

Ras GTPase-activating protein-associated p62 is a major v-Src-SH3-binding protein

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Abstract Oncogenic transformation by v-Src is accompanied by marked morphological changes and cytoskeletal reorganization. Yet, the cytoskeleton-associated proteins with which v-Src interacts are largely unknown. We have studied the binding of v-Src-SH3 domain to cellular proteins utilizing a blot overlay procedure with a GST-v-Src-SH3 fusion protein as probe. A major 62–64 kDa v-Src-SH3-binding protein, present in detergent-insoluble cellular fractions, was identified as p21^{ras}-GTPase-activating protein-associated p62 (GAPA62). In non-transformed cells, including NIH 3T3 cells, GAPA62 was present in both the RIPA-soluble and RIPA-insoluble fractions, but only the latter form was tyrosine-phosphorylated. In contrast, in polyoma middle T antigen-transformed 3T3 cells, GAPA62 was present only in the RIPA-insoluble fraction, where it was highly phosphorylated. It is suggested that tyrosine phosphorylation of GAPA62 may be an important determinant of its cellular localization and its possible function as a mediator of v-Src actions.

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Key words: Src; Tyrosine kinase; Cellular transformation; p62; SH3 domain; Cytoskeleton

1. Introduction

The tyrosine kinase activity of c-Src is increased in vivo during mitosis and in response to stimulation by growth factors [1]. The role of Src in cellular growth control is further illustrated by the cell transformation caused upon transfection with v-Src, the viral counterpart of c-Src [2–5]. However, despite an extensive study, the mechanism(s) by which Src exerts its effect and transforms cells are not fully understood. The interaction of Src with other proteins is mediated by its Src homology (SH) 2 and SH3 domains. Src associates with tyrosine-phosphorylated proteins through its SH2 domain, enabling its SH3 domain to interact with downstream effectors. The SH3 domain is a 50–60 amino acid domain [6,7], found in various eukaryotic proteins [8,9]. The SH3 domain is hydrophobic in nature, and is comprised of a few highly conserved motifs. Determination of the 3-dimensional structure of a number of SH3 domains revealed that the SH3 domain is a compact β -barrel in which many of the conserved amino acid residues are located closely together on its surface to form the binding site for the ligand [8,10–12], a proline-rich sequence of about 10 amino acids [12–14].

Cellular transformation by the oncogene v-Src is accompanied by marked morphological changes and cytoskeletal reorganization; indeed, Src is found in association with the Triton

X-100-resistant cytoskeleton [15–21]. In this study we have examined the binding of the v-Src-SH3 domain to cellular proteins by utilizing a blot overlay procedure. We show that p21^{ras}-GTPase-activating protein-associated p62 (GAPA62) is one of the main targets of v-Src-SH3 and that it is enriched in detergent-insoluble fractions where it is phosphorylated on tyrosine residues.

2. Materials and methods

2.1. Materials

Dithiothreitol, glutathione, glutathione cross-linked to agarose, Triton X-100, Nonidet P-40, isopropyl β -thiogalactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were obtained from Sigma. Sodium deoxycholate was purchased from Fluka. Sodium dodecyl sulfate was obtained from BDH. Horseradish peroxidase (HRP)-linked antibodies were purchased from Zymed or Biomakor (Nes-Tsiona, Israel). Anti-GAPA62 antibody was obtained from Transduction Laboratories and anti-phosphotyrosine (α Py) was from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) reaction kit was purchased from Amersham. Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS) and supplemented antibiotics are products of Biological Industries, Kibbutz Beth HaEmek, Israel.

2.2. Cell lines and tissue culture

HT1080 cells, derived from a metastatic lesion of human fibrosarcoma, and NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC). NG108-15 cells are routinely maintained in our laboratory. V4 cells are a kind gift from Dr. L.C. Cantley (Harvard Medical School, Boston, MA, USA). All cell lines were maintained in DMEM containing 10% FCS and supplemented with 1000 U/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained at 37°C. The experiments were carried out with cells which had been serum-deprived by an overnight incubation in DMEM containing 0.1% BSA.

