Identification of a Candidate Human Spectrin Src Homology 3 Domain-binding Protein Suggests a General Mechanism of Association of Tyrosine Kinases with the Spectrin-based Membrane Skeleton

(Received for publication, October 23, 1997, and in revised form, January 30, 1998)

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Spectrin is a widely expressed protein with specific isoforms found in erythroid and nonerythroid cells. Spectrin contains an Src homology 3 (SH3) domain of unknown function. A cDNA encoding a candidate spectrin SH3 domain-binding protein was identified by interaction screening of a human brain expression library using the human erythroid spectrin (a1) SH3 domain as a bait. Five isoforms of the a1 SH3 domain-binding protein mRNA were identified in human brain. Mapping of SH3 binding regions revealed the presence of two a1 SH3 domain binding regions and one Abl-SH3 domain binding region. The gene encoding the candidate spectrin SH3 domain-binding protein has been located to human chromosome 10p11.2 → p12. The gene belongs to a recently identified family of tyrosine kinase-binding proteins, and one of its isoforms is identical to e3B1, an eps8-binding protein (Biesova, Z., Piccoli, C., and Wong, W. T. (1997) Oncogene 14, 233–241). Overexpression of the green fluorescent protein fusion of the SH3 domain-binding protein in NIH3T3 cells resulted in cytoplasmic punctate fluorescence characteristic of the reticulovesicular system. This fluorescence pattern was similar to that obtained with the anti-human erythroid spectrin αIΣ/βIΣ antibody in untransfected NIH3T3 cells; in addition, the anti-αIΣ/βIΣ antibody also stained Golgi apparatus. Immunofluorescence obtained using antibodies against αIΣ/βIΣ spectrin and Abl tyrosine kinase but not against αII/βII spectrin colocalized with the overexpressed green fluorescent protein-SH3-binding protein. Based on the conservation of the spectrin SH3 binding site within members of this protein family and published interactions, a general mechanism of interactions of tyrosine kinases with the spectrin-based membrane skeleton is proposed.

Erythroid spectrin is the predominant component of the two-dimensional protein network called the membrane skeleton, underlying the lipid bilayer of red cells (for recent reviews, see Refs. 1–3). Formation of the membrane skeleton involves multiple protein-protein interactions among integral membrane proteins. Interactions of spectrin with other membrane proteins such as ankyrin, protein 4.1, and adducin provide a linkage of spectrin either to the plasma membrane or among spectrin tetramers. Many hereditary anemia mutations affect interactions of these integral membrane proteins, resulting in increased fragility and shortened lifespan of erythrocytes. In hereditary elliptocytosis and pyropoikilocytosis, the mutations have been localized in the a- and β-subunits of spectrin (reviewed in Refs. 4 and 5). Many of these proteins, including spectrin, which were first identified in red cells, have isoforms expressed in nonerythroid cells, but the structure and regulatory processes of the nonerythroid membrane skeleton are less well understood (reviewed in Refs. 1–3, 6, and 7). Functional differences between the membranes of erythroid and nonerythroid cells argue against the simple erythrocyte model of the membrane skeleton. Major differences between the erythroid model and other cells include differences in the expression of spectrin (8–11) and ankyrin isoforms (12–15) (reviewed in Ref. 16), interactions of spectrin and ankyrin with additional proteins (17–21), localization of spectrin in the cytoplasm as well as in the plasma membrane (10, 11, 22), and the potential for dramatic rearrangements of spectrin’s cellular location (23, 24) (reviewed in Refs. 2 and 7).

Several studies have demonstrated that both erythroid and nonerythroid spectrins are expressed in brain tissue (8–11, 25). Neuronal compartmentalization of brain spectrin isoforms into axons and presynaptic terminals (nonerythroid spectrin) and into cell bodies and dendrites (erythroid spectrin) (10, 25) suggests that brain spectrin isoforms may perform related but distinct functions in neuronal cells. It has been suggested that nonerythroid spectrin performs a more general, constitutive role, while erythroid spectrin takes part in more specialized activities of differentiated cells (26). The a-subunit of erythroid spectrin, αI (27), and the a-subunit of nonerythroid spectrin, αII (28, 29), each contains a unique SH3 domain. Distinct protein interactions are likely to involve these domains, and they may be important for specific distribution and specialized roles of brain spectrin isoforms.

* This work was supported by National Institutes of Health NINDS Grant R29 NS32874 (to L. K.) and the New York State Office of Mental Retardation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U87166.

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1 Nomenclature for spectrin isoforms used in this paper is according to Winkelmann and Forget (6).

2 The abbreviations used are: SH3, Src homology 3; GST, glutathione S-transferase; FISH, fluorescence in situ hybridization; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PCR, polymerase chain reaction; DIG, digoxigenin; PAC, P1 artificial chromosome; mAb, monoclonal antibody; BS, binding site; kb, kilobase pair(s); GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; TBS, Tris-buffered saline with Tween 20.
The SH3 domain was originally identified in the regulatory region of Src and Src-like tyrosine kinases (Abl, Fps) and then identified in other proteins, including Raf, phospholipase C, Ras GTPase-activating protein, and phosphatidylinositol 3'-kinase, all involved in transmitting signals within cells (reviewed in Refs. 30–32). The SH3 domains are involved in protein interactions thought to control signaling pathways. In Src and Abl, oncogenic mutations have been identified in the SH3 domain, indicating that this region might have a negative regulatory effect on transformation (33). The SH3 domains of several tyrosine kinases were found to bind to short proline-rich sequences containing a PXXP motif (34, 35) and a general model for the SH3-ligand complex has been proposed based on NMR studies (36, 37). The list of SH3-containing and SH3-binding proteins is rapidly growing (reviewed in Ref. 32). Diversity in SH3 domains and in their ligand binding sites indicates that their binding specificities are variable and thus mediate different protein-protein interactions. In addition to αI and αII spectrin, several unrelated cytoskeletal or membrane proteins have been shown to contain the SH3 domain, including a major pulmotoxylated erythrocyte membrane protein p55 (38), several isoforms of myosin Ib, a yeast actin-binding protein implicated in the regulation of cytoskeletal assembly (39), and yeast protein BEM1, which is involved in cell polarization (40).

