filaments.

MATERIALS AND METHODS

cDNA Construct Encoding AFAP-120—A λgt11 cDNA expression library (Clontech, Palo Alto, CA) created from the mRNA of a 10-day-old chick embryo was screened as described previously (12). A single cDNA clone, p2.1, was isolated by hybridization using an amino-terminal probe (12). Phage from this positive plaque was purified by four rounds of plaque purification. The cDNA insert was isolated and subcloned into pBluescript KS+ (Stratagene, San Diego, CA). DNA sequence analysis was carried out using the dideoxy sequencing strategy (24). Sequence analysis of clone p2.1 uncovered a sequencing error in the fibroblast homologue AFAP-110 (12). Two codons were omitted that predict an Asn-Asn after Ser^{358}, rather than a single Ile at position 359, and an additional Lys residue is predicted after Gly^{555} (12). These amino acids are conserved in clone p2.1 and have no effect on the SH2- or SH3-binding motifs predicted for AFAP-120 or AFAP-110. The sequence for AFAP-120 as well as the corrected sequence of AFAP-110 (accession number L20303) have been communicated to GenBank. A full-length version of AFAP-120 was constructed by substituting the *Bgl*II-*Bgl*II fragment of p2.1 into the cDNA encoding AFAP-110. This clone is referred to as FLC2.1. DNA and amino acid sequence were analyzed by using the sequence analysis software DNASIS and PROSIS (Hitachi software, Brisbane, CA).

The pCMV-1 vector was obtained from the laboratory of Dr. E. H. Humphries and was used to express cloned proteins in COS-1 cells via the CMV promoter (25). The cDNAs encoding AFAP-110 and the FLC2.1 were excised from pBluescript ks+ with *Hin*cII and *Sma*I digestion and subcloned into pCMV-1 via the unique *Sma*I restriction site in the multiple cloning site.

Cell Culture—Primary cultures of chick embryo fibroblast (CE) cells were prepared from day 9 embryos (Spafas, Norwich, CT) and cultured as described previously (26). COS-1 cells were also maintained in DMEM containing 5% fetal calf serum and 1% penicillin/streptomycin. Transfection of pCMV-1 plasmid constructs into COS-1 cells was carried out according to the method of Chen and Okayama (27).

Protein Characterization and Analysis—The polyvalent antibody (Ab) F1 (12) and monoclonal antibody (mAb) 4C3 (28) were prepared as described previously. Cell lysates were prepared using modified RIPA as described previously (26). Tissue lysates were obtained from either a day 16 chick embryo (chick brain) or a 7-week-old adult chicken. Immune complexes were isolated by adding either 1.2 μg of Ab F1 or 1 μl of mAb 4C3 ascites to 500 μg of RIPA cell lysate (1 μg/μl) containing protease inhibitors (50 μg/ml leupeptin, 1 mM sodium vanadate, 0.5% aprotinin, and 1 mM phenylmethylsulfonyl fluoride) for 2 h at 4 °C, followed by immobilization of the immune complex with 100 μl of protein A-sepharose (Pharmacia), 50% (w/v), for 2 h at 4 °C. The immobilized immune complexes were washed and resuspended in boiling Laemmli sample buffer. Western transfer and Western blot analysis with Ab F1 or mAb 4C3 were done as described previously (26). Bound primary antibody was quantitated using a horseradish peroxidase-conjugated secondary antibody followed by detection using chemiluminescence.

The novel 258-bp insert is surrounded by sequence that define *Sau*3AI sites. This *Sau*3AI fragment encodes Ser^{510} to Ser^{593} and does not include the three carboxyl-terminal amino acids of the NINS that predict Val^{594}, Ser^{595}, or Gln^{596}. This sequence was subcloned into the novel *Bam*HI site in pGEX-2T and transfected into DH5-α bacteria. This GST-encoded fusion protein (GST-NINS for *NINS*) was expressed as described previously (12). The preparation of bacterial lysates containing these fusion proteins, and immobilization on glutathione-Sepharose beads, were done according to the method of Smith and Johnson (29). Immobilized GST fusion proteins were incubated with 500 μg of chick embryo day 16 brain tissue lysate for 1.5 h at 4 °C. The bound proteins were washed two times with RIPA and two times with Tris-buffered saline, pH 7.5. Adsorbed brain tissue proteins were removed by incubating the beads in boiling 2 × Laemmli sample buffer for 2 min, centrifuging, and resolving the supernatant by 8% SDS-PAGE. The resolved brain lysate proteins were analyzed by ammoniacal silver staining according to the method of Harlow and Lane (32).