2.3. Expression and purification of GST and GST-fused SH3 domains

The pGEX-2T expression system (Pharmacia) was used to express SH3 domains as a fusion with glutathione S-transferase (GST) in *E. coli* DH5 α strain [22]. Overnight bacterial cultures were diluted 1:10 in LB medium. After 2 h shaking at 37°C IPTG was added to a final concentration of 0.1 mM and the incubation proceeded for 4 h. The bacteria were harvested by centrifugation (10 min at 4225 $\times$ g) and then resuspended in 1/10 volume of Ca²⁺/Mg²⁺-free PBS supplemented with 1 mM PMSF and 10 μ g/ml leupeptin. To extract the GST-SH3 fusion protein from insoluble inclusion bodies, the cells were lysed by sonication for 2.5 min and then centrifuged for 20 min at 38700 $\times$ g. In order to solubilize the included proteins, the pellet was resuspended in 4.5 ml of a buffer containing 8 M urea, 25 mM Tris-HCl pH 7.5, 1 mM DTT, and 1 mM EGTA. Renaturation of the proteins was accomplished by dialysis for 2 h against 4 M urea, 1 mM Tris-HCl pH 5.0, 1 mM DTT. Further overnight dialysis with one change was accomplished against 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM MgCl₂, 0.2 mM DTT. 0.5 ml (1/10 of the volume) of 10 $\times$ MIX solution (10% Triton X-100, 1% Tween 20, 100 mM DTT) was added, and the dialysate was cleared by an additional centrifugation for 6 min at 4300 $\times$ g. The native GST-SH3 fusion protein was purified on glutathione-agarose column (2 ml) equilibrated with 1 $\times$ MIX prior to the loading of the bacterial extract.

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Following loading and after 10 min agitation at room temperature the glutathione-agarose column was washed twice with $1 \times \text{MIX}$ in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and twice with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS. Protein was eluted with 10 ml of 5 mM glutathione in 50 mM Tris-HCl pH 8.0. Fractions of 1 ml were collected and the GST or GST-fusion proteins were located at fractions 2 and 3 by SDS polyacrylamide gel electrophoresis and immunoblot analysis with anti GST antibodies. The GST-Fyn-SH3 domain used as a control was kindly provided by Dr. R. Kappeler.

2.4. Cell fractionation and blot overlay analysis

Serum-deprived cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and lysed with lysis buffer (50 mM HEPES pH 7.0, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl_2 , 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM NaVO_4 , 1 mM PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin). Cells were scraped, incubated with the lysis buffer on ice for 5 min, centrifuged (5 min) and the Triton X-100-soluble (TS) fraction was collected. The Triton X-100-insoluble (TI) pellet was washed with lysis buffer and then solubilized with RIPA buffer ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM NaF, 10 mM Na-pyrophosphate, 1–2 mM NaVO_4 , 1 mM PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin). Following an additional centrifugation the RIPA-soluble (RS) fraction was separated from the RIPA-insoluble (RI) pellet. The RI pellet was extensively washed with RIPA and solubilized in $2 \times \text{SDS}$ sample buffer (250 mM Tris pH 6.8, 8% SDS, 40% glycerol and 20% 2-mercaptoethanol). In some experiments, the RI pellet was further solubilized by resuspension in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and a 30 s sonication, followed by centrifugation. In a typical experiment carried out with NIH 3T3 cells, the TS, RS and RI fractions comprised 56.9%, 38.6% and 4.5% of total cellular protein, respectively. Blot overlay with GST-v-Src-SH3 was carried out as follows. The TS, RS and RI fractions (3–6 μg protein/well) were separated on SDS-polyacrylamide gels, blotted onto nitrocellulose and blocked for 3 h with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS containing 0.05% Tween-20 (T-PBS) and 10% milk (homogenized, 1% fat). Next the blots were incubated for 2 h in T-PBS, 0.5% BSA, containing 0.5–1.0 $\mu\text{g}/\text{ml}$ GST-fused SH3 probes. After washing with T-PBS the blots were incubated for 1.5 h with rabbit anti-GST (R α GST) serum diluted 1:15 000 in T-PBS, 0.5% BSA, 5% milk. Blots were washed as before and then incubated for 1.5 h with HRP-linked goat anti-rabbit IgG (G α R-HRP) diluted 1:15 000 in T-PBS, 5% milk and immunoreactive bands were visualized by ECL reaction.