Using interaction cloning, we identified a cDNA encoding a candidate human αI spectrin SH3 domain-binding protein. Five isoforms of the candidate mRNA were identified in human brain. Using the recombinant polypeptides, we located the αI SH3 domain and Abl-SH3 domain binding regions. Immunofluorescence studies suggest association of the αI spectrin SH3 domain-binding protein and Abl tyrosine kinase with an erythroid-like spectrin in transfected NIH3T3 cells. The candidate αI SH3-binding protein belongs to a recently identified family of tyrosine kinase-binding proteins (41–44), and one of its isoforms is identical to εβ1, an eps8-binding protein (44). Based on conservation of the spectrin SH3 binding site within members of this protein family and published data (41–44), a general mechanism of interactions of tyrosine kinases with the spectrin-based membrane skeleton is proposed.

Experimental Procedures

Expression of the αI and αII SH3 Domains in Yeast, and Expression Library Screening—The sequence encoding the αI spectrin SH3 domain (nucleotides 3115–3291 of the αSpI cDNA; Ref. 37) and the αII spectrin SH3 domain (nucleotides 2995–3177 of the αSpII cDNA; Ref. 29) were amplified using specific primers (see Table I) and subcloned into the plasmid pGAD4-1 (isolated from the human brain expression library) and subcloning of the αI spectrin SH3 domain-binding protein and Abl tyrosine kinase with an erythroid-like spectrin in transfected NIH3T3 cells. The candidate αI SH3-binding protein belongs to a recently identified family of tyrosine kinase-binding proteins (41–44), and one of its isoforms is identical to εβ1, an eps8-binding protein (44). Based on conservation of the spectrin SH3 binding site within members of this protein family and published data (41–44), a general mechanism of interactions of tyrosine kinases with the spectrin-based membrane skeleton is proposed.

Rapid Amplification of cDNA Ends and PCR Cloning of hssh3bp1/e3B1 Isoforms—Marathon-Ready human brain cDNA and Klentak DNA polymerase mix (CLONTECH) were used to clone the 5’ (primer A3; 5’-TATGATTGGTGGAGGAACATCTG-3’) and 3’ (primer T25; Table I) ends of the hssh3bp1/e3B1 mRNA and to clone its isoforms. Although no additional sequence was obtained at the 5’ end, the 3’-untranslated region was extended to 1044 base pairs; it contained a poly(A) addition signal, NAAATAA, located 17 base pairs from the 3’ poly(A) tail. At the 5’ end of the hssh3bp1/e3B1 cDNA, the first ATG codon was located 81 base pairs downstream from the 5’ EcoRI restriction site. No additional ATG codons were found following the TATA box identified in the genomic DNA fragment that hybridized to the 5’ end sequence (data not shown); therefore, it is likely that the first ATG identified in the original cDNA clone represents the translation initiation site of the candidate mRNA. The amplified DNA fragments were cloned into M13mp18 or 19 or into pGEX2T vector (Amersham Pharmacia Biotech) and sequenced (46). Each hssh3bp1/e3B1 isoform was sequenced from two independent clones. The variable alanine residue (see Fig. 4A) was present in five of eight subclones. Expression of Glutathione S-Transferase (GST) Fusion Proteins: αI and αII SH3 Domain—The desired regions of αI and αII spectrin were obtained by amplification using specific primers and subsequently cloned into the pGEX-2T vector using BamHI and EcoRI restriction sites incorporated into amplification products by primers. All primer sequences used for expression plasmids are listed in Table I. The amplified αI spectrin αSpI encodes residues 977–1062 of the αI spectrin that includes the SH3 domain (residues 3115–3370 in the αI cDNA; Ref. 27). In this case, the termination codon was present in the vector sequences, and this resulted in addition of residues EFVTVT to the C terminus of the spectrin sequence. The construct GST-F-SH3 encodes residues 965–1025 of αI spectrin (nucleotides 2995–3177 of αSpII cDNA sequence; Ref. 29). The nucleotide sequences of all plasmid constructs were confirmed by DNA sequencing. pGEX-2T plasmids expressing SH3 domain isoforms of αI, Crk, Src and n-Src (34, 35) were obtained from Dr. Bruce J. Mayer (Howard Hughes Medical Institute, Children’s Hospital, Boston, MA).

Clone 4-1—The 1.63-kb EcoRI fragment was obtained from the clone pGAD4-1 (isolated from the human brain expression library) and subcloning of the αI spectrin SH3 domain-binding protein and Abl tyrosine kinase with an erythroid-like spectrin in transfected NIH3T3 cells. The candidate αI SH3-binding protein belongs to a recently identified family of tyrosine kinase-binding proteins (41–44), and one of its isoforms is identical to εβ1, an eps8-binding protein (44). Based on conservation of the spectrin SH3 binding site within members of this protein family and published data (41–44), a general mechanism of interactions of tyrosine kinases with the spectrin-based membrane skeleton is proposed.

Green Glutathione S-Transferase Protein (GST) Fusions—The coding sequence of hssh3bp1/e3B1 isoform 1 was obtained by PCR amplification (primers M5’ and NGFP413’) and was subcloned into the plasmid pEGFP-N3 (BglII and EcoRI sites) (CLONTECH) so that the GST sequences were located at the C terminus of hssh3bp1/e3B1 (plasmid N3–1). The αI SH3 domain containing the same region of αI spectrin as clone 4-1 was amplified using primers M5’ and NGFP413’ and this resulted in addition of residues EFIVTD to the C terminus of αI spectrin (nucleotides 2995–3177 of αSpII cDNA; Ref. 27). The desired regions of αI and αII spectrin were obtained by amplification using specific primers and subsequently cloned into the pGEX-2T vector using BamHI and EcoRI restriction sites incorporated into amplification products by primers. All primer sequences used for expression plasmids are listed in Table I. The amplified αI spectrin αSpI encodes residues 977–1062 of the αI spectrin that includes the SH3 domain (residues 3115–3370 in the αI cDNA; Ref. 27). In this case, the termination codon was present in the vector sequences, and this resulted in addition of residues EFVTVT to the C terminus of the spectrin sequence. The construct GST-F-SH3 encodes residues 965–1025 of αI spectrin (nucleotides 2995–3177 of αSpII cDNA sequence; Ref. 29). The nucleotide sequences of all plasmid constructs were confirmed by DNA sequencing. pGEX-2T plasmids expressing SH3 domain isoforms of αI, Crk, Src and n-Src (34, 35) were obtained from Dr. Bruce J. Mayer (Howard Hughes Medical Institute, Children’s Hospital, Boston, MA).