Northern Blot Analysis—Total cellular or tissue RNA was prepared by the method of Chomczynski and Sacchi (30). Twenty μg of RNA was mixed in a 20-μl volume of 20 mM MOPS (Sigma), pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 50% formamide, and 2.2 M formaldehyde, were resolved on a 1.5% agarose gel (SeaKem ME, FMC BioProducts), transferred to Immobilon-N membrane (Millipore Corp., Bedford, MA), and hybridized with a nick-translated DNA probe using [^{32}P]dCTP (ICN Biochemicals, Irvine, CA).

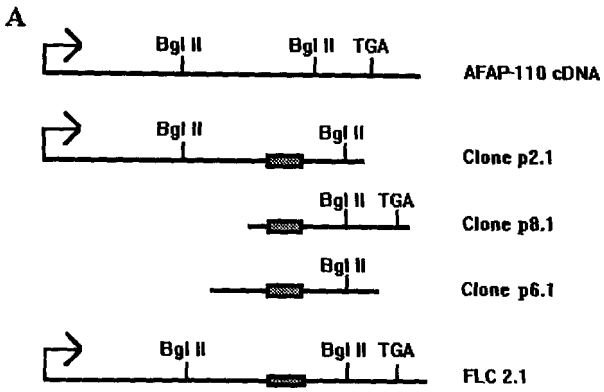
RESULTS AND DISCUSSION

Isolation of a cDNA Clone Encoding a 258-bp Insert in AFAP-110—AFAP-110 cDNA clones were isolated from λgt10 and gt11 cDNA libraries generated from the mRNA of day 10 whole chick embryos and from chick embryo fibroblast (CE) cells, as described previously (12). One of the 5′ clones isolated from the gt11 whole chick embryo library (p2.1) encoded a 2068-bp cDNA clone that contained a 258-bp novel insert (NINS) located between AFAP-110 coding sequence encoding bp 1549 and 1550 (12). A 247-bp DNA fragment corresponding to the NINS was isolated by *Sau*3AI digestion and used to probe the same gt11 library. Two additional cDNA clones, p8.1 and p6.1, were isolated that correspond to the 3′ coding and noncoding sequence common to AFAP-110 (Fig. 1*A*). Clone p8.1 begins at bp 1444 that is predicted for AFAP-110 and encodes sequence homologous to clone p2.1, including the NINS. The clone p8.1 terminates at bp 2252, which is 59 bp downstream of the 3′ termination codon predicted for AFAP-110. Clone p6.1 begins further upstream at bp 1327, predicted for AFAP-110, and encodes the NINS. Clone p6.1 terminates at bp 2129 within the coding region common to AFAP-110 and clone p2.1. The fact that the NINS was detected in three different cDNA clones and exists between sequence that define AFAP-110 indicate that the NINS is an authentic biological entity.

The 258-bp insert encodes 86 amino acids and retains the integrity of the serine residue encoded at amino acid position 510 (Fig. 1*B*). In addition, the 258-bp insert does not disrupt the downstream reading frame predicted from the AFAP-110 encoded phenylalanine 511 (Fig. 1*B*). Interestingly, a second variant cDNA has been isolated that diverges from AFAP-110 cDNA sequence at exactly the same bp (bp 1549); however, this variant encodes 19 novel amino acids after serine 510, followed by an opal stop codon and 3′-noncoding sequence.[2] Because two different variants of AFAP-110 have been isolated that diverge at exactly the same site, we speculate that AFAP-120 may have arisen by alternative splicing. The predicted amino acid sequence of the 258-bp insert revealed a novel sequence that was not homologous with any proteins in the GenBank data base. The 86-amino-acid sequence contains a disproportionate number of serine residues (15 serine residues, or 17% of the insert) and a short proline-rich motif (*boxed*). To characterize the NINS, a full-length version of this variant was created by substituting the *Bgl*II to *Bgl*II fragment of p2.1 (bp 1162–2056) into the same *Bgl*II to *Bgl*II restriction site of full-length AFAP-110 (Fig. 1*A*). This clone is referred to as full-length clone p2.1 (FLC2.1) and encodes 723 amino acids with a predicted molecular mass of 81,458 Da.