2.5. Western blotting

Western blotting was carried out on cell fractions prepared as described above. Following protein transfer, the nitrocellulose membranes were blocked for 3 h in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS containing 0.05% Tween-20 (T-PBS) and 10% milk. Next the blots were incubated for 2 h in T-PBS, 0.5% BSA, 5% milk, containing the first antibody at the indicated concentration. The blots were washed with T-PBS and incubated for 1.5 h with G α R-HRP or HRP-linked goat anti-mouse IgG (G α M-HRP) diluted 1:15 000 in T-PBS, 5% milk. Immunoreactive bands were visualized by ECL reaction.

2.6. Immunoprecipitation

Lysates were incubated with agarose-linked αPY overnight at 4°C and then the beads were washed three times with HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycer-

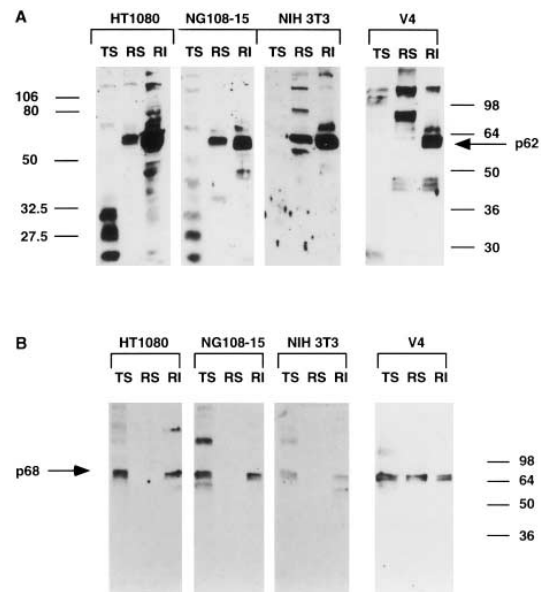


Fig. 1. GST-vSrc-SH3 binding proteins in TS, RS and RI fractions of various cell lines. TS, RS and RI fractions of the indicated cell lines were electrophoretically separated on 10% SDS-polyacrylamide gel and blotted on a nitrocellulose membrane. The ability of the blotted proteins to associate with either GST-v-Src-SH3 (A) or GST-Fyn-SH3 (B) was examined by blot overlay with these probes as described in Section 2. Association of SH3 domains was detected by incubation first with R α GST and then with G α R-HRP, and visualized by ECL.

ol). The precipitated proteins were recovered with $2 \times \text{SDS}$ sample buffer and separated on SDS gels.

3. Results

Interactions of the intracellular protein tyrosine kinase c-Src with other proteins are believed to be mediated by two modules within it, the SH2 and SH3 domains. Whereas the SH2 domain associates with phosphorylated tyrosines of substrate proteins [5,23,24], the SH3 domain binds to proline-rich sequences of target proteins [9,12–14,25–27]. In order to study the interaction of v-Src-SH3 with its targets, a 154 bp fragment (nucleotides 352–505) coding for 51 amino acids of the Schmidt-Ruppin D strain v-Src-SH3 domain [28] was expressed in fusion with GST and used to probe v-Src-SH3-binding proteins by utilizing a blot overlay procedure. To compare the pattern of SH3-binding proteins in different cells, lysates from several cell lines were analyzed with GST-v-Src-

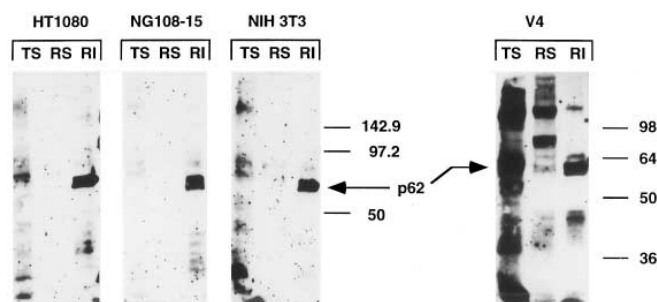


Fig. 2. Tyrosine phosphorylation of the 62 kDa protein. TS, RS and RI fractions of HT1080, NG108-15, NIH 3T3 and V4 cells were immunoblotted with αPY mAb. Immunoreactivity was detected with G α M-HRP and visualized by ECL.