Expression Fusion Proteins and Filter Binding Assay—The affinity purification of GST fusion proteins on glutathione-Sephrose was followed by gel filtration using Sephacryl S-100 (47). Protein concentrations were estimated using the biocinchoninic acid assay (Pierce). Biotinylation of recombinant peptides was performed using 6-[biotinamino]hexanoylamino]hexanoic acid, succinimidyl) ester (Biotin-XX; SE; Molecular Probes, Eugene, OR), and the filter binding assay was performed essentially as described (34, 35). All GST fusion proteins were expressed in BL21 strain of Escherichia coli. Inductions of GST recombinant polypeptides were performed as described (47). In each case, bacterial lysates from 50 ml of induced cell cultures were solubilized and separated on SDS-Tricine polyacrylamide gels and blotted onto polyvinylidenefluoride membranes. After the transfer, blots were blocked in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, v/v) containing 2% nonfat dried milk for 1 h at room temperature. The blots were then incubated in the same buffer with biotinylated recombinant fusion proteins (0.4 μg/ml). Following incubation for 1 h at 20 °C, the blots were extensively washed with TBST buffer and incubated with a streptavidin-alkaline phosphatase conjugate at 1:5,000 dilution (Boehringer Mannheim) in 2% milk TBST for 1 h. After washing, the blots were developed with nitro blue tetrazolium/5-bromo-
national Inc., Temecula, CA (20). The polyclonal antibody raised to GST-hssh3bp1/e3B1,3 followed by FITC-conjugated antibody against GST was raised at the IBR Antibody Facility using standard techniques (49).

Chromosomal Localization of the hssh3bp1/e3B1 Gene—A human P1 artificial chromosome (PAC) (50) library was screened using the EcoRI cDNA fragment from clone pGAD4-1 (BIOS Laboratories, New Haven, CT). The primers used for chromosomal mapping by PCR amplification were primer BS15 (see Table I) and primer Y3 (5′-CAA ATA TG CTA TGT TTA TAA GTG GC 3′). The sequence of the primer BS15 was present within an exon identified in the PAC clone 102.J.10. The sequence of the primer Y3 was derived from an intron downstream from the BS15 sequence (data not shown). Each PCR amplification was performed in 25 μl using 50 ng of template DNA and Advantage PCR polymerase mix (CLONTECH) with 7 mM MgCl₂; after an initial denaturation step (94 °C for 60 s), 32 cycles of PCR were performed: 92 °C for 15 s, 55 °C primed method (46) using digoxygenin-11-dUTP (SIG; Boehringer

4-chloro-3-indolyl phosphate substrates. The band located between the 19.8- and 33.5-kDa molecular mass markers, present on all blots developed with the streptavidin-alkaline phosphatase conjugate, most likely represents a naturally occurring biotinylated bacterial protein and has been observed previously (34, 35). To quantify the intensity of bands, the scans were quantitated using Scan Analysis software (Biosoft, Ferguson, MO).

**Table I**

| Plasmid | Primer name | Primer sequence |
|---------|-------------|-----------------|
| pAS-Sp  | YSSH5'      | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
|         | YSSH3'      | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
| pAS-F   | YFSH5'      | 5′-GGCCATTTAAAAATCCCTCTCAGTGTC-3′ |
|         | YFSH3'      | 5′-GGCCATTTAAAAATCCCTCTCAGTGTC-3′ |
| GST-E-SH3 | SHS1' | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
| GST-F-SH3 | SHS1' | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
| N3-1    | M5'         | 5′-GGGGATCTGAGTTGAGATTTTTG-3′ |
|         | NGFP413'    | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
| C1–2E-1 | SHS1'      | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
|         | ZEHS3'      | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
| C4      | T15'        | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
|         | E3'         | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
| C5      | T25'        | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
|         | E3'         | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
| C6 and C7 | T15' | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
|         | T33'        | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |
| C8      | BS35'       | 5′-AGAGGATCTGGATGATGAGATTTTTG-3′ |
|         | T33'        | 5′-AGAGGATCTGGATGATGAGATTTTTG-3′ |
| C9      | BS45'       | 5′-AGAGGATCTGGATGATGAGATTTTTG-3′ |
|         | T33'        | 5′-AGAGGATCTGGATGATGAGATTTTTG-3′ |
| C10     | BS15'       | 5′-GAGAGGATCTGGATGATGAGATTTTTG-3′ |
|         | T33'        | 5′-GAGAGGATCTGGATGATGAGATTTTTG-3′ |
| C11     | WS15'       | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
|         | T33'        | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
| C12     | BS65'       | 5′-AGAGGATCTGGATGATGAGATTTTTG-3′ |
|         | T33'        | 5′-AGAGGATCTGGATGATGAGATTTTTG-3′ |
| C15     | WS15'       | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
|         | T33'        | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
| C16     | BS15'       | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
|         | T33'        | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
| C17     | BS23'       | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
|         | T33'        | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
| C18     | BS15'       | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
|         | T33'        | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
| C19     | BS15'       | 5′-ATCGGATCCACAGTTCTGGATGATG-3′ |
|         | G3'         | 5′-GGAATCCATGCTGGAAGAAAGGTTC-3′ |

* L. Kotula and K. S. Kim, unpublished data.
Mannheim). Labeled DNA probes were solubilized at 5 ng/µl in Hybrid 7 containing blocking DNA as indicated (Onco, Gaithersburg, MD). Anonymous material remaining from clinical whole blood samples was cultured according to a previously described protocol (51). Metaphases were banded with a trypsin-Giemsa protocol and digitized using a PSI Genetec image analyzer. These pre-identified metaphases were then printed for retrospective chromosome identification (52) of FISH preparations. The slide preparations were destained by immersing them for 2 min each in three changes of 3:1 methanol-acetic acid. They were then treated according to a commercial protocol for in situ chromosome hybridization of unique sequence probes (Onco catalog no. ES150), and the DIG-labeled probes 102.J.10 and 305.I.23 were hybridized to them. Subsequently, the preparations were counter-stained with propidium iodide (orange) for FITC detection (yellow) of the probes on the hybridized chromosomes (see Fig. 2).

Sequence Analyses—were performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Madison, WI) (53) and the BLAST algorithm, National Center for Biotechnology Information (Bethesda, MD) (54).

