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Specificity of protein interactions with highly related SRC homology (SH) domains of FGR and FYN protein–tyrosine kinases

Octavio M. Rivero-Lezcano, Keith C. Robbins*

Laboratory of Cellular Development and Oncology, National Institute of Dental Research, National Institutes of Health, 9000 Rockville Pike, Building 30, Room 211, Bethesda, MD 20892, USA

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Abstract

As an approach toward identification and isolation of cellular proteins that may act as substrates or effectors of the SRC-family of protein-tyrosine kinases, fusion proteins containing noncatalytic elements of two highly related SRC-family members were tested for their ability to recognize distinct molecules present in lysates of cells known to normally express both enzymes. Our results demonstrate differences of protein binding between the SH2 elements of FYN and FGR kinases, but do not discriminate proteins binding to their SH3 domains.

Key words: Proto-oncogene; Protein binding; Tyrosine phosphorylation

1. Introduction

The SRC family of oncogenes consists of cytoplasmic protein-tyrosine kinases predominantly expressed in hematopoietic cells. These enzymes all share the ability to induce malignant transformation when expressed ectopically in murine NIH 3T3 cells, and emerging evidence suggests that they normally play a critical role in transducing signals from the extracellular environment to intracellular pathways. A catalytic domain which phosphorylates proteins exclusively on tyrosine residues, and three noncatalytic elements [1], believed to regulate enzyme action, represent the primary structural features of the family. Noncatalytic elements, designated SRC homology domains 2 and 3 (SH2 and SH3, respectively) are highly related within the SRC family, and more distantly related versions are found in other proteins [2]. SH2 and SH3 domains coexist in phospholipase C-γ (PLC-γ) [3], RAS GTPase-activating protein (GAP) [4], and the p85 subunit of phosphatidylinositol-3-kinase [5], as well as a number of proteins having no known enzymatic activity [6–9]. SH2 and SH3 elements also appear independently.

The function of SH2 appears to involve mediating the physical association of SH2 containing molecules to tyrosine phosphorylated proteins [10–12]. On the other hand, a recently described protein, 3BP-1, which binds SH3 of the ABL tyrosine kinase [13], has sequence similarity to BCR and GAP-rho, suggesting a linkage between G-protein regulated pathways and protein tyrosine kinases [14]. In the present report, we have directly investigated the specificity of SH2 and SH3 interactions. Our results demonstrate that even closely related SH2 and SH3 elements specifically recognize distinct proteins.

2. Materials and methods

2.1. Cells and antibodies

Propagation of NIH 3T3 murine fibroblasts transformed with FYN has been described previously [15]. HL60 cells [16] were maintained in RPMI 1640 medium containing 10% FBS. Exponentially growing HL60 cells were subcultured at a density of 5×10^4 cells/ml and where indicated, were treated with retinoic acid for 48 h at a concentration of 10^{-6} M to induce their differentiation. Cells were lysed in phosphate buffered saline (PBS), pH 7.2, which also contained 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 5 μg/ml aprotinin and 50 μg/ml leupeptin. Lysates were clarified by centrifugation at $14{,}000 \times g$ for 20 min, and were biotinylated at room temperature for 30 min with gentle shaking by incubation with 20 μg *N*-hydroxysuccinimidobiotin dissolved in 10 μl *N*,*N*-dimethylformamide per mg of cell protein. The reaction was quenched by the addition of 2 μl ice cold 1 M Tris-HCl, pH 7.5, per mg cell protein. Biotinylated PY20 (ICN Biochemicals, CA), 4G10 (UBI Inc., NY) and 1G2 (Boehringer Manheim, IN) monoclonal antibodies capable of recognizing phosphotyrosine residues were used for immunoblots.

2.2. Fusion proteins

Protein elements were expressed in *Escherichia coli* HB 101 transformed with pGEX-2T based vectors [17]. Fusion proteins consisted of the amino-terminal region of glutathione-*S*-transferase linked to FGR or FYN SH2 or SH3 elements. DNAs encoding SH domains were obtained by gel purification of human FGR cDNA components [18]. *Hin*dII–*Sty*I and *Bst*EII–*Hin*dII fragments of human c-FGR represented SH2 (residues 137–253) and SH3 (residues 79–136) domains, respectively. Similarly, the *Hin*dII–*Xho*I fragment of human FYN cDNA [19] contained its SH2 domain, (residues 142–262). A PCR

*Corresponding author.