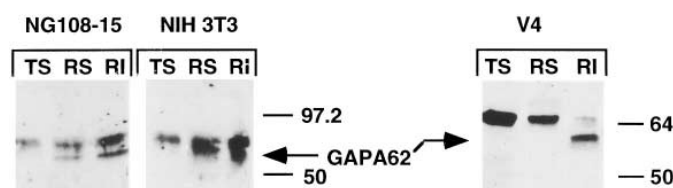


Fig. 3. Western blot analysis of cell fractions with anti-GAP62. Samples from TS, RS and RI fractions of NG108-15, NIH 3T3 and V4 cells were subjected to SDS-PAGE and then immunoblotted with anti-GAP62. Immunoreactivity was detected with G α M-HRP and visualized by ECL.

SH3 after being solubilized with lysis buffer and RIPA. Except for minor differences, the general pattern of labeling was repeated in most cells examined. The most intense labeling appeared as a doublet in the molecular weight range of 62–64 kDa in both the RS fraction and mainly in the RI fraction of NIH 3T3, NG108-15 and HT1080 cells (Fig. 1A). However, in the polyoma middle T antigen-transformed 3T3 cell line (V4), the 62 kDa protein appears only in the RI fraction. The Fyn-SH3 interacted with a limited number of bands, none of which corresponded to a 62 kDa protein, and its main target was a slightly heavier protein in the 68 kDa region (Fig. 1B). That association was much weaker than that of the Src-SH3 domains and a longer exposure was required for its detection. The p68 band is most likely to be a protein designated Sam68, which was recently reported to associate with the Fyn-SH3 [29].

The above results indicate that the 62 kDa protein is a major v-Src-SH3-binding protein and therefore it was of interest to identify and characterize it. To determine whether the 62 kDa protein is phosphorylated, cell fractions of non-stimulated NG108-15, HT1080, NIH 3T3 and V4 cells were immunoblotted with α PY antibody. As expected, in non-transformed cells the basal level of phosphorylation was relatively low, whereas in the V4 cells a high level of phosphorylation was evident. However, in all cell lines the 62–64 kDa doublet is heavily phosphorylated on tyrosine (Fig. 2). Interestingly, only the RI form was found to be phosphoryl-

ated on tyrosine residues. A similar pattern was obtained also in V4 cells in which the phosphorylation level of the 62 kDa band was higher than that of other proteins (Fig. 2).

It has been shown previously that in mitotically arrested Src-overexpressor NIH 3T3 cells there is an increase in the phosphorylation of Sam68 [29,30]. Sam68 is highly homologous to the Ras-GAP-associated p62 (GAP62) and was suggested to be an alternatively spliced form of the same mRNA [30,31]. Like the 62 kDa protein (cf. Fig. 2), Sam68 was shown to be phosphorylated while background tyrosine phosphorylation was very low. On the basis of these similarities it was decided to examine whether the 62 kDa band is GAP62. Cell fractions were immunoblotted with anti-GAP62 antibody, revealing a band that migrated to the same location as the v-Src-SH3 binding 62 kDa protein (Fig. 3).

In order to examine the possibility that the tyrosine phosphorylated protein is a 62 kDa protein other than the GAP62, an effort was made to solubilize the 62 kDa protein from the RI pellet. As shown in Fig. 4, a 30 s sonication of the RI pellet prepared from V4 cells released a 62 kDa tyrosine phosphorylated protein, which retained the capacity to bind the GST-v-Src-SH3 and was immunoreactive with anti-GAP62 (Fig. 4). Fyn-SH3 did not associate with GAP62 but bound a 68 kDa protein that cross-reacted with anti-GAP62, and is likely to be Sam68.