RESULTS

Yeast Two-hybrid Screening for Spectrin SH3 Domain-binding Proteins—The yeast two-hybrid system was used to screen the human brain cDNA expression library for spectrin SH3 domain-binding proteins. Approximately 1.9 × 10⁹ yeast transformants were screened with pAS-SP (αI SH3 domain construct) on drop-out synthetic medium lacking tryptophan, histidine, and leucine. All yeast colonies larger than 1 mm (over 550 colonies) were tested for β-galactosidase activity using the filter assay, and 17 clones were positive as indicated by blue colonies. These clones were selected on -leucine and -cycloheximide (-leu, -cyh) medium to retain only the pGAD-cDNA library fusion plasmids. After purification, each of the library fusion plasmids was retransformed separately with the appropriate pAS2-SH3 construct into yeast, and a liquid β-galactosidase assay was performed. Clone pGAD-1 showed over 33-fold above background β-galactosidase activity when transformed with the construct containing the αI spectrin SH3 domain, indicating an interaction (see Table II). The αII SH3 domain plasmid, pAS-F, showed only background values when cotransformed with the clone pGAD-1. Relatively high β-galactosidase activity was observed when the pAS-F plasmid was transformed with the unmodified library pGAD plasmid. This intrinsic transcriptional activity of pAS-F prevented the screening of two-hybrid system expression libraries using the αII SH3 domain. Control assays for induction of β-galactosidase activity of the pGAD-4 clone alone and when cotransformed with the unmodified library plasmid pGAD or the non-related plasmids pLAM and pVA3 were negative, indicating a specific interaction with the αI SH3 domain. Therefore, we designated this clone human spectrin SH3-binding protein 1, or hssh3bp1 (Fig. 1A).

Characterization of the hssh3bp1/e3B1 Isoforms—Northern blotting analysis using the EcoRI insert of the clone pGAD-1 indicated the presence of alternative transcripts. The PCR amplification using human brain cDNA as a template and primers ‘T15’ and ‘E3’ (Table I) resulted in simultaneous amplification of five DNA fragments that differ in size (data not shown). Sequence analyses indicated that the fragments represent alternatively spliced forms of the candidate cDNA and were named isoforms 1 (the largest isoform) through 5 (the smallest isoform) (Fig. 1B). 5’ and 3’ rapid amplification of cDNA ends was used to clone the ends of the hssh3bp1 cDNA (see “Experimental Procedures”). Data base searches using BLAST revealed that hssh3bp1 (Fig. 1A) and e3B1 represent alternatively spliced isoforms of the same cDNA. e3B1 was recently reported as the eps8 SH3 domain-binding protein (44). The predicted amino acid sequence of e3B1 is identical to isoform 2 of hssh3bp1. Subsequently, hssh3bp1 will be referred to as hssh3bp1/e3B1.

The Human Spectrin SH3 Domain-binding Protein 1

Table II

Liquid β-galactosidase assay

| Transformation | pGADX/ pAS-Y | β-galactosidase assay (Miller units) mean ± S.D. |
|---------------|-------------|-----------------------------------------------|
| pGAD/pAS      | 0.01        | pGAD/pAS-Sp                                   | 0.17 ± 0.04 |
| pGAD/pAS-Sp   | 0.17        | pGAD/pAS-F                                    | 1.71 ± 0.73 |
| pGAD/pAS−F    | 0.24        | pGAD/pAS−F-Sp                                 | 0.55 ± 0.01 |
| pGAD/pAS−F−S  | 0.13        | pGAD/pAS−F−S−F                                | 5.23 ± 0.69 |
| pGAD1−1/pAS−F−S | 9.50       | pGAD1−1/pAS−F−S−F                             | 2.37 ± 0.25 |
| pGAD1−1/pAS−F−S−F | 5.09   | pGAD1−1/pAS−F−S−F−F                          | 4.31 ± 2.39 |
| pGAD1−1/pAS−F−S−F−F | 16.98  | pGAD4−1/pAS−F−S−F−F                          | 40.84 ± 8.88 |
| pGAD1−1/pAS   | 1.66        | pGAD4−1/pAS−F−S−F                            | 8.88 ± 5.55 |
| pGAD4−1/pAS   | 0.16        | pGAD4−1/pAS−F−S−F                            | 0.05 ± 0.01 |
| pGAD4−1/pLAM  | 0.10        | pGAD4−1/pLAM                                  | 0.09 ± 0.01 |
| pGAD4−1/pVA3’ | 0.23        | pGAD4−1/pVA3’                                 | 0.31 ± 0.07 |
| pGAD4−1/pVA3’ | 0.10        | pGAD4−1/pVA3’                                 | 0.10 ± 0.01 |
| pGAD       | 0.10        | pGAD1−1                                      | 0.11 ± 0.01 |
| pGAD1−1      | 0.10        | pGAD4−1                                      | 0.10 ± 0.01 |
| pVA3/pTDI*   | 160.65      | pVA3/pTDI*                                    | 9.63 ± 8.96 |

The sizes of hssh3bp/e3B1 cDNAs, between 2394 base pairs for isoform 1 and 2049 base pairs for isoform 5, corresponded well to mRNA sizes represented by the lower bands observed on Northern blots, ranging from approximately 2.3 to 2.7 kb (data not shown; Ref. 44). The mRNA of 3233 bases reported for e3B1 corresponds to higher bands on Northern blots, which range between approximately 3.3 and 3.7 kb (44). Apparently, the hssh3bp1/e3B1 mRNA contains two functional polyadenylation consensus signals (data not shown), which result in alternative splicing of the 3‘-untranslated region. Alternatively spliced forms correspond to the size of either of the bands observed on Northern blots.

The hssh3bp1/e3B1 gene is closely related to a group of recently identified genes encoding tyrosine kinase-binding proteins (see Refs. 41–44). Sequence comparisons using the predicted amino acid sequence of isoform 1 showed 86% identity to Abi-1 (41), and 65% identity to Abi-2 (42). The above proteins were identified as Abi-binding proteins: Abi-1 as a protein binding to the Abl C-terminal proline-rich region, and Abi-2 as a protein binding to the Abi SH3 domain. Abi-1 and Abi-2 were shown to play a role in regulating the transforming ability of Abl; Abi-2 is also a substrate of Abl tyrosine kinase (42). Isoform 1 showed 76% identity to Arg protein-tyrosine kinase-binding protein, which is likely to be a splice isoform of Abi-2 (43), and 70% identity to a 35-kDa proline-rich protein from Xenopus Xlan 4 (55).

