Identification of SH3-domain Binding Proteins from Cell and Tissue Extracts by Using Peptide-Mass Fingerprinting

Masaho Ishino[1)], Hiroshi Aoto[1)], Hiroko Sasaki[1)],
Fumio Matsumura[2)], Shigeko Yamashiro[2)], and Terukatsu Sasaki[1)]

[1)] *Department of Biochemistry, Cancer Research Institute, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku Sapporo 060-8556, Japan*
[2)] *Department of Molecular Biology and Biochemistry, Rutgers University, Bush Campus, Piscataway, New Jersey, 08855, USA*

ABSTRACT

Efs is a Fyn-binding protein with an SH3 domain and multiple tyrosine phosphorylation sites, and it functions as a possible modulator in intracellular signal transduction. To better understand signaling through Efs, we searched for Efs-binding proteins by using immobilized SH3 domains fused to glutathione S-transferase (GST). Proteins which bound to the affinity matrices from cell and tissue extracts were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). By this method, it was possible to identify multiple proteins at one time. However, special care was necessary to eliminate nonspecific binding of cytoskeletal proteins, including actin, myosin and tropomyosin; the amounts of these proteins present in the cell lysate were large and significant amounts were found to bind to the affinity matrices. The preclearing process did not work well to eliminate these cytoskeletal fractions and a specific elution procedure was necessary to selectively identify signaling proteins.

Key words: Signal transduction, SH3 domain, MALDI-TOF-MS, Peptide-mass fingerprinting

INTRODUCTION

Src homology domain 3 (SH3) is composed of about 50 amino acids and associates with short amino acid sequence motifs that are rich in proline[1,2)]. This association has been shown to be very important for protein-protein interactions of signaling molecules, which modify and/or link intracellular signal transduction pathways. Efs was first found as the Fyn-SH3 binding molecule[3)], and its structure is closely related to those of p130Cas and HEF1. Although the latter two molecules have been shown to have important roles in signal transduction of integrin and other cell surface receptors[4)], we do not have any significant evidence so far on the function associated with Efs. We have observed, however, the characteristic expression of Efs, in which elevated expression of Efs is only seen in several restricted tissues such as brain, skin and bone. Our

Corresponding author: M Ishino
Department of Biochemistry, Cancer Research Institute, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku Sapporo 060-8556, Japan

extensive effort to screen Efs-binding proteins by utilizing a phage display library and yeast two-hybrid system failed. One of the possible reasons for this failure is that these in vitro systems did not properly reconstitute the associations of signaling proteins. Protein-protein interactions that occur naturally and can only be observed in living cells can be studied by a system that directly analyzes the association of proteins within cells. However, high sensitivity is required to identify proteins that are only present in very limited amounts within cells. The recently developed method of peptide fingerprinting combined with protein identification by mass spectrometric analysis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and by database searching is a very effective approach to quickly identify very small amounts of proteins[5,6]. In this study, we have applied this strategy to our system and identified mutiple proteins that were bound to affinity matrices of GST-SH3 proteins. Problems in handling these systems and their possible solutions are discussed.

MATERIALS AND METHODS

Recombinant adenovirus

The BstUI-StuI fragment of human Efs1[7] containing the entire coding sequence was inserted into the SwaI site of the adenovirus cosmid cassette, pAxCAwt[8]. A recombinant adenovirus for expression of hEfs1, AxCAShEfs1, was obtained by transfecting 293 cells with the above cosmid together with adenoviral DNA cut with EcoT22I, according to the COS-TPC method[9]. For expression of Efs, the medium was removed from HeLa cells growing at 75% confluence and cells were infected with AxCAShEfs1 in 1ml of culture medium per 10 cm dish at a moi of 10. The cells were supplied with 10 ml of culture medium 1 h after infection, and used 2 days later.