To determine whether the solubilized phosphorylated 62 kDa protein is also the v-Src-SH3 binding protein, tyrosine-phosphorylated proteins were immunoprecipitated from sonicated RI fraction of V4 cells with α PY. Following separation on SDS-polyacrylamide gel and blotting the recovered proteins were analyzed by blot overlay with either GST-v-Src-SH3 or GST-Fyn-SH3. The phosphorylated 62 kDa protein

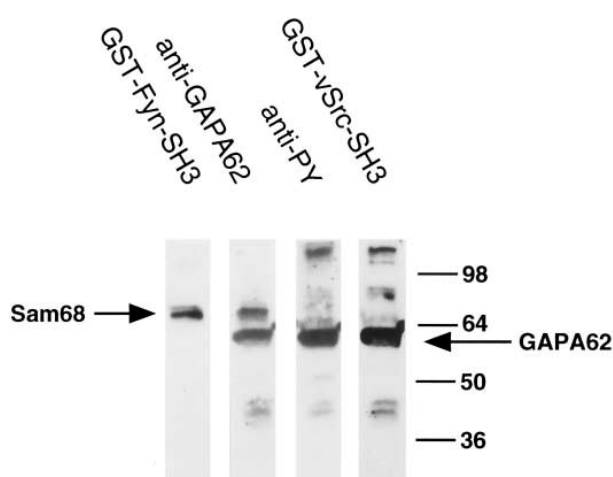


Fig. 4. Solubilization of RI proteins by sonication. RI pellet of V4 cells was resuspended in Ca^{2+} / Mg^{2+} -free PBS and then sonicated for 30 s. Following centrifugation the supernatant was subjected to SDS-PAGE and the solubilized proteins were assayed by immunoblotting with either anti-GAP62 or α PY, as well as by blot overlay with GST-v-Src-SH3 or GST-Fyn-SH3 followed by incubation with R α GST. Immunoreactive bands were visualized by ECL.

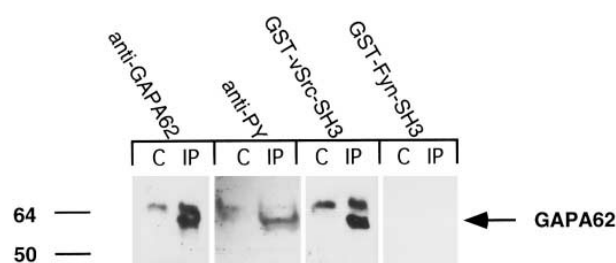


Fig. 5. Precipitation of GAP62 from solubilized RI pellet. The GAP62 from RI fraction of V4 cells was solubilized by sonication and immunoprecipitated with agarose-linked α PY. The precipitated proteins were eluted with 2 $\times$ SDS sample buffer and electrophoretically separated on SDS gels. The proteins were immunoblotted with either anti-GAP62 or α PY. Other blots were assayed by blot overlay with R α GST. Control immunoprecipitations consisted of agarose beads carrying α PY that were similarly treated with 2 $\times$ SDS sample buffer. IP, immunoprecipitates; C, controls.

has the capacity to associate with the v-Src-SH3 but not with the Fyn-SH3 (Fig. 5). Finally, the immunoprecipitate was immunoblotted with anti-GAPA62, revealing that the precipitated protein is indeed the GAPA62 (Fig. 5). This experiment took advantage of the protein being tyrosine-phosphorylated and therefore was conducted using V4 cells that express the highest tyrosine phosphorylation state (cf. Fig. 2). In other experiments, conducted on NIH 3T3 cells, GST-v-Src-SH3 immobilized on GSH-agarose was used to affinity-precipitate v-Src-SH3 binding proteins solubilized from the RI fraction by sonication. The recovered proteins were electrophoretically separated and immunoblotted with anti-GAPA62 antibody. Only one protein, a 62 kDa band, appeared to be immunoreactive (data not shown). These results strongly suggest that the 62 kDa protein is GAPA62.