Prominent features of all hssh3bp1/e3B1 isoforms are the presence of PEST sequences (56, 57) and numerous stretches of proline-rich sequences containing the SH3 binding consensus...
sequence, PXXP (Fig. 1A) (35–37). Overall, 13% of the residues in isoform 1 are prolines. The proline-rich region of hssh3bp1/e3B1 undergoes alternative splicing, suggesting that regulation of its binding specificities may be mediated by differential expression of proline containing sequences. All hssh3bp1/e3B1 isoforms contain an SH3 domain, residues 446–505.

Chromosomal Localization of the hssh3bp1/e3B1 Gene—The chromosomal location of the hssh3bp1/e3B1 gene was deter-
mined with PAC (50) clones 102.J.10 and 305.I.23, isolated using the hssh3bp1/e3B1 cDNA. Southern blotting and sequence analyses indicated that the 102.J.10 clone contains the entire hssh3bp1/e3B1 gene while clone 305.I.23 contains only the 3' end portion of the gene (data not shown). Each of the PAC clones contained at least 50 kb of human genomic DNA. In FISH analysis, the probe 102.J.10 hybridized with the same intensity to chromosomes 10 and 18 at bands 10p11.2 → p12 (gene symbol SSH3BP1) and 18q11.2 → q12.1 (gene symbol SSH3BP2), respectively (Fig. 2, upper panel). This was observed in a total of 19 metaphases that were pre-G-banded and digitized using the method described under “Experimental Procedures.” Probe 305.I.23, however, hybridized only to chromosome 10p11.2 → p12 in a total of 16 metaphases (Fig. 2, lower panel). In both cases, 100% hybridization efficiency was observed.

Primers specific to the hssh3bp1/e3B1 gene were used for PCR amplification analysis of a monochromosomal somatic cell hybrid panel (BIOS Laboratories). PCR amplification using primers BS15' and Y3' yielded a 1.2-kb DNA fragment in the reaction containing human chromosome 10 DNA as a template. A product of identical size was obtained by PCR amplification with either total human DNA or the clone 102.J.10 DNA as a template. PCR amplification using DNA from human chromosomes other than 10 as templates, and control reactions with mouse or hamster genomic DNA, did not result in amplification of the 1.2-kb fragment. These data located the hssh3bp1/e3B1 gene on human chromosome 10.

Hybridization of clones 102.J.10 and 305.I.23 to human chromosome 10 in FISH analysis is consistent with mapping of hssh3bp1/e3B1 to chromosome 10 by PCR amplification. However, since duplication of the coding region alone would be insufficient for FISH detection, the pattern observed in situ with 102.J.10 as the probe suggests the presence of a duplication of genomic DNA in these regions of chromosomes 10 and 18. Chromosome 18 may contain an hssh3bp1/e3B1-related gene. However, the homology between the related sequences on chromosomes 10 and 18 does not extend to the 3' end of the hssh3bp1/e3B1 gene since 305.I.23 did not hybridize to chromosome 18.

Mapping of the Spectrin SH3 Domain Binding Region—A number of GST-hssh3bp1/e3B1 mutants (Fig. 3) were created to map the spectrin SH3 domain binding region of hssh3bp1. Mapping was performed using GST fusion polypeptides derived from isoform 4 cDNA with the exception of clone C7 in which the desired cDNA region was obtained from isoform 5. The first set of mutants enabled us to localize the aI SH3 domain binding to the proline-rich region, residues 152–431 (Fig. 4). The binding was stronger, as indicated by the intensity of bands, to the aI SH3 domain than to the aII SH3 domain (compare lanes 2, 3, 5, and 6 in the GST-E-SH3 and GST-F-SH3 panels). It should be noted that the expression of GST-hssh3bp1/e3B1 fusions was represented by several bands in each lane (anti-GST panel, lanes 2–7), suggesting that GST-hssh3bp1/e3B1 fusions were rapidly degraded in bacterial lysates.

Further mapping of aI SH3 binding was performed using GST fusion polypeptides containing a series of N-terminal and C-terminal deletions of the hssh3bp1/e3B1 proline-rich region. GST fusion polypeptides containing the Abl SH3 domain and the Src SH3 domain were included in this experiment to study the specificity of the proline region for interaction with other SH3 domains (Fig. 5, A–D). GST-E-SH3 and GST-Abl-SH3 domain showed different binding specificities to GST.
FIG. 3. Diagram of the GST-hssh3bp1/e3B1 polypeptides. The top line illustrates schematically the restriction enzyme map of the EcoRI fragment of the pGAD4-1 clone isolated by the two-hybrid screening and corresponds to isoform 4 of the cDNA. The translation initiation codon, ATG (arrow above the black dot), and termination codon, TAA (arrow above crossed lines), are indicated. Restriction enzymes are named above their positions as indicated by vertical lines. The boxed area above the top line represents the alternatively spliced region not present in isoforms 3 and 5 of the hssh3bp1/e3B1 cDNA. Lines below represent regions of the hssh3bp1/e3B1 cDNA expressed as GST fusion polypeptides. Names of the plasmids are indicated on the left (C1–C19). The three letters in uppercase at each end of the lines indicate the three predicted amino acids in the hssh3bp1/e3B1 polypeptide; the three letters in lowercase indicate the additional amino acids resulting from expression of pGEX sequences. C7 contains DNA sequences from isoform 5, and deletion of the region corresponding to the boxed area is indicated. Thick lines under the top line represent the SH3 domain-binding regions BS1, BS2, and BS3 (see Fig. 1A). The asterisk indicates a table summarizing relative binding of hssh3bp1/e3B1 polypeptides to GST-E-SH3 (E-SH3), and to Abl tyrosine kinase (Abl-SH3) as observed on blots. The location of + and − in the table correspond to diagrams of plasmids on the left. ++++, very strong binding; ++ strong binding; + weak binding; −, no binding.

