A Novel Group IIA Phospholipase A\textsubscript{2} Interacts with v-Src Oncoprotein from RSV-transformed Hamster Cells*

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We have isolated a novel isoform of phospholipase A\textsubscript{2}. This enzyme was designated srPLA\textsubscript{2} because it was discovered while analyzing the proteins interacting with different forms of the v-Src oncoproteins isolated from Rous sarcoma virus-transformed hamster cells. It contains all the functional regions of the PLA\textsubscript{2} group IIA proteins but differs at its C-terminal end where there is an additional stretch of 8 amino acids. The SrPLA\textsubscript{2} isoform was detected as a 17-kDa precursor in cells and as a mature 14-kDa form secreted in culture medium. A direct interaction of the 17-kDa precursor with the Src protein was observed in lysates of transformed cells. Both the 17- and 14-kDa forms were found to be phosphorylated on tyrosine. To our knowledge, this is the first report of a PLA\textsubscript{2} group II protein that is tyrosine phosphorylated. We surmise that srPLA\textsubscript{2} interacts with the Src protein at the cell membrane during the process of its maturation.

Previous work in our laboratories has focused on several transformed hamster cell lines named HET-SR, HET-SR-1, and HET-SR-8. Those lines were isolated as a result of independent infection of primary hamster fibroblasts with different stocks of Rous sarcoma virus (RSV).\textsuperscript{1} All lines displayed high EMA (experimental metastatic activity) when injected intravenously in adult hamsters, but differed remarkably in SMA (spontaneous metastatic activity) when injected subcutaneously. HET-SR-1 and HET-SR-8 cells were highly metastatic (HM) and HET-SR cells were low metastatic (LM) (1). Each line has one integrated RSV provirus and expresses the Src-specific tyrosine kinase (2, 3). Those lines secrete prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), which inactivates the cytotoxic activity of NK cells (1). The production of PGE\textsubscript{2} is in strict correlation with v-src gene expression, with tumor growth, and with the EMA potential of the transformed cells (3, 4).

src genes from both cell lines were cloned and sequenced. It was found that v-src genes in both HM and LM cells had significant structural changes that were not previously observed in other members of the src family. The most interesting feature was that v-SrcHM and v-SrcLM differed from each other by several amino acids. Cells transformed with retroviral vectors carrying srcHM and srcLM display differences in morphology and in vivo metastatic activities, and we showed that structural changes in the C-terminal region of the v-src proteins account for these differences in the metastatic properties of the corresponding transformed cells (5).

To assess the molecular mechanisms underlying the v-srcHM-dependent high metastatic activity (or lack of such an activity) in v-srcLM-transformed cells, we tried to identify the products of cellular genes associated with the unique C-terminal regions of both v-Src variants, using a two-hybrid approach and a cDNA library made from low metastatic parental hamster fibroblasts. Several proteins interacting with v-Src via its C-terminal fragments were identified that were not characterized previously as v-Src protein partners.

We describe here one of these proteins related to the IIA group of phospholipase A\textsubscript{2} enzymes, srPLA\textsubscript{2} (Src-associated phospholipase A\textsubscript{2}). This protein contains all the conserved functional residues typical for IIA PLA\textsubscript{2} enzymes and, in addition, 8 amino acids at its C terminus. In vitro, srPLA\textsubscript{2} protein interacts with v-SrcHM as well as with v-SrcLM oncoproteins. The 17-kDa precursor version of srPLA\textsubscript{2} was identified by co-immunoprecipitation in lysates of RSV-transformed cells in a complex with the v-Src protein; it was found to be phosphorylated on a tyrosine.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Spontaneously transformed Syrian hamster fibroblasts and Syrian hamster embryo fibroblasts transformed independently in vitro by different stocks of RSV SR-D (from the Russian Cancer Research Center viral collection) were used (1). RSV-transformed lines had a typical transformed phenotype, were highly tumorigenic in syngenic hamsters, but differed in their SMA levels: HET-SR cells, low metastatic cell line; HET-SR-1 and HET-SR-8, high metastatic cell lines; HET-SR-28C, high metastatic line selected from metastatic nodules in vivo. Transfectants HET-SR-N-Ras c.9 and HET-SR-N-Ras c.34 lines were obtained by supertransfection of HET-SR and HET-SR-28C, respectively, with an activated N-ras gene mutated in the 12th codon (Gly→Asp). Transfection of the cells by the N-ras oncogene was shown to induce the suppression of v-Src activity, coinciding with the decrease of prostaglandin secretion (4, 6). NIH3T3-c-Src cells expressing the c-src gene were obtained after NIH3T3 cell transfection with a vector containing avian c-src (7). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 μg/ml gentamycin, and 2 mM glutamine in the presence of 5% CO\textsubscript{2} at 37 °C.