[1] The abbreviations used are: GST, glutathione *S*-transferase; CMV, cytomegalovirus; CE, chick embryo; mAb, monoclonal antibody; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; MOPS, morpholinepanesulfonic acid.

[2] D. C. Flynn, unpublished data.



B

1453	TCC	ACA	AAT	CCA	TAC	CGG	GGA	AGC	ACT	GCC	AAT	GGC	TAT	GCA	TGT
479	S	T	N	P	Y	R	G	S	T	A	N	G	Y	A	C
1498	CCG	AGT	GGA	ATG	GCA	CTT	CAT	TAC	GAT	GAT	GTT	CCC	TGC	ATA	AAT
494	P	S	G	M	A	L	H	Y	D	D	V	P	C	I	N
1543	GGA	TCG	TGG	GAA	CCA	GAA	GAT	GGC	TTT	CCT	TCT	TCT	CGT	AGC	AGG
509	G	S	W	E	P	E	D	G	F	P	S	S	R	S	R
1588	AAC	ATG	GGA	GAA	GAA	ATG	CTT	TAT	GAT	AAC	GCA	GGC	CTG	TAC	GAT
524	N	M	G	E	E	M	L	Y	D	N	A	G	L	Y	D
1633	AAC	TTG	CCG	TCT	CCA	AAA	ATC	TTT	GCG	CGC	TAT	CCG	CCA	GCT	GAC
539	N	L	P	S	P	K	I	F	A	R	Y	P	P	A	D
1678	AGG	AAA	ACC	AAT	AGG	TTA	TCT	ACT	GAC	AAA	CTC	TCC	TCT	AAC	CAT
554	R	K	T	N	R	L	S	T	D	K	L	S	S	N	H
1723	TAT	AAA	CAC	CCT	GTC	TCA	TCC	AAT	CTG	TCT	TCT	GCT	CAG	TCT	GTC
569	Y	K	H	P	V	S	S	N	L	S	S	A	Q	S	V
1768	ACT	AAT	ACC	TCT	GCT	GTG	GGG	AGG	GGA	TCT	GTC	TCA	CAG	TTC	AAA
584	T	N	T	S	A	V	G	R	G	S	V	S	Q	F	K
1813	GGC	AAG	AAA	CCT	CCT	GCA	ACT	GCT	AAT	GGG	ATC	ACT	GGA	AAA	GTG
599	G	K	K	P	P	A	T	A	N	G	I	T	G	K	V
1858	AGA	ACA	TTA	AAT	AGC	CAA	CCG	AAA	AAA	CCT	GAA	TCA	GTG	TCA	TGT
614	R	T	L	N	S	Q	P	K	K	P	E	S	V	S	C

FIG. 1. **Analysis of clone p2.1 and construction of a full-length version of clone p2.1.** *A*, clone p2.1 was sequenced on both strands. The cDNA sequence of the NINS was isolated by *Sau*3AI digestion and used to isolate two additional clones, p8.1 and p6.1, which were sequenced completely on one strand. Clones p8.1 and p6.1 encode the NINS, as well as sequence identical to AFAP-110 and clone p2.1. A full-length version of clone p2.1 (FLC2.1) was created by subcloning the *Bgl*II to *Bgl*II fragment of clone p2.1, which encodes the novel 258-bp insert, into the cDNA encoding AFAP-110. This *Bgl*II to *Bgl*II fragment encodes Leu^{383} to Asp^{680}, predicted for clone p2.1. *B*, the predicted amino acid sequence of AFAP-120 from bp 1453 (common to AFAP-110 and AFAP-120) to bp 1902 (bp 1644 for AFAP-110). The cDNA sequence of the novel 258-bp insert is *underlined*, along with the predicted amino acid sequence. Sequence information that is not underlined represents sequence common to both AFAP-110 and clone p2.1. A proline-rich region is *highlighted* in the *box* defining Pro^{541} to Pro^{551}.