FIG. 4. Localization of the spectrin SH3 domain binding to the proline-rich region of the hssh3bp1/e3B1 polypeptide. Equal amounts of isopropyl 1-thio-β-D-galactopyranoside-induced bacterial lysates of the GST-hssh3bp1/e3B1 fusion polypeptides were solubilized in Laemmli buffer and separated on 7% SDS-Tricine polyacrylamide gels. Identical blotted transfers of gels were probed with biotinylated GST-SH3 fusion proteins or anti-GST mAb as indicated: GST-E-SH3 represents GST-al SH3 domain; GST-F-SH3 represents GST-αI SH3 domain; GST represents glutathione S-transferase alone. The intensity of the bands stained with the anti-GST antibody show the expression levels of the GST fusion proteins in bacterial lysates. The gel on the far left was stained with Coomassie Brilliant Blue. Lanes 1–7 represent bacterial lysates transformed with the following plasmid constructs: lane 1, pGEX-2T; lane 2, C1; lane 3, C2; lane 4, C3; lane 5, C6; lane 6, C4; lane 7, C5.
The Human Spectrin SH3 Domain-binding Protein 1

Using interaction cloning, we identified a cDNA clone encoding a candidate human spectrin SH3 domain-binding protein. We have characterized five isoforms of its mRNA in human brain; each isoform contains: 1) an alternatively spliced proline-rich region in the middle of the molecule, and 2) an SH3 domain at the C terminus.

The SH3 domain spectrin binding site was mapped to two regions of hssh3bp1/e3B1, one spanning residues 360–372 (BS2) and the other spanning residues 390–431 (BS3). Both regions contain a PXPF SH3 binding consensus sequence (34–36). All five isoforms of hssh3bp1/e3B1 contain BS3; isoforms 1, 2, and 4 contain both spectrin binding sites. Our data indicate that isoforms 4 and 5 can interact with the αI spectrin SH3 domain. Isoform 5 lacks BS2, yet it binds to αI SH3 domain, indicating that BS3 alone is sufficient for the interaction; therefore, all isoforms of hssh3bp1/e3B1 are likely to bind to the αI SH3 domain. BS2 may provide an additional binding site in isoforms 1, 2, and 4. The fact that BS2 is a part of an alternatively spliced region in hssh3bp1/e3B1 and the fact that the sequence N-terminal to BS2 is required for its activity suggest that different isoforms of hssh3bp1/e3B1 bind to the αI SH3 domain with different affinities. On the other hand, the

hssh3bp1/e3B1-derived polypeptides (see the polypeptides indicated by the lower arrow in Fig. 5A). The GST fusion peptides encoded by clones C13 and C17 bound to the αI spectrin recombinant polypeptide GST-E-SH3 (see Fig. 3, lower panel, and Fig. 5). No binding of the polypeptides encoded by clones C18 and C19 to αI SH3 domain was observed. These results together with the data presented in Fig. 4 locate the αI SH3 domain binding region to residues 360–372 and residues 390–431 of hssh3bp1/e3B1 (BS2 and BS3, respectively; Fig. 1A). Clone C15 did not bind to GST-E-SH3, but it contains the residues 360–372. This indicates that the region 360–372 requires sequences located N-terminal to it for its binding activity. Clone C7, which is derived from isoform 5 of hssh3bp1 and contains BS3 only, also bound to αI SH3 domain. The AbI SH3 domain bound to hssh3bp1/e3B1 polypeptides encoded by clones C17 and C19 with similar affinities (Fig. 6); thus, the AbI SH3 binding region is located within the polypeptide encoded by clone C19, residues 144–260 (BS1; Fig. 1A). Quantitative blots indicated that the BS1 binds specifically to the AbI SH3 domain, and that BS2 and BS3 bind specifically to the αI SH3 domain (Fig. 6). No binding of these regions to the αI SH3 domain and to the Src SH3 domain was observed. All of the hssh3bp1/e3B1 SH3-binding regions contain numerous prolines and the SH3-binding consensus sequence, PXPF.

Spectrin SH3-binding Protein Colocalizes with the Erythroid-like Spectrin in Transfected NIH3T3 Cells—No relevant bands representing hssh3bp1/e3B1 were detected by Western blotting in human erythrocyte ghosts using several anti-hssh3bp1/e3B1 polyclonal and monoclonal antibodies (data not shown). To study whether hssh3bp1/e3B1 interacts with erythroid spectrin *in vivo*, we tested several cell lines for expression of erythroid isoforms of spectrin. Immunostaining obtained with antibodies to erythroid spectrin indicated expression of an erythroid-like spectrin in NIH3T3 cells. Staining with the anti-HS antibody to αII/βII spectrin (Fig. 7) resulted in perinuclear staining characteristic of Golgi apparatus, and in cytoplasmic punctate fluorescence, suggesting vesicular staining (Fig. 8a). The Golgi staining was very similar to that observed with anti-Golgi spectrin βII* antibody (Fig. 8b). No cytoplasmic punctate staining was observed with the anti-βII* antibody. Anti-αII/βII-specific antibody 992 strongly stained cell edges, suggesting association of nonerythroid spectrin with the plasma membrane (Fig. 8f). These data indicated specific distribution of erythroid and nonerythroid isoforms of spectrin in NIH3T3 cells and enabled us to study the interaction of hssh3bp1/e3B1 with spectrin in transfected NIH3T3 cells. Expression of the green fluorescence protein fusion of hssh3bp1/e3B1 (GFP-hssh3bp1/e3B1) in NIH3T3 cells resulted in cytoplasmic punctate fluorescence. This pattern was similar to that observed with the anti-HS antibody in untransfected cells, but a lower number of vesicular structures per cell and no Golgi staining were observed (Fig. 8e). In many transfected cells, vesicular structures were fused and produced tubulovesicular structures (Fig. 8g) or larger vesicles (Fig. 9a) or resulted in a reticular-like pattern very similar to that observed in cells transfected with GFP fusion protein containing the αI spectrin SH3 domain (Fig. 8d). In cells transfected with GFP-hssh3bp1/e3B1, regardless of the observed expression pattern, immunofluorescence of the fusion protein was coincident with the staining obtained with the anti-HS antibody (Fig. 8, g and h), but not with the staining obtained with the 992 antibody (Fig. 8, e and f). In addition, the characteristic perinuclear Golgi staining was partially disrupted (Fig. 8, compare a with h). The pattern of hssh3bp1/e3B1 expression was also coincident with that of the antibody against AbI tyrosine kinase (Fig. 9, a and b, respectively). These data indicate colocalization of hssh3bp1/e3B1 with the erythroid-like spectrin and AbI tyrosine kinase *in vivo* in NIH3T3 cells.