**Hamster cDNA Fusion Library**—mRNA was isolated from Syrian hamster fibroblasts HET-SR cell line using an RNA extraction kit.
Hybridization was performed using /H9251 with glyceraldehyde-3-phosphate dehydrogenase end-labeled with either anti-Src polyclonal Ab (Santa Cruz Biotechnology) or srPLA2 by a filter were selected for histidine prototrophy and tested for LacZ expression. Yeast growth and two-hybrid procedures were handled according to standard protocols (9, 10, 11). Double transformants were selected for histidine prototrophy and tested for LacZ expression by a filter /H9253

Sequencing of the Hamster cDNA Library—Several plasmids containing the coding sequence of different parts of v-SrcHM and v-SrcLM, inserted in frame with the LexA DNA-binding domain coding sequence, were constructed in the bact plasmid pVJL1 (a derivative of pBTM116 with a modified polylinker): pLexA-v-SrcHM, pLexA-v-SrcLM, pLexA-v-SrcHMN (corresponding to the N-terminal part of v-SrcHM: amino acids 1–521) and pLexA-v-SrcHMC (C-terminal part of v-SrcHM: amino acids 522–546) and pLexA-v-SrcLHM (C-terminal part of v-SrcLM). The hamster cDNA library was screened as described (8).

The Saccharomyces cerevisiae L40 reporter strain (Mat a his 3D200 leu2–3, 112 ade 2 LYS2::lexAop–URA3::lexAop–lacZ GAL4) was used. Yeast growth and two-hybrid procedures were handled according to manufacturer’s instructions. The expressed proteins were extracted from bacteria by sonication in modified lysis buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM PMSF) and purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to manufacturer’s procedure. Purity and integrity of GST fusion proteins were assessed using SDS–PAGE and Coomassie Blue staining on gels. For in vitro binding studies, 2 mg of protein extracts, obtained as described above, were incubated with glutathione-Sepharose 4B-bound proteins GST-srPLA2, or GST in binding buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.3 mM Na2VO4, 0.02% NaN3, 1 mg/ml aprotinin, 1% ovalbumin (18). Following four washes in lysis buffer and boiling in SDS sample buffer, eluted proteins were separated by SDS–PAGE.

Measurement of Phospholipase A2 Activity—PLA2 activity was assayed directly in the beads prepared as indicated above. Briefly, the beads were resuspended in the reaction mixture containing 140 mM NaCl, 30 mM KCl, 20 mM ATP, and 20 mM HEPES pH 7.5. Lipid kinase assays were initiated by the addition of the mixture of 10 μM phosphatidylcholine in TIFF stock with 0.5 μCi/ml 32P-ATP (Amersham Pharmacia Biotech). Reaction mixtures were incubated at room temperature for 40 min, then lipids were extracted by a chloroform/methanol mixture (65:35:6:3). The solvent was run up to 2 cm from the top of the plate. Plates were air dried, fixed, and autoradiographed to detect labeled lipids. Lipids were located by autoradiography or visualized with iodine vapor.