Affinity binding and SDS-PAGE

The vector for the expression GST-FynSH3 was as described[3]. HeLa cells infected with and without AxCAShEfs1 were harvested at 2 days postinfection and lysed in RIPA buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 25mM HEPES pH 7.2, 150 mM NaCl, 1mM EDTA, 0.3 mg/ml benzamidine, 1μg/ml leupeptine, 10 μg/ml aprotinin, 0.1 mg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1.5 mM Na_3VO_4). Soluble extracts were mixed at 4℃ overnight with GST-FynSH3 protein (30 μg) in the presence of glutathione-agarose beads (SIGMA). The beads were washed 3 times with RIPA buffer and proteins binding to the beads were eluted in 3X sample buffer (187.5 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 0.03% phenol red, 125 mM DTT) by heating for 1 h at 60℃. Electrophoresis was performed in 10% polyacrylamide gels in the presence of 0.1% SDS. To obtain placental extract, 2.5 g of human placenta was homogenized in 10 ml of RIPA buffer and centrifuged at 16000 g for 10 min. Soluble lysate was used for affinity preparations. GST and GST-EfsSH3 columns were prepared by binding 2 mg of the protein to 1 ml of CNBr-activated Sepharose beads (Pharmacia). Eluted samples were concentrated with microconcentrators (Amicon, Beverly, MA, USA) for the separation by SDS-PAGE.

In-gel tryptic protein digestion

All steps were done at room temperature unless otherwise specified. After visualization by Coomassie blue staining, gels were washed twice with water for 15 min. Protein bands were excised, diced and put in siliconized 0.5 ml centrifuge tubes. The gels were washed twice in water, once in 50% acetonitrile, and then once in pure acetonitrile. Each of these three washes was followed by incubation for 15 min. Acetonitrile was discarded and 40 μl of 100 mM ammonium bicarbonate was added to the gels, which were then incubated for 5 min,

after which the same amount of acetonitrile was added to make 50% acetonitrile/50 mM ammonium bicarbonate. After another incubation for 15 min, the solution was pulled off and samples were dried completely in a vacuum centrifuge. For tryptic digestion of proteins within gels, 40 μl of 12.5 μg/ml trypsin solution in digestion buffer (50 mM ammonium bicarbonate and 5 mM $CaCl_2$) was added to the dried gels. After incubation for 45 min on ice, excess solution was pulled off and the gels were soaked in digestion buffer without trypsin. The samples were incubated overnight at 37℃ and supernatants were saved in different tubes. Peptide fragments of digested proteins within gels were extracted as follows. To the gels, 25 mM ammonium bicarbonate was added and then incubation for 15 min was followed by addition of the same amount of acetonitrile, and incubation again for 15 min. Then the supernatants were pooled with the first supernatant. Next, 5% formic acid was added to the gels, which were then incubated for 15 min, followed by addition of the same amount of acetonitrile, another incubation for 15 min, and pooling of supernatants again. This step was repeated twice. Then 10mM DTT solution was added to the pooled solutions to make 1mM DTT, which was completely dried in a vacuum centrifuge. Collected proteins were resuspended in 5% formic acid. Samples were bound to ZipTip (Millipore) and eluted with 50% acetonitrile saturated with α-cyano-4-hydroxycinnamic acid. Samples were dried again and MS analysis was carried out using a Voyager DE-STR MALDI time-of-flight mass spectrometer (ABI). For interpretation of the MS spectra of protein digests, we used the MS-Fit program available at the World Wide Web site of the University of California, San Fransisco (http://rafael.ucsf.sdu/cgi-bin/msfit).