Chick Brain Expresses a 120-kDa Variant of AFAP-110—The 86 amino acids have a predicted molecular mass of 9534 Da. Therefore, it was hypothesized that the protein encoded by FLC2.1 would have a molecular mass of 120 kDa. Because clone p2.1 was isolated from a λgt11 library created from the RNAs of a whole chick embryo, it was further hypothesized that a variant form of AFAP-110 of 120 kDa might be detected in a specific organ by Western blot analysis. Tissues were extracted from a 7-week-old chicken, lysed in RIPA, 25 μg of cellular proteins resolved by 8% SDS-PAGE, and transferred to polyvinylidene fluoride by Western transfer. The transfer was then probed with mAb 4C3 (28). The data reveal that all tissues contain a 110-kDa protein that is AFAP-110 (Fig. 2, *lanes 1–11*). Liver and kidney (*lanes 7* and *8*) contain very low levels of AFAP-110 that can be detected by extended exposure to film.[2] However, in tissue lysates derived from brain, a 120-kDa isoform was also identified (Fig. 2, *lane 2*). These data indicate that a larger 120-kDa variant of AFAP-110 is represented in brain. Furthermore, the steady-state levels of expression appear to be significantly higher in brain than the other non-neural tissues. Treatment of the 120-kDa protein from brain with potato acid phosphatase indicated that the increased molecular mass could not be entirely accounted for by increased phosphorylations.[2] This larger variant form of the AFAP-110 protein detected in brain lysates is referred to as AFAP-120, for Actin Filament Associated Protein-120 kDa.

Expression of FLC2.1 in COS-1 Cells—The effect of the novel 258-bp insert was examined with respect to changes in the molecular mass. The cDNAs encoding AFAP-110, or FLC2.1, were subcloned from pBluescript KS^+ into the expression vector pCMV-1 for transient expression in COS-1 cells. These new constructs, pCMV-FLC2.1 and pCMV-AFAP-110, as well as pCMV-1, were transfected into COS-1 cells and the cells lysed with RIPA lysis buffer 60 h post-transfection. The transiently expressed proteins were immunoprecipitated with mAb 4C3, which is avian specific (28) and would preferentially recognize these avian proteins rather than the COS-1 (African green monkey) cellular form of AFAP-110 (Fig. 3, *lane 5*). For comparison, AFAP-120 and AFAP-110 were immunoprecipitated with mAb 4C3 from day 16 chick embryo brain and CE, (Fig. 3, *lanes 1* and *2*, respectively). The data demonstrate that the protein encoded by FLC2.1 has a molecular mass of 120 kDa

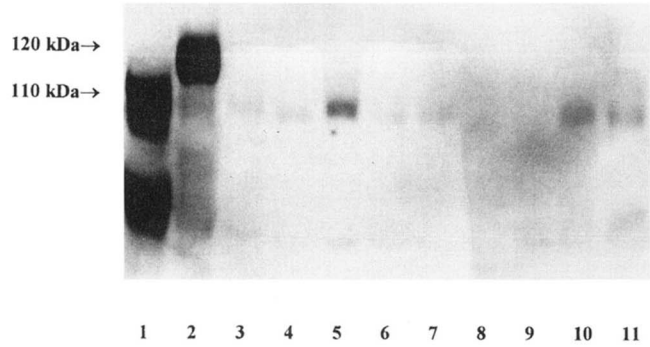


FIG. 2. **Detection of a 120 kDa form of AFAP-110 in chick brain.** Twenty-five μg of tissue lysates were diluted with RIPA to 1 μg/μl and resolved by 8% SDS-PAGE. The cellular proteins were transferred to nitrocellulose by Western transfer and probed for the presence of AFAP-110 using mAb 4C3. Tissues include CE cells (*lane 1*), brain (*lane 2*), eye (*lane 3*), heart (*lane 4*), lung (*lane 5*), muscle (*lane 6*), kidney (*lane 7*), liver (*lane 8*), intestine (*lane 9*), bladder (*lane 10*), spleen (*lane 11*). The data are representative of three independent experiments.