Results

Two-hybrid Screening—A two-hybrid system was used to search for novel cellular proteins associated with the v-SrcHM and v-SrcLM (19). Full-length v-SrcHM and v-SrcLM and their C-terminal parts (v-SrcHMC and v-SrcLMC) were used as baits to screen the hamster cDNA library (8). The majority of the specific clones contained DNA-encoding proteins not known as v-Src partners. Here we present detailed analysis of one gene identified as a result of screening with v-SrcHMC.

The ability of this clone to interact with two different regions of Src proteins v-SrcHMN (with the HMC region), v-SrcHMC, and v-SrcLMC was assessed by a two-hybrid test. The specific plasmid was introduced into yeast cells in the presence of pLexA-v-SrcHMN, pLexA-v-SrcHMC, pLexA-v-SrcLHM, or pLexA-lamin (negative control), and /H9254

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Sequence Analysis of the srPLA₂ Gene and Protein—The cloned cDNA was sequenced. A search in the GenBank data base revealed a homology with the 3’ parts of different members of the phospholipase A₂ group II genes. The missing 5’ section of the gene (150 nucleotides of coding region) was obtained as a result of polymerase chain reaction, using cDNA library and primers corresponding to the pGAD3S2X section of the gene (150 nucleotides of coding region) was observed with the first 394 coding nucleotides of the rat PLA₂ gene from the IIA group of phospholipases A₂ (21). The homology observed between these two proteins was less obvious than the homology found at the DNA level (Fig. 1B).

Comparison of the srPLA₂ protein structure with amino acid sequences of other known members of group II phospholipases A₂, including IIA (21), IIB (22), IIC (23), IID (24), IIE (25), and IIF (26) confirmed that this protein is related to the group IIA enzymes.

The protein encoded by the newly identified gene contains essential structural characteristics typical of the PLA₂ group II family of proteins: a signal peptide with a potential transmembrane helix (positions 1–21); the PLA₂ histidine (63–71), and aspartic acid (109–119) in the active enzyme site; the calcium binding loop (45–52) and all conserved 14 cysteine residues typical for this group of proteins (Fig. 1A and B). The amino acid differences are located in almost all regions of the protein except in the functional zones situated in the middle of the protein (Fig. 1B). The main difference between srPLA₂ and the majority of the other known PLA₂ proteins (with the exception of group IIF) is the existence of an additional 8-amino acid fragment at the C-terminal end of the hamster protein (Fig. 1B). This peptide appears as a result of changes in the last exon of srPLA₂ encoding DNA in comparison with PLA₂ IIA from phylogenetically related rodent cells. In the hamster cDNA, the stop codon present in the rat and mouse PLA₂ genes is missing, and the gene is terminated by a TAG codon localized further downstream. These structural changes were identified in all tested Syrian hamster DNAs isolated from different cell lines and animals (data not shown). Despite differences in the structure, our data strongly suggest that this novel srPLA₂ is a member of the so-called “secreted” PLA₂ gene family and, most probably, belongs to the IIA group of phospholipases A₂.

In Vitro Protein Binding—To further demonstrate the interaction existing between srPLA₂ and v-Src, binding ability was tested in vitro. A fragment of srPLA₂ (srPLA₂(50–154)) iso-

### Table I

| Binding domain | Activation domain | β-galactosidase activity (arbitrary units) |
|----------------|-------------------|----------------------------------------|
| pLexA-lamin    | pGAD-srPLA₂       | 0.000                                  |
| pLexA-Ras      | pGAD-Raf          | 1.000                                  |
| pLexA-v-srcHMC  | pGAD               | 0.000                                  |
| pLexA-v-srcLMC  | pGAD               | 0.000                                  |
| pLexA-v-srcHMM  | pGAD-srPLA₂       | 0.250                                  |
| pLexA-v-srcLMC  | pGAD-srPLA₂       | 0.250                                  |