4. Discussion

In order to study SH3-mediated interaction of Src, the SH3 domain of v-Src was expressed in bacteria as a fusion with GST and used to probe SH3-binding proteins. The v-Src-SH3 was found to associate with several blotted protein bands, but mainly with a 62–64 kDa doublet. Determination of phosphotyrosines revealed that the 62 kDa protein was tyrosine phosphorylated. The ability of the 62 kDa protein to associate with an SH3 domain and its phosphorylation on tyrosine residue(s) were similar to another protein, Sam68, raising the possibility that the p62 is the Sam68 homolog GAPA62. Indeed, the v-Src-SH3-binding 62 kDa protein co-migrated on SDS gels with GAPA62, and immunoprecipitation with α PY antibodies followed by immunoblotting with anti-GAPA62 or blot overlay with v-Src-SH3, identified the tyrosine phosphorylated 62 kDa band as GAPA62 and the v-Src-SH3 binding protein. Our results are in apparent contrast to other studies that failed to identify GAPA62 as a major SH3-binding protein [29–31]. Those studies were carried out on a soluble fraction of cells lysed with RIPA buffer, and binding of GAPA62 to SH3 was tested with GST-Fyn-SH3. Three factors may have prevented the detection of GAPA62 in those studies. First, as we show here, the GAPA62 does not associate with the Fyn-SH3 and therefore the Fyn-SH3 cannot be used as a probe for detection of GAPA62 or for its precipitation. Second, proteins recovered after affinity-purification with immobilized SH3 were subjected to immunoblotting with α PY [30]. It is illustrated here that the RIPA-soluble form of GAPA62 is not tyrosine-phosphorylated. Last, in non-transformed cells GAPA62 is localized to RIPA-soluble and RIPA-insoluble fractions. However, in transformed cells exhibiting high level of basal tyrosine phosphorylation, GAPA62 is restricted to the RIPA-insoluble fraction, explaining why it was not detected in RIPA-soluble fraction of Src-overexpressor NIH 3T3 cells. Thus, it may be assumed that non-transformed cells exhibited the basal pattern in which GAPA62 is located in both RIPA-soluble and RIPA-insoluble fractions. The RIPA-insoluble GAPA62 form is tyrosine-phosphorylated and it is the main form detected in transformed cells. It should be noted, however, that both phosphorylated and non-phosphorylated forms bind v-Src-SH3.

The significance of identifying GAPA62 as the major target for the v-Src-SH3 lies in its unique features. The GAPA62 contains five putative SH3-binding sites, a K homology (KH)-like domain and high tyrosine content in its C-terminal

portion [32,33]. GAPA62 was previously shown to interact with poly(U) RNA and with total cell mRNA [32,34]. It was also demonstrated to interact with various SH2- and SH3-containing proteins such as Ras-GAP, Grb2, the p85 regulatory subunit of the PI 3-kinase, PLC γ 1 and Src [33,35–39]. Tyrosine phosphorylation of GAPA62, possibly by Src, has a dual effect. It increases GAPA62 association with SH2-containing proteins [36] and decreases its interaction with RNA [31,34]. The dissociation of GAPA62 from mRNA may enable a rapid, stimulus-dependent and Src-mediated modulation of mRNA transcription and/or translation [40–42]. On the other hand, tyrosine-phosphorylated GAPA62 may have a modulatory effect on various components of signal transduction pathways with which it interacts. Furthermore, association of GAPA62 with two or more proteins may bring them into close proximity, turning GAPA62 into an adaptor protein such as Grb2 or Shc [9,43,44]. Thus, GAPA62 may represent a new family of adaptor proteins that associate with various proteins as well as mRNA and DNA, whose ligand-binding properties and the nature of its current substrate are modulated by tyrosine phosphorylation. The present study suggests an additional role for tyrosine phosphorylation of GAPA62 in controlling its subcellular localization. The RI fraction is a nuclear-enriched fraction as was detected by the exclusive presence of the A1 nuclear protein [45] in RI fraction of NIH 3T3 cells (data not shown). Therefore, it may be speculated that the target of phosphorylated GAPA62 depends on the type of tyrosine phosphorylation. While phosphorylation of certain tyrosine residues enhances the interaction with cytosolic or cytoskeletal proteins, phosphorylation of another subset of tyrosines promotes interactions with nuclear proteins. Moreover, the possible recruitment of all the GAPA62 into the nucleus of the transformed V4 cells (evinced by its exclusive localization in the RI fraction; Fig. 3) implies a role for GAPA62 in cell transformation by the middle T antigen and supposedly other oncogenes as well.

The results presented here suggest a new pathway by which a signal may be transmitted downstream of Src, by phosphorylation of GAPA62 and modulation of its interactions with other proteins and mRNA/DNA. Therefore, GAPA62 may be a mediator of the effects of Src on various intracellular events. Further study is required in order to elucidate mechanism(s) regulating Src-GAPA62 interactions and to characterize the exact role(s) of GAPA62 in mediating the physiological and pathophysiological effects of Src.

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