DISCUSSION

Using interaction cloning, we identified a cDNA clone encoding a candidate human spectrin SH3 domain-binding protein. We have characterized five isoforms of its mRNA in human brain; each isoform contains: 1) an alternatively spliced proline-rich region in the middle of the molecule, and 2) an SH3 domain at the C terminus.

The αI SH3 domain spectrin binding site was mapped to two regions of hssh3bp1/e3B1, one spanning residues 360–372 (BS2) and the other spanning residues 390–431 (BS3). Both regions contain a PXPF SH3 binding consensus sequence (34–36). All five isoforms of hssh3bp1/e3B1 contain BS3; isoforms 1, 2, and 4 contain both spectrin binding sites. Our data indicate that isoforms 4 and 5 can interact with the αI spectrin SH3 domain. Isoform 5 lacks BS2, yet it binds to αI SH3 domain, indicating that BS3 alone is sufficient for the interaction; therefore, all isoforms of hssh3bp1/e3B1 are likely to bind to the αI SH3 domain. BS2 may provide an additional SH3 binding site in isoforms 1, 2, and 4. The fact that BS2 is a part of an alternatively spliced region in hssh3bp1/e3B1 and the fact that the sequence N-terminal to BS2 is required for its activity suggest that different isoforms of hssh3bp1/e3B1 bind to the αI SH3 domain with different affinities. On the other hand, the
Data from the filter binding assay and results of a \(\beta\)-galactosidase assay in which hssh3bp1/e3B1 bound to the \(\alpha\) SH3 domain but not to the \(\alpha I\) SH3 domain suggested that in fact \(\alpha I\) spectrin may be the preferred partner for hssh3bp1/e3B1 in vivo. Although we were able to precipitate \(\alpha I\) spectrin from human erythrocyte ghosts using GST-hssh3bp1/e3B1, hssh3bp1/e3B1 was not detected in erythrocyte ghosts. These data suggest that the protein does not have a role in mature red cells. More comprehensive biophysical studies using GST-hssh3bp1/e3B1 were not possible owing to limited stability of the fusion polypeptides in solution (see anti-GST panels in Figs. 4 and 5). This is likely to be a result of several PEST sequences present within hssh3bp1/e3B1; PEST sequences have been reported to function as signals for rapid intracellular proteolysis and are usually present in proteins with short half-lives (56, 57).

Several laboratories have demonstrated expression of isoforms of erythroid membrane proteins in nonerythroid cells. Erythroid-like Golgi spectrin (22) and cytoplasmic forms of ankyrins (13, 14, 64) are likely to play a structural role in the membrane skeletons of the internal membranes of endoplasmic reticulum, Golgi, and Golgi-associated vesicles (reviewed in Refs. 2 and 7). In this report, we provide immunostaining evidence for expression of the erythroid-like spectrin in the cytoplasm of NIH3T3 cells. The antibody against human \(\alpha I\Sigma I/\beta I\Sigma I\) spectrin stained Golgi membranes and a large number of cytoplasmic vesicular structures. In cells overexpressing the GFP-hssh3bp1/e3B1 fusion protein, the erythroid-like spectrin colocalized with the spectrin SH3-binding protein, as indicated by coincident pattern of immunofluorescence. These data suggest interaction of hssh3bp1/e3B1 with the erythroid spectrin in NIH3T3 cells. The perinuclear Golgi staining observed with the anti-\(\alpha I\Sigma I/\beta I\Sigma I\) antibody in untransfected cells was partially disrupted in cells transfected with the \(\alpha I\) SH3 domain-binding protein. In some cases, only cytoplasmic vesicular structures were observed with the anti-\(\alpha I\Sigma I/\beta I\Sigma I\) antibody in transfected cells.\(^5\) Disruption of Golgi staining was observed in cells overexpressing centrinactin; however, a different change in Golgi morphology was noted (63). Alternatively, an increased expres-
sion of hssh3bp1/e3B1 cause redistribution of the erythroid-like spectrin from Golgi membranes into the cytoplasmic vesicles. Further work is necessary to identify the role of hssh3bp1/e3B1 in Golgi function. Different patterns of hssh3bp1/e3B1 fluorescence observed in cells may reflect different expression levels of the protein in transiently transfected cells. It should be noted that the shape of cells did not change, suggesting that the plasma membrane skeleton is not affected by the overexpression of hssh3bp1/e3B1.

The staining of Golgi membranes with anti-αI spectrin antibodies: a, anti-HS; b, anti-Golgi spectrin. Cells were transiently transfected with plasmids: c, pEGFP-C1 containing unmodified GFP; d, C1–2E1 containing the GFP-αI SH3 domain fusion protein; e–h, N3–1 containing the GFP-hssh3bp1/e3B1 fusion protein. e and f and g and h represent pairs of the same cell from which double fluorescence micrographs were obtained. c–e and g show green fluorescence from GFP fusion. f shows fluorescence obtained with antibody 992; h shows fluorescence with antibody anti-HS. Red fluorescence was obtained using Texas Red-linked secondary antibody. The pattern of red fluorescence obtained with anti-HS coincides with the green fluorescence of GFP-hssh3bp1/e3B1, indicating colocalization of the erythroid-like spectrin with hssh3bp1/e3B1 in transfected NIH3T3 cells.

The sequence of Golgi spectrin has not yet been reported as of date of this publication, but it is likely to be a homolog of erythroid β spectrin, βI2+. Our immunocolocalization data suggest that either Golgi spectrin contains an SH3 domain similar to the αI spectrin SH3 domain and/or that there is an erythroid α-like spectrin associated with Golgi βI2+ spectrin. A protein immunoreactive to the anti-αI spectrin SH3 domain and the anti-HS antibodies can be copurified with hssh3bp1/e3B1 from untransfected NIH3T3 cells. In addition, immunofluorescence patterns of the αI SH3 domain and of hssh3bp1/e3B1 expression in these cells were very similar (Fig. 8, compare d with e and g), indicating similar intracellular localization of hssh3bp1/e3B1 and a protein containing the αI SH3 domain.