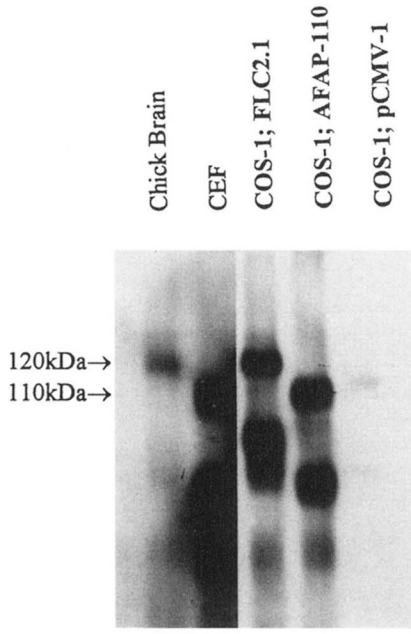


FIG. 3. **Expression of FLC2.1 and AFAP-110 in COS-1 cells.** Twenty-five μg of day 16 chick embryo brain lysate (*lane 1*), CE lysate (*lane 2*) and COS-1/pFLC2.1 (*lane 3*), COS-1/pAFAP-110 (*lane 4*), or COS-1/pCMV-1 (*lane 5*) transfected COS-1 cell lysates were resolved by 8% SDS-PAGE, Western transferred, and probed for the presence of AFAP-110 or AFAP-120 with mAb 4C3. Reactive proteins were quantitated with sheep anti-mouse antibodies conjugated to horseradish peroxidase and detected by chemiluminescence. *Lanes 1* and *2* represent a 2-min exposure, while *lanes 3–5* represent a 10-s exposure. The data are representative of three independent experiments.

(Fig. 3, *lane 3*) and is equivalent in size to AFAP-120 immunoprecipitated from chick brain (Fig. 3, *lane 1*). In addition, the transiently expressed AFAP-110 (Fig. 3, *lane 4*) has a molecular mass equivalent to the 110-kDa AFAP-110 immunoprecipitated from CE cells (Fig. 3, *lane 2*). These data indicate that the 86-amino-acid insert encoded in FLC2.1 is sufficient to give rise to the 120-kDa brain form of AFAP-110, referred to as AFAP-120.

Interestingly, an immunoreactive polypeptide of 92 kDa is detected in pCMV-FLC2.1-transfected COS-1 cells and an 82-kDa immunoreactive polypeptide is detected in pCMV-AFAP-110-transfected COS-1 cells, (Fig. 3, *lanes 3* and *4*, respectively). It is likely that these lower molecular weight forms are the

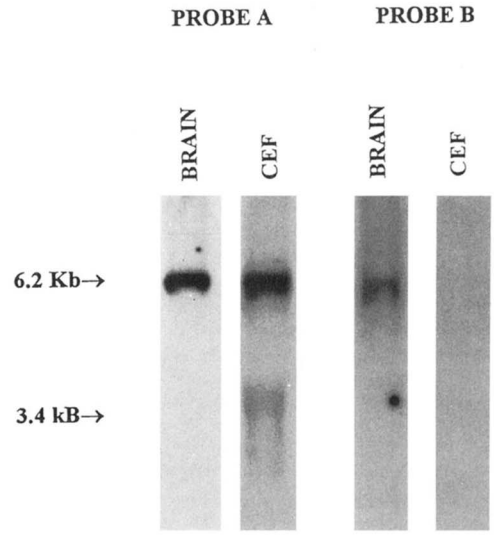


FIG. 4. **Northern blot analysis.** Twenty μg of total RNA was isolated from chick brain and CE, resolved on a 1.5% agarose gel, transferred to Immobilon, and probed with *probe A* for the presence of sequence common to AFAP-110 and AFAP-120 (*lanes 1* and *2*) or with *probe B* to detect the novel 258-bp insert (*lanes 3* and *4*).

products of proteolytic degradation. Similarly sized polypeptides are detected in cell and tissue lysates (see Fig. 2) and have been described earlier and postulated to be proteolytic degradation products (13).