RESULTS AND DISCUSSION

Proteins bound to GST-FynSH3 in Efs-expressed HeLa cells

Protein-protein interactions have been studied using a variety of experimental systems. Although some in vitro systems have been proved to be effective, natural association of proteins within cells is best reproduced by experimental systems that can utilize the cellular environment for the detection of binding. To study binding partners of Efs, cDNA of Efs was introduced in HeLa cells via an adenovirus vector designed to express excessive amount of Efs protein. In this experiment, Efs-binding protein was precipitated together with Efs by an immobilized ligand of Efs. Because the most effective ligand of Efs known so far is the SH3 domain of Fyn (Fyn-SH3), we used GST-Fyn-SH3 immobilized to glutathione-agarose beads to pull down the expressed Efs protein. Although GST-Fyn-SH3 should also bind authentic Fyn-binding proteins from HeLa cell extracts, we expected extra proteins pulled down together with Efs from the extract of HeLa cells that expressed exogenous Efs. We found, as expected, several additional proteins pulled down from the extract of Efs-introduced cells (Fig 1, lane 2, band E1, E2). Interestingly, at least one protein was seen in a larger amount in the control precipitate (lane 1, band C1). We identified these proteins bound to GST-Fyn-SH3 by analyzing their tryptic digests with MALDI-TOF-MS. The major protein specifically pulled down from the Efs-introduced cells (band E1) was found to be Efs itself, as expected, while the prominent protein pulled down from the control cell (band C1) was Sam68[10-13].

The results showed that Fyn-SH3 bound to Sam68 endogenously present in control cells, but that Sam68 was replaced by Efs when Efs was present. Because Sam68 is expressed rather ubiquitously and abundantly among different cells and tissues, Fyn may commonly bind Sam68. It is likely, however, that the binding affinity of Fyn to Sam68 is in itself not very

high, because Sam68 does not have the typical consensus sequence of the Fyn-SH3 binding site[13], whereas Efs does[3]. It is thus possible that, although Fyn usually binds Sam68, Efs could easily, if present and close to Fyn, replace Sam68 as a binding partner of Fyn. This hypothesis concerning Efs may also be applica ble to p130Cas, which also have authentic proline-rich binding motifs for Fyn and Src SH3 domains[3,14,15]. Thus, high-affinity association of these SH3 domains to p130Cas and FAK is also anticipated.

Actin and myosin were major proteins binding to GST fusion protein immobilized to glutathione agarose beads

There was at least one clear band of protein other than that of Efs, which was found specifically in the Efs-introduced cells (Fig. 1, lane 2, band E2). This protein was revealed to be fascin. Fascin is an actin binding protein that bundles actin filaments[16,17]. However, we were not able to detect any in vivo or in vitro binding of fascin to either Efs-SH3 or Fyn -SH3. This implied that the apparently specific binding of fascin was an artifact. We also identified proteins in the bands seen in both lanes (lanes 1 and 2) of SDS-PAGE analysis of samples with and without expression of Efs and it was revealed that the most prominent bands (band N2 and N3) were the myosin heavy chain and actin bands, respectively. Other bands present were: filamin, β-spectrin, myosin I β, alpha-actinin, lipocortin and the calpactin I heavy chain: all of which are actin and actin-associated proteins. Control experiments revealed that the GST moiety was sufficient to precipitate actin and myosin (data not shown). The results suggested that GST or the matrices on which GST was immobilized were potent binders of an actin-related cytoskeletal protein, which could be either actin itself or myosin. It was thus likely that fascin was coprecipitated with the actin complex. Because Efs is a member of the p130Cas family of proteins, which are related to the process of actin rearrangement[18–20], we postulated that Efs influenced some process of actin reorganization within cells expressing excessive amounts of Efs. This effect of Efs may have caused enhanced binding of fascin to actin and then the pulling down of fascin with an actin complex by GST matrices.