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reaction using the oligonucleotide 5'-GCGTGGATCCGCCCTT-TATGAC-3' created a *Bam*HI restriction site at FYN nucleotide 635. The *Bam*HI–*Hin*dII fragment of this construct, containing the FYN SH3 domain (residues 89–141) was gel purified and ligated to *Bam*HI–*Sma*I cut pGEX-2T DNA. Other SH encoding DNAs were blunt-end ligated into pGEX-2T. For the expression of the fusion protein, cultures were processed by the technique described by Lavan, et al. [20]. Yields were 4–8 μg of fusion protein/μl packed agarose beads as measured by elution of bound protein with 4% SDS. Bacterial fusion protein (12–15 μg) were incubated for 1 h at 4°C with 1 mg of mammalian cell lysate unless otherwise indicated. Beads were washed twice with a buffer containing 0.2 M Tris-HCl, pH 7.5 and 150 mM NaCl before elution of proteins binding to SRC homology domains by boiling in Laemli loading buffer.

2.3. Protein blotting and detection

Proteins were fractionated by electrophoresis in polyacrylamide gels, transferred by electroblotting to polyvinylidene difluoride (PVDF) (Millipore, MA) membranes, and blocked by incubation with a buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (TN) and 4% bovine serum albumin fraction V (BSA). Streptavidin coupled to horseradish peroxidase (HRP) (4 μg/ml) (Pierce, IL) was added to fresh blocking buffer, and filters were incubated an additional hour at room temperature. After extensive washing in TN buffer with 0.4% BSA, proteins were detected by enhanced chemiluminiscence (ECL) (Amersham, UK) following the manufacturer's instructions.

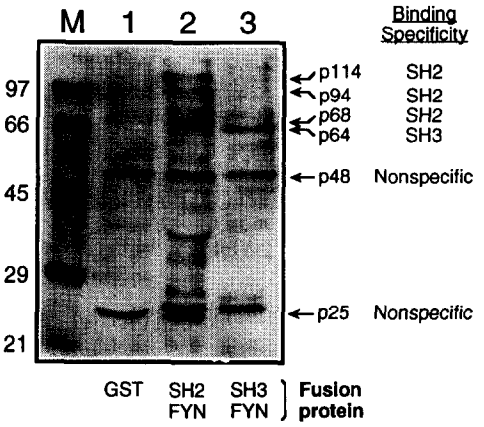

Fig. 1. Specificity of cellular protein binding to noncatalytic elements of $p59^{FYN}$ expressed in bacteria as a GST-fusion protein. Lysates from FYN-transformed cells were labeled with biotin, incubated in the presence of FYN SH2 or SH3 domains or GST and analyzed as described in Section 2. Prominent binding proteins are indicated at the right together with their corresponding molecular masses. Specificity of binding to SH elements as well as nonspecific binding for each of these prominent proteins is also shown.

3. Results

3.1. Detection of cellular proteins that specifically associate with SH2 and SH3 domains

To explore the possibility that protein–protein interactions could be used directly for the identification of candidate substrates or effectors for SRC-family kinases, whole cell lysates from FYN transformed cells were labeled with *N*-hydroxysuccinimidobiotin. Fusion proteins bearing FYN SH2 or SH3 domains were prepared from bacterial extracts, coupled to agarose beads and incubated with biotinylated cell extracts. Nonspecific binding in the assay was defined as those cellular proteins that bound only the GST portion of the fusion protein. As shown in Fig. 1 (lane 1) proteins associating with GST included predominant bands of 48 and 25 kDa as well as a number of other minor proteins. When these same lysates were incubated with a fusion protein containing FYN SH2 (Fig. 1B, lane 2), specific binding proteins of 114, 94 and 68 kDa, among others, were detected. Similarly, a band of 64 kDa was observed as a specific FYN SH3 binding protein (Fig. 1, lane 3). Thus, proteins identified as SH2-binding differed in size from SH3-binding species. We conclude that this procedure is capable of scoring cellular proteins that specifically bind to FYN SH2 or SH3 domains.