Northern Blot Analysis Demonstrates That the 258-bp Insert Is Represented in Brain—Total RNA was isolated from the brain of a day 2 chick hatchling, as well as from CE cells. Twenty μg of total RNA was resolved on a formaldehyde gel, transferred to Immobilon, and probed by Northern hybridization using a probe representing the 5′ 452 base pairs common to both AFAP-110 and clone p2.1 (*probe A*), or a probe specific to the NINS representing the 247 base pair within the NINS that are surrounded by *Sau*3A1 restriction sites (*probe B*). The data demonstrate that probe A, common to both AFAP-110 and FLC2.1, detects an RNA of 6.2 kilobases in both brain and CE (Fig. 4, *lanes 1* and *2*), as well as a smaller RNA of 3.4 kilobases in CE cells (Fig. 4, *lane 2*). However, probe B only recognized a 6.2-kilobases RNA in brain (Fig. 4, *lane 3*) and did not hybridize to any RNA isolated from CE cells (Fig. 4, *lane 4*). These data indicate that the NINS is represented in brain but not in CE cells. Furthermore, the RNA detected by probes A and B are the same size in brain, suggesting that they likely hybridize to the same RNA. Taken together, these data indicate that (*a*) a larger 120-kDa variant of AFAP-110 can be detected in brain, (*b*) the novel 258-bp sequence is sufficient to increase the molecular mass of AFAP-110 to 120 kDa, and (*c*) the NINS can be detected in brain RNA, but not in CE cellular RNA. Thus, the FLC2.1 construct does appear to be representative of AFAP-120.

The NINS May Facilitate Binding to Cellular Proteins—The structure of the 86 amino acids encoded by the NINS predict a very hydrophilic domain,[2] based on the Kyte and Doolittle hydropathy parameter set (31). In addition, the NINS contains a disproportionate number of serine residues (15/86 residues or 17%). Although no sites for phosphorylation have been mapped, 7 of these serine residues are adjacent to Arg or Lys residues, which could predict a site for protein kinase C phosphorylation (33). In addition, the NINS encoded within FLC2.1 does not disrupt the predicted SH2- or SH3-binding motifs (12). The NINS exists in the carboxyl terminus, while the predicted SH2-binding motifs are left intact, and the predicted SH3-binding motif exists further upstream, in the amino terminus