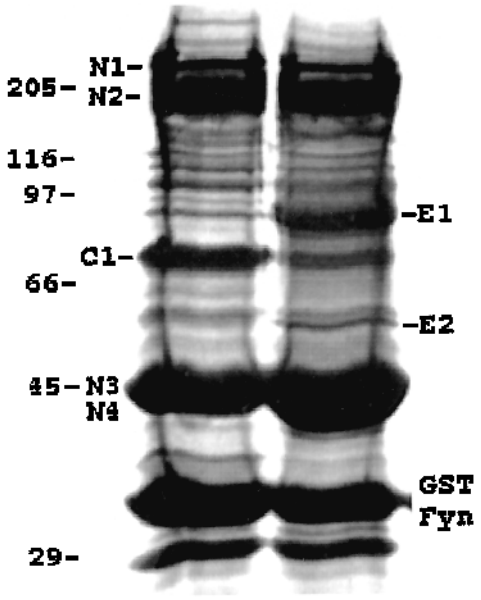

Fig. 1 HeLa cells with (for lane 2) and without (for lane 1) infection by the recombinant adenovirus for the expression of human Efs1 were harvested, lysed, and subjected to an affinity precipitation with Fyn-SH3 matrices, as described in Materials and Methods. Samples were analyzed by SDS-PAGE with 10% gel and the gel was stained with Coomassie Blue R-250.

Effective identification of signaling proteins utilizing affinity column

Protein on the subpicomol order could be identified by the method combining peptide fingerprinting, MALDI-TOF-MS and computer -based data base searching. However, when dealing with signaling proteins, which are usually the minor components within cells, for reliable identification it is necessary to collect these proteins on a larger scale. Though significant amounts of actin and myosin are present in cell extracts, it seemed inappropriate to apply the protocol of affinity precipitaion to a large-scale system. Thus, we used an affinity column to analyze a RIPA-soluble lysate of hu-

man placenta. Human placenta was selected as a source of a larger amount of cellular proteins. Preclearing of cytoskeletal proteins was attained with a GST-Sepharose column of an appropriate size. The flow-through was then applied to a GST-Efs-SH3-Sepharose column. After washing the column with PBS, proteins were eluted from the column with 7mg/ml poly -L-proline in PBS. Several bands of protein were seen in the elute. (Fig. 2 lane 1). However, when we used a high-salt buffer to elute more protein, we again had many proteins coming out of the affinity column (lane 2); some of the major bands of which (L and N in lane 2) turned out to represent tropomyosine, a typical cytoskeletal protein. The result indicated that elution was best attained with a specific binding-competitor. In contrast, the use of a general elution protocol utilizing a high-salt buffer may result in significant elution of non-specifically bound cytoskeletal proteins, even after the preclearing process.

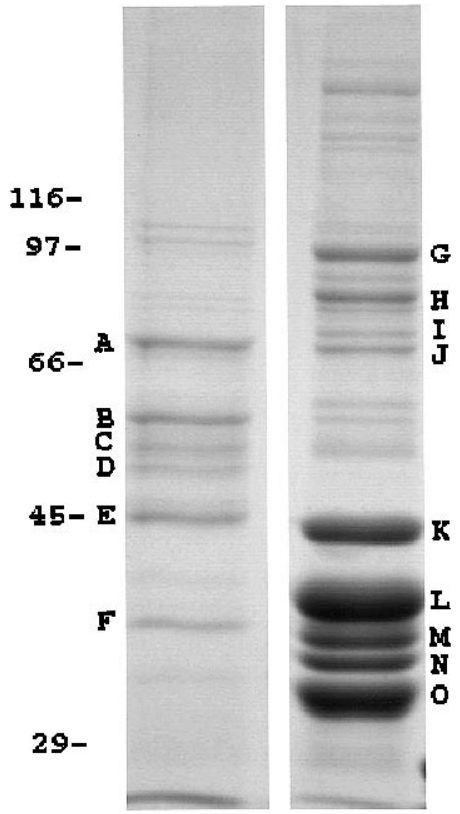

Fig. 2 Human placenta tissue was lysed and homogenized in RIPA buffer as described in Materials and Methods. The supernatant was precleared with a GST column and the proteins which did not bind to the column were applied to a GST-Efs -SH3 column. The second column was washed with PBS and bound proteins were eluted first with 7mg/ml polyproline (lane 1) and then with 1M NaCl (lane 2). Eluted proteins were separated by SDS-PAGE with 10% gel.