3.2. Differential binding to SH2 and SH3 domains of FYN and FGR gene products

The protein product of the FYN proto-oncogene is expressed in a wide variety of cell types, including fibroblasts and hematopoietic cells [21]. In contrast, the FGR gene product is normally expressed only in mature myelomonocytic cells [22,23]. To determine whether specific association with analogous domains of these highly related proteins could be demonstrated, we examined binding in lysates from HL60 cells, a lineage where both FYN and FGR proteins are normally expressed. Expression of $p55^{c\text{-}FGR}$ only in HL60 cells induced to differentiate [23] prompted us to examine lysates from untreated as well as retinoic acid treated cells. When proteins binding to FYN or FGR SH2 were compared, we observed bands in common as well as proteins binding specifically to the FYN SH2 domain. As shown in Fig. 2A (lanes 6 to 9), a doublet of 95 kDa appeared in lanes containing either FYN or FGR SH2 binding proteins. Protein bands of 60 and 70 kDa appearing in lane 6 were not reproducibly observed and therefore are not categorized as specific FGR SH2 binding proteins. In contrast, specific FYN SH2 binding proteins p114, p70 and p51 were observed. None of these proteins was detected in association with only the GST portion of the fusion protein. Although no major change in the pattern of binding proteins was observed in lysates of untreated as compared to retinoic acid treated cells, the signal intensities of p95 and p51 were stronger when differentiated cell lysates were analyzed (compare lanes 7 and 9).

The pattern of proteins observed to associate with FGR or FYN SH3 domains was substantially different from that observed with SH2 domains (Fig. 2A). However, no distinct bands could be observed to associate specifically with either FYN or FGR SH3 domains in differentiated or undifferentiated cells. FYN SH3 binding proteins appeared to be identical to those associating with FGR SH3 and a protein of 60 kDa was the most intense of the SH3 binding proteins.

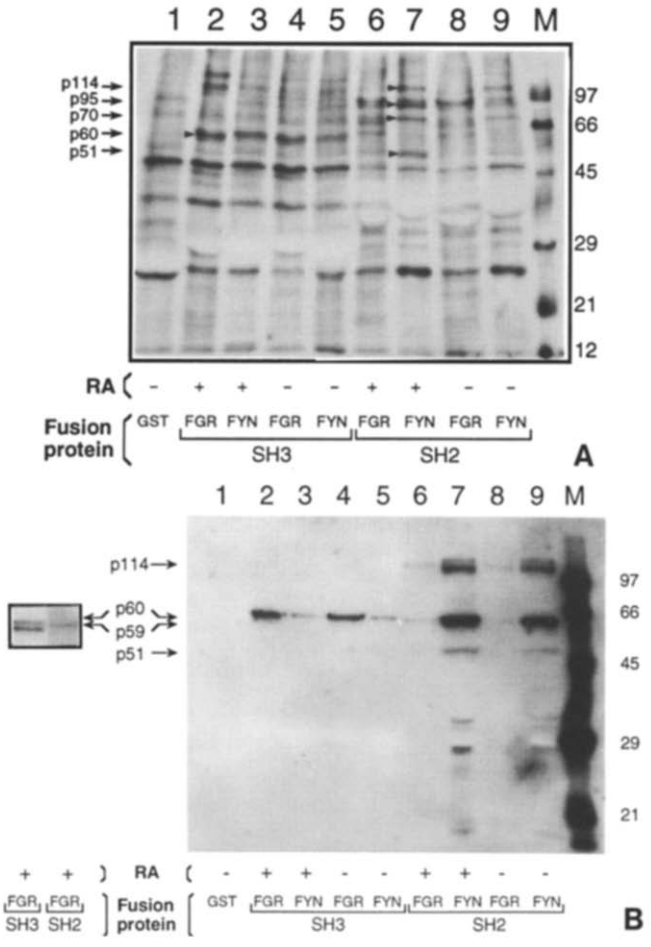

Fig. 2. (A) Identification of HL60 cell proteins that specifically bind FYN and FGR SH2 and SH3 elements. Protein extracts of untreated (−) or retinoic acid (RA) treated (+) HL60 cells were biotinylated, incubated with fusion proteins and analyzed as described in Section 2. Molecular masses of biotinylated standards are shown in kilodaltons at the right. Arrowheads between lanes indicate the more prominent SH-element binding proteins which are shown with their corresponding molecular masses. (B) Nonbiotinylated protein extracts from the same cultures described in Panel A were incubated with the fusion proteins shown and visualized by immunoblotting with biotinylated anti-phosphotyrosine antibody. Molecular size standards are shown at the left in kilodaltons. Arrows at the right indicate prominent SH-element binding proteins which are shown with their corresponding molecular masses.