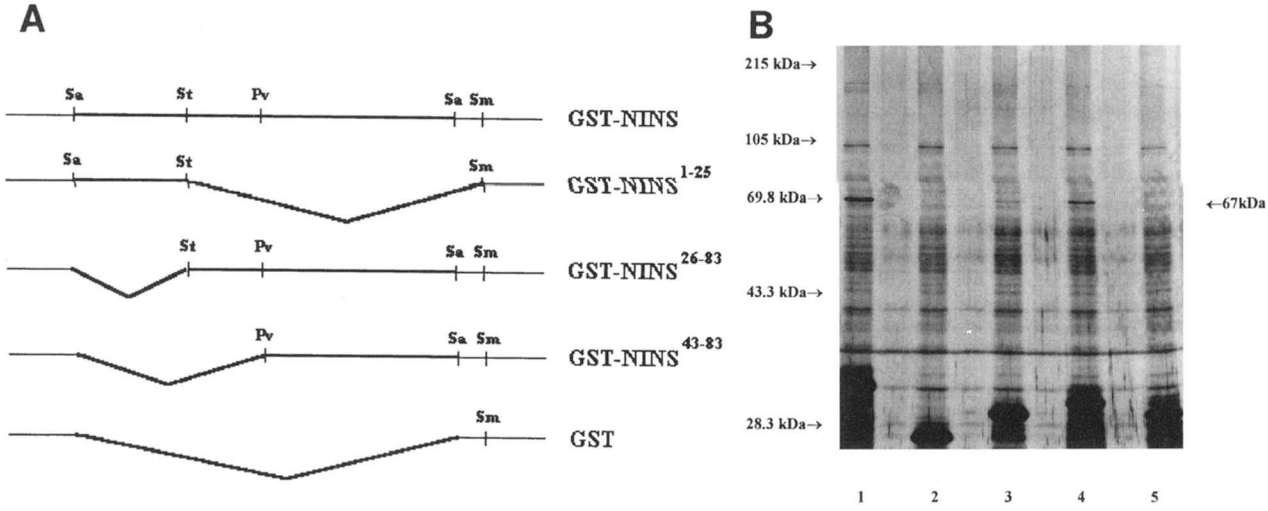


FIG. 5. **Description of a potential cellular binding partner for the NINS.** *A*, GST-NINS encodes Ser^{510} to Ser^{593}. Three deletion variants of GST-NINS were constructed. GST-$NINS^{1-25}$ encodes the first 25 amino acids of the NINS (Ser^{510} to Gly^{535}). GST-$NINS^{26-83}$ encodes Leu^{536} to Ser^{593}. GST-$NINS^{43-83}$ encodes Asp^{553} to Ser^{593}. *B*, 250 μg of chick brain lysate (from a day 9 embryo) were preincubated with GST immobilized on Sepharose beads, precleared, and then incubated with GST-NINS (*lane 1*), GST (*lane 2*), GST-$NINS^{1-25}$ (*lane 3*) GST-$NINS^{26-83}$ (*lane 4*), or GST-$NINS^{43-83}$ (*lane 5*). Adsorbed brain lysate proteins were eluted with boiling Laemmli sample buffer, resolved by 8% SDS-PAGE, and analyzed by silver stain. The abundant protein bands between 26 and 36 kDa represent the GST-encoded fusion proteins. These data are representative of three independent experiments.

between amino acids 61–85 (12). For this reason, it was hypothesized that the SH3 binding properties of AFAP-120 would be unaffected. These data were confirmed by the adsorption of AFAP-120 and AFAP-110 with the c-Src SH3 domain, while neither protein was capable of interacting with c-Src+ SH3 domain.[2] These data indicate that AFAP-120 could represent an important binding partner for c-Src within the brain. AFAP-120 may contain sufficient information to be an important binding partner for other cellular proteins, as well.

Interestingly, it was noted that a proline-rich region which resembles an SH3-binding motif is encoded in the NINS between amino acids 541 and 551 (23). This proline-rich motif exists in a region of the NINS that has a high hydrophilicity prediction.[2] It has already been established that AFAP-110 has two potential SH3-binding motifs in the amino terminus and that AFAP-110 is an SH3-binding partner for Src and Fyn (12). Because it has been hypothesized that the function of AFAP-110 is to facilitate interactions between actin filaments and SH2- and/or SH3-signaling proteins, it was predicted that the NINS might facilitate interactions with additional cellular proteins. To test this hypothesis, the NINS was subcloned into pGEX-2T by isolating a 247-bp fragment that defines the 5′ and 3′ borders of the insert and subcloning it into the novel *Bam*H I site of pGEX-2T. GST-NINS and GST were used to adsorb potential binding proteins from day 16 chick embryo brain lysates, and the molecular mass of these proteins evaluated by silver stain. To evaluate specificity in binding, three deletions were constructed in NINS (Fig. 5*A*). A *Stu*I-*Sma*I deletion eliminates amino acids 536–593 predicted within the NINS. This binding protein is referred to as GST-$NINS^{1-25}$ and encodes the first 25 amino acids of the NINS (Ser^{510} to Leu^{536}). In addition, the sequences encoding Leu^{536} to Ser^{593}, or Asp^{553} to Ser^{593}, were subcloned into pGEX-2T and pGEX-1, respectively. These GST fusion proteins, GST-$NINS^{26-83}$ and GST-$NINS^{43-83}$, encode the carboxyl-terminal 55 or 41 amino acids of the NINS, respectively. These data reveal that a 67-kDa protein is adsorbed by GST-NINS (Fig. 5*B*, *lane 1*) and GST-$NINS^{26-83}$ (Fig. 5*B*, *lane 4*), but not with GST or GST-$NINS^{43-83}$ (Fig. 5*B*, *lanes 2* and *5*, respectively). These data indicate that the amino acid sequence between Tyr^{537} and Asp^{553}, which encodes the proline-rich motif, is critical for binding p67. There appears to be a small amount of a 67-kDa protein absorbed by GST-$NINS^{1-25}$ (Fig. 5*B*, *lane 4*), indicating that sequence interactions defined between Ser^{510} and Leu^{536} may also contribute to binding.