Hssh3bp1/e3B1 binds to the Abl-SH3 domain in vitro (this report and Ref. 44). The Abl-SH3 domain binding site of hssh3bp1/e3B1 is separate from the αI SH3 domain binding sites of hssh3bp1/e3B1 (see Fig. 1A), suggesting the possibility of simultaneous interactions of spectrin and Abl with hssh3bp1/e3B1. Another possible interaction site of hssh3bp1/e3B1 with Abl tyrosine kinase would be the SH3 domain of hssh3bp1/e3B1. It is closely related to the SH3 domain of Abi-1, which has been demonstrated to bind to the proline-rich C-terminal region of Abl tyrosine kinase (41). A GST fusion protein of Abl SH3 interacts with isoform 2 of hssh3bp1/e3B1, as indicated by precipitation experiments (44). Our immunostaining data suggest an association of Abl tyrosine kinase with

**FIG. 8.** Colocalization of hssh3bp1/e3B1 with erythroid-like spectrin in NIH3T3 cells. Cells were fixed with methanol and processed for immunofluorescence as described under "Experimental Procedures." Untransfected NIH3T3 cells were stained with anti-spectrin antibodies: a, anti-HS; b, anti-Golgi spectrin. Cells were transiently transfected with plasmids: c, pEGFP-C1 containing unmodified GFP; d, C1–2E1 containing the GFP-αI SH3 domain fusion protein; e–h, N3–1 containing the GFP-hssh3bp1/e3B1 fusion protein. e and f and g and h represent pairs of the same cell from which double fluorescence micrographs were obtained. c–e and g show green fluorescence from GFP fusion. f shows fluorescence obtained with antibody 992; h shows fluorescence with antibody anti-HS. Red fluorescence was obtained using Texas Red-linked secondary antibody. The pattern of red fluorescence obtained with anti-HS coincides with the green fluorescence of GFP-hssh3bp1/e3B1, indicating colocalization of the erythroid-like spectrin with hssh3bp1/e3B1 in transfected NIH3T3 cells.

**FIG. 9.** Colocalization of hssh3bp1/e3B1 with Abl tyrosine kinase in NIH3T3 cells. Cells were transfected with plasmid NG-1 containing hssh3bp1/e3B1 sequences, and immunofluorescence was obtained as described under "Experimental Procedures." The pattern of fluorescence obtained with hssh3bp1/e3B1 (a) is coincident with that obtained with anti-Abl tyrosine kinase (b), indicating colocalization of these two proteins in transfected NIH3T3 cells.
hssh3bp1/e3B1. Abi-1, Abi-2, ArgBP1, XLAN4, and hssh3bp1/e3B1 represent a family of nonreceptor tyrosine kinase-binding proteins. The spectrin SH3 binding sites, BS2 and BS3, are well conserved among these proteins (see sequence comparison in Fig. 10); therefore, it is possible that other members of the family also bind to the cλ spectrin SH3 domain. Spectrin molecules provide a number of SH3-ligand binding sites for hssh3bp1/e3B1 and possibly for other members of this family of tyrosine kinase-binding proteins. These proteins in turn could target various tyrosine kinases to spectrin-containing membranes, as we observed for hssh3bp1/e3B1 and Abi tyrosine kinase.

Overexpression of isoform 2 of hssh3bp1/e3B1 in NIH/EGFR fibroblasts inhibits cell growth (44). Previous studies by the same group showed enhanced mitogenic response and enhanced cell growth in fibroblastic and hematopoietic cells that overexpressed ep8 (65). hssh3bp1/e3B1 and ep8 can therefore be considered negative regulators of each other’s functions, both playing a regulatory role in cell growth. Our data suggest that hssh3bp1/e3B1 may be important for the intracellular distribution of spectrin molecules in NIH3T3 cells and in this way plays a role in assembly of the erythrocyte spectrin-membrane skeleton. Although the role of hssh3bp1/e3B1 and its isoforms in formation of the membrane skeleton has yet to be established, inhibition of cell growth following the membrane skeleton assembly would be a logical possibility.

The hssh3bp1/e3B1 gene has been located to the human chromosome 10p11.2 → p12 by PCR amplification and FISH. These data indicate that hssh3bp1/e3B1 is not a human homolog of mouse Abi-1 (in mouse, the gene for Abi-1 has been localized to the region of chromosome 2, which, based on synteny, corresponds to human chromosome 9q32 → q34; Ref. 41). The signal on human chromosome 18 (18q11.2 → q12.1) observed in FISH raises a possibility that an hssh3bp1/e3B1-related gene is present on chromosome 18.

Acknowledgments—we thank Drs. Peter J. Curtis (Wistar Institute, Philadelphia, PA) and Marshall Elzinga and Henry M. Wisniewski (New York State Institute for Basic Research in Developmental Disabilities, NYS IBR, Staten Island, NY) for helpful discussions. We thank Drs. Bernard G. Forget (Yale University, New Haven, CT) and Randall T. Moon (University of Washington, Seattle, WA) for providing the cDNA clones of cλ and cλ spectrin and Dr. Bruce J. Mayer (Howard Hughes Medical Institute, Children’s Hospital, Boston, MA) for providing pGEX-2T plasmids expressing SH3 domains of Ab, Crk, Ssrc, and n-Src. We are grateful to Dr. David W. Speicher (The Wistar Institute, Philadelphia, PA) for providing antibodies against cλ spectrin and to Dr. Steven R. Goodman (University of Alabama, Mobile, AL) for providing some of the anti-spectrin antibodies used in the preliminary studies. Dr. Michal Tarnawski’s (NYS IBR, Staten Island, NY) help in statistical analysis is acknowledged. We thank Dr. Carl Dobkin (NYS IBR, Staten Island, NY) for providing Southern blots containing human DNA and for discussions during this study and Dr. George S. Merz (NYS IBR, Staten Island, NY) for help in immunochemistry. We thank Dr. Patrick G. Gallagher (Yale University, New Haven, CT) for critical review of the manuscript.

Addendum—While this manuscript was in preparation, another group reported cloning of e3B1, an eps8 SH3 domain-binding protein (44). The predicted amino acid sequence of e3B1 is identical to isoform 2 of the candidate spectrin SH3-binding protein reported here. Since this is the first report of a spectrin SH3 domain-binding protein, we propose to use the name human spectrin SH3 domain-binding protein 1/e3B1 or the abbreviated form, hssh3bp1/e3B1. This name recognizes both functions identified by the two laboratories independently.

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