Yeast two-hybrid analysis of srPLA₂-src interaction

N- and C-terminal parts of v-SrcHM fusion to LexA and srPLA₂ fusion to pGAD were co-expressed in yeast strain L40. β-galactosidase activity reflecting proteins interaction was measured by a quantitative β-galactosidase solution assay. Yeast co-transformed with pLexA-lamin and pGAD-srPLA₂ were used as negative controls; yeast co-transformed with pLexA-Ras and pGAD-Raf were used as positive controls, and the data were normalized according to this interaction arbitrarily considered as a standard (value 1).
labeled by two-hybrid screening was expressed as a GST fusion protein and prepared from E. coli. Protein extracts from low metastatic HET-SR and high metastatic HET-SR-8 RSV-transformed cell lines were incubated with purified GST-srPLA2 or GST proteins bound to the glutathione-Sepharose 4B beads, and retained proteins were analyzed by SDS-PAGE; mAbs against c-Src/v-Src were used for immunodetection. Previously, it was found that the proteins of both the HM and LM v-Src isoforms had a molecular mass of 62 kDa. This unusual molecular size (62 kDa instead of 60 kDa) is due to an insertion of 20 additional amino acids in the unique domain of the oncoproteins (5). Fig. 2A shows approximately the same level of v-Src and c-Src proteins in RSV-transformed cells, independent of their metastatic activity (lanes 1 and 2). In both cell lines, GST-srPLA2 binds with the v-Src protein (Fig. 2A, lanes 5 and 6), whereas GST does not (Fig. 2A, lanes 3 and 4).

The truncated srPLA2, isolated by two-hybrid screening, lacked the first amino acids that constitute the N-terminal α-helix and the calcium-binding loop, both regions important structurally and functionally. To test the ability of full-length srPLA2 to interact with the Src protein, GST-full-length srPLA2 fusion protein was used in protein binding assay in vitro. Fig. 2B shows that the fusion protein carrying full-length srPLA2 protein also effectively binds v-Src protein from transformed cells.

The phospholipase activity of bacterially expressed GST-srPLA2 protein was measured. Fig. 3 demonstrates that the purified bacterial fusion protein carrying full-length srPLA2 stimulates the formation of arachidonic acid after incubation with phosphatidylcholine substrate: i.e. this fusion protein preserves its enzymatic function. According to quantitative evaluation, 1 mg of total bacterial protein contains approximately 20 units of PLA2 activity.

In RSV-transformed hamster cells expressing v-Src, no interaction between the c-Src protein and srPLA2 was found (Fig. 2A, lanes 5 and 6). The interaction of the fusion GST-srPLA2 with c-Src proteins from cells not expressing v-Src, was analyzed (Fig. 2C). NIH3T3 cells overproducing avian c-Src protein and RSV-transformed hamster cells with depressed v-Src gene transcription (HET-SR-N-ras c34) were compared (Fig. 2C, lanes 1 and 2). In cell lines overproducing exogenous avian c-Src, a minimal amount of p60 was found to be associated with srPLA2 (Fig. 2C, lane 5). The binding of p60csrc with GST-srPLA2, from HET-SR-N-ras c34 cells producing only the c-Src protein was not observed (Fig. 2C, lane 6). The interaction between GST-srPLA2 and c-Src from spontaneously transformed hamster fibroblasts (STHE) producing high level of Src kinase activity was also observed (data not shown). The protein binding data suggests that both v-Src and c-Src proteins can potentially interact with the srPLA2 protein, but the affinity of the latter is significantly higher for the viral oncoprotein.

srPLA2 Expression in RSV-transformed Cells—To detect the srPLA2 protein and its interaction with Src proteins in cells, rabbit anti-srPLA2 serum elicited against a peptide in the C-terminal part of the protein (amino acids 131–145) was prepared. The chosen peptide includes the residues from the amino acid extension at the C terminus and shares less homology with the same region of other known phospholipases (Fig. 1B).