GST-NINS and GST-$NINS^{43-83}$ were used to probe other avian tissue lysates to determine whether there might be specificity in the interaction between p67 and the NINS.[2] The data indicate that an equivalent amount of p67 can be adsorbed from brain, lung, muscle, liver, and kidney. Theses data support a hypothesis that p67 may not be a specific binding partner for the NINS. However, the data would indicate that the proline-rich region contained within the NINS may be responsible for facilitating protein-protein interactions in the brain. It is likely that p67 contains a structure that is favorable for interacting with the NINS proline-rich motif. Taken together, these data indicate that the function of the NINS might be to facilitate protein-protein interactions in brain via an internal proline-rich motif.

Ren *et al.* (23) predicted that a consensus SH3-binding motif would be approximately 10 amino acids long and contain proline residues at peptide positions 2, 7, and 10 that would be critical for binding. In addition, a proline residue at position 9 is frequently identified as a component of an SH3-binding motif, and an alanine residue at position 1 appears to be important for binding. Lastly, a hydrophobic amino acid at peptide position 8 may contribute to stability in SH3 binding. The NINS contains a region of 11 amino acids that are almost identical to the consensus SH3-binding motif predicted by Ren *et al.* (23). This sequence contains proline residues at peptide positions −1, 2, 9, and 10, as well as a hydrophobic tyrosine residue at position 8 and serine at position 1 (a conserved change). However, this stretch of amino acids contains an arginine residue rather than a proline at peptide position 7. A proline residue at peptide position 7 was identified as being very important for stable SH3 binding (23). This variation could be required for some specific interaction with another cellular protein. Because the proline-rich region in the NINS resembles an SH3-binding motif, it is possible this structure could facilitate additional protein-protein interactions. It is also possible that the function of the NINS is not related to facilitating protein-protein binding. However, given that the

hypothesized function of AFAP-110 is as an adaptor molecule that facilitates interactions between SH2- and/or SH3-containing proteins with the cytoskeleton (12), it would not be unrealilistic to hypothesize that the NINS may contribute a similar function to AFAP-120, thus enhancing its diverse interactions between cellular proteins and the cytoskeleton.

The function of c-Src has not been discerned; however, it is likely that this proto-oncogene has a specialized function within the brain. This hypothesis is based on several observations which indicate that (*a*) the c-Src tyrosine kinase is activated in brain (34–37, 43); (*b*) the c-Src proto-oncogene product is developmentally regulated in neurons, being expressed initially at the onset of neuronal differentiation and then maintained at high levels in fully differentiated neurons in the adult central nervous system (21, 22, 37–42); (*c*) this proto-oncogene product is alternatively spliced only in neurons (20, 44); and (*d*) upon activation, is capable of inducing neuronal differentiation in cultured neurons, indicating a role in the signal transduction processes that modulate neuronal differentiation (19). Thus, signaling by c-Src in the brain may be specific and significant.

SH2/SH3 interactions between AFAP-110 or AFAP-120 and c-Src may represent an important link in signal transduction by Src. It is possible that $pp60^{c\text{-}src}$ may have a unique function in affecting the integrity of the cytoskeleton (15–17, 45–48). Localization studies in neurons indicate that $pp60^{c\text{-}src}$ is enriched in growth cones, a structure that is also rich in actin filaments (49, 50). Further, $pp60^{c\text{-}src}$ isolated from these growth cones have a high tyrosine-specific kinase activity (49). Thus, $pp60^{c\text{-}src}$ may have a specialized function in growth cone extension, which would not be inconsistent with previous observations that demonstrate a role for activated forms of Src in modulating cell-cell contacts or cell-substratum contacts.

One potential function for AFAP-120 would be to facilitate interactions between SH2/SH3-containing proteins and the cytoskeleton. AFAP-120 does retain an association with actin structures, as detected by immunofluorescence with Ab F1 in COS-1 cells expressing FLC2.1.[2] The function of AFAP-120 in signal transduction is not clear; however, given that nonreceptor tyrosine kinases like $pp60^{c\text{-}src}$ and $pp59^{c\text{-}fyn}$ are expressed at relatively high levels in brain (18), it would appear likely that AFAP-120 would be positioned to interact with, and possibly mediate a signal transduction event through, these tyrosine kinases. Lastly, the NINS may play a unique role in facilitating a similar interaction with other cellular proteins. These stable complexes may play a functionally important role in modulating the integrity of actin filaments in response to specific cell signaling events in the brain.

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