In summary, we successfully identified cellular proteins that bind to the SH3 domain, prepared as a recombinant fusion protein. The protein identification method combining in-gel tryptic digestion, MALDI-TOF-MS analysis and database searching worked well in identifying proteins of the subpicomol order. However, specific care is necessary to avoid contamination by cytoskeletal proteins, which are present in large quantities within cells.

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REFERENCES

1. Ren R, Mayer BJ, Cicchetti P, Baltimore D. Identification of a ten-amino acid proline-rich SH3 binding site. Science 1993, 259: 1157-1161.
2. Feng S, Chen JK, Yu H, Simon JA, Schreiber SL. Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. Science 1994, 266: 1241-1247.
3. Ishino M, Ohba T, Sasaki H, Sasaki T. Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn. Oncogene 1995, 11: 2331-2338.
4. Cottrell JS. Protein identification by peptide mass fingerprinting. Pept Res 1994, 7: 115-124.
5. O'Neill GM, Fashena SJ, Golemis EA. Integrin signalling: a new Cas(t) of characters enters the stage. Trends Cell Biol 2000, 10: 111-119.
6. Gevaert K, Vandekerckhove J. Protein identification methods in proteomics. Electropho-

resis 2000, 21: 1145-1154.
7. Ishino M, Ohba T, Inazawa J, Sasaki H, Ariyama Y, Sasaki T. Identification of an Efs isoform that lacks the SH3 domain and chromosomal mapping of human Efs. Oncogene 1997, 15: 1741-1745.
8. Kanegae Y, Lee G, Sato Y, Tanaka M, Nakai M, Sakaki T, Sugano S, and Saito I. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res 1996, 23: 3816-3821
9. Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, Tokuda C, and Saito I. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. Proc Natl Acad Sci USA 1996, 93: 1320-1324.
10. Shen Z, Batzer A, Koehler JA, Polakis P, Schlessinger J, Lydon NB, Moran MF. Evidence for SH3 domain directed binding and phosphorylation of Sam68 by Src. Oncogene 1999, 18: 4647-4653.
11. Fusaki N, Iwamatsu A, Iwashima M, Fujisawa Ji. Interaction between Sam68 and Src family tyrosine kinases, Fyn and Lck, in T cell receptor signaling. J Biol Chem 1997, 272: 6214-6219.
12. Fumagalli S, Totty NF, Hsuan JJ, Courtneidge SA. A target for Src in mitosis. Nature 1994, 368: 871-874.
13. Taylor SJ, Shalloway D. An RNA-binding protein associated with Src through its SH 2 and SH3 domains in mitosis. Nature 1994, 368: 867-871.
14. Polte TR, Hanks SK. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc Natl Acad Sci U S A 1995, 92: 10678-10682.
15. Nakamoto T, Sakai R, Ozawa K, Yazaki Y, Hirai H. Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase. J Biol Chem 1996, 271: 8959-8965.
16. Yamashiro S, Yamakita Y, Ono S, Matsumura F. Fascin, an actin-bundling protein, induces membrane protrusions and increases cell motility of epithelial cells. Mol Biol Cell 1998, 9: 993-1006.
17. Edwards RA, Bryan J. Fascins, a family of actin bundling proteins. Cell Motil Cytoskeleton 1995, 32: 1-9.
18. Kirsch KH, Georgescu MM, Ishimaru S, Hanafusa H. CMS: an adapter molecule involved in cytoskeletal rearrangements. Proc Natl Acad Sci USA 1999, 96: 6211-6216.
19. Honda H, Oda H, Nakamoto T, Honda Z, Sakai R, Suzuki T, Saito T, Nakamura K, Nakao K, Ishikawa T, Katsuki M, Yazaki Y, Hirai H. Cardiovascular anomaly, impaired actin bundling and resistance to Src -induced transformation in mice lacking p 130Cas. Nat Genet 1998, 19: 361-365.
20. Nakamura I, Jimi E, Duong LT, Sasaki T, Takahashi N, Rodan GA, Suda T. Tyrosine phosphorylation of p130Cas is involved in actin organization in osteoclasts. J Biol Chem 1998, 273: 11144-11149.

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