3.3. *Tyrosine phosphorylation of proteins binding to non-catalytic domains*

Previously, proteins binding to SH2 elements have been shown to require phosphorylation on tyrosine residues [10,11]. To determine whether proteins binding to FYN or FGR SH2 domains were tyrosine phosphorylated, associating proteins were fractionated by electrophoresis and immunoblotted using a biotinylated, affinity purified anti-phosphotyrosine antibody (PY20) as a probe. As shown in Fig. 2B, most of the biotinylated proteins detected as SH2 binding were also scored by anti-PY. No differences were found when compared with the anti-PY monoclonal antibodies 1G2 and 4G10 (data not shown). SH2 binding proteins of 114 and 51 kDa (lanes 1 and 3) as well as lower molecular weight proteins were detectably phosphorylated on tyrosine. The p51 molecule appeared to be more highly phosphorylated in differentiated as compared to untreated cells. In contrast, p70 and p95, the most prominent SH2 binding proteins observed in Fig. 2A, were not detected as tyrosine phosphorylated species. Furthermore, a phosphoprotein of 59 kDa, not recognizable as a biotinylated SH2 binding protein, was readily detected by anti-PY. Taken together, these findings provide evidence that not all SH2 binding proteins are tyrosine phosphorylated. Recent studies of BCR association with ABL SH2 [24] have shown that its phosphorylation on serine residues is important for binding to ABL SH2. A similar mechanism may account for the binding of molecules like p70 and p95 to the FYN and FGR SH2 domains.

The only phosphorylated SH3 binding protein detected was a protein of 60 kDa that does not bind SH2 despite its tyrosine phosphorylation. This protein appears to have higher affinity for FGR SH3 than for FYN SH3 (Fig. 2B). Extended electrophoresis (Fig. 2B, at left) revealed that p60 migrates as a doublet, which could represent either two forms of the same protein or two different proteins. Although we cannot formally rule out the possibility that p59 is a component of the p60 band shown in Fig. 2A, we conclude that the p59 phosphoprotein is distinct from p60.

4. Discussion

Previous methods utilized for the study of proteins that physically interact with noncatalytic SH2 and SH3 domains have relied either on the availability of antibodies recognizing the protein under study [25] or have required the use of directly or indirectly labeled SH domains for binding to cellular proteins immobilized on filters [12]. We addressed the question of specific binding by incubating labeled cell lysates with SH-fusion proteins coupled to agarose beads. As a detection method, we chose to label cellular lysates with biotin for several reasons. First, our interest focused upon the entire complement of proteins expressed in cells at steady state. Metabolic labeling approaches were deemed unsatisfactory because of their inherent problems providing molecules of uniform detectability. In addition, it was important for us to avoid labeling tyrosine residues, since tyrosine has been implicated in SH2 domain recognition. Thus, we used acylating agents like *N*-hydroxysuccinimide esters, which are reactive toward amines, mainly on lysines. This approach can be used with ^{125}I or biotine [26,27]. Theoretically both labeling techniques label the proteins with the same efficiency since each utilizes the same mechanism for coupling. Furthermore, the sensi-

tivity of detection with the ECL reagent is comparable to ^{125}I [28].

The array of bands observed to bind SH3 domains was similar whether SH3 was derived from FYN or FGR and appeared to depend little on the source of cellular protein. We view it likely that the high degree of amino acid identity between SH3 elements from FGR and FYN proteins (83%) accounts for the similarity of patterns observed. A molecule of 60 kDa was identified as a specific SH3 binding protein, but surprisingly was found to be phosphorylated on tyrosine residues in HL60 cells. Thus, an abundant, widely distributed protein capable of specifically binding to SH3 elements of SRC family kinases is phosphorylated on tyrosine but does not bind to SH2 domains.

Distinct proteins associating with the SH2 domains of either FGR or FYN kinases demonstrated binding specificity between highly related SH2 elements. A 51 kDa molecule was associated with the FYN SH2 element but not with that of FGR, and represented the major difference found between immature and differentiated HL60 cells. We cannot determine at this juncture whether enhanced expression of p51 in differentiated cells or a change in its affinity for FYN SH2 accounts for its increased detectability in our assay. Other examples of binding specificity included p114 and p70, both of which bound FYN but not FGR SH2. Whereas binding of tyrosine phosphorylated proteins to SH2 elements is well established [10,11], the novelty of this finding relates to the degree of binding specificity observed between two such closely related SH2 domains.

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