This family of enzymes is referred as “secreted PLAs” (26). We compared the srPLA2 protein in HET-SR culture medium and in cell lysates. The molecular mass of srPLA2 in cell lysates corresponds to the precursor forms of the PLA2 II group of proteins (about 17 kDa) (Fig. 4A, lane 5). The extracellular protein has a molecular mass of 14 kDa corresponding to the mature form of PLA2 proteins without the N-terminal signal peptide (Fig. 4A, lane 4). Total cell lysate and culture medium (M) immunoprecipitated with normal rabbit antiserum were used as negative controls (Fig. 4A, lanes 1 and 2). The immunodetection by anti-srPLA2 antibodies was specific because incubation of the serum with the antigenic peptide precluded detection of the 17-kDa band (Fig. 4B). The absence of detectable 14-kDa srPLA2 in the total cell lysate blot was probably due to low amounts of this protein in cytosolic fractions (Fig. 4A, lane 5).

A direct association between the v-Src protein and the precursor form of srPLA2 in transformed cells was detected. After immunoprecipitation of cell lysates with anti-Src antibodies, a protein (17 kDa) recognized by anti-srPLA2 antibodies was observed (Fig. 4A, lane 3).

The v-Src protein contains tyrosine kinase activity. We surmised that the interaction between srPLA2 and Src could bring about phosphorylation of srPLA2 on tyrosine. To examine this hypothesis, the blots reacting with anti-srPLA2 anti-serum were stripped and reprobed with the anti-phosphotyrosine monoclonal antibody PY20. Fig. 4C shows that both 17- (Fig. 4C, lane 3) and 14-kDa forms (Fig. 4C, lane 4) are phosphorylated on tyrosine. The phosphorylation of srPLA2 protein was confirmed using the reciprocal procedures: the total phosphotyrosine-containing proteins were precipitated from culture medium and cell lysates by anti-phosphotyrosine antibodies and tested by anti-srPLA2 serum. Among the tyrosine-phosphorylated proteins, the 17- and 14-kDa bands were identified in culture medium and cell lysates, correspondingly (Fig. 4D, lanes 1 and 2). A complex formed between the v-Src protein and the tyrosine-phosphorylated 17-kDa form of srPLA2 was also detected in lysates of high metastatic cell lines (data not shown).

The steady-state level of srPLA2-specific RNA was analyzed.
in high and low metastatic cell lines. A single 0.8-kilobase RNA transcript was identified in cell cultures expressing the v-src gene. In cells with a depressed v-src gene expression, the amount of srPLA2-specific messenger was decreased (Fig. 5). In normal hamster embryo fibroblasts, this RNA was practically undetectable (data not shown).

**DISCUSSION**

By analyzing the protein partners of various isoforms of the v-Src oncoprotein (v-SrcLM and v-SrcHM), we identified a novel gene encoding a low molecular weight phospholipase A2. This gene (srPLA2) has a high degree of homology with the group II PLA2 genes from different species, including rodent, human, and viper.

The nearest evolutionary rat IIA group PLA2 exhibits 70% amino acid sequence identity with srPLA2. srPLA2 contains all the functional areas of the known group II PLA2 including the signal peptide region, histidine-aspartate active sites, the calcium binding loop, and the 14 conserved cysteine residues involved in the disulfide bonds (Fig. 1, A and B). As in the other members of group II, srPLA2 contains amino acids localized identically in PLA2 from groups IB and V (27). No homology was observed with PLA2 from different groups in the C-terminal part of the srPLA2 protein (Fig. 1B). The most unusual structural alteration in srPLA2 was the presence of an additional eight amino acid residues at the C-terminal end of the protein. The function exerted by these amino acids remains, at present, unclear.

We show here that there is a direct interaction of this novel srPLA2 with the v-Src oncoprotein. This was shown by different methodological approaches in vitro as well as in vivo: (i) by using the yeast two hybrid system (ii) by the association of a GST-srPLA2 fusion protein with the v-Src protein in lysates of transformed cells, and (iii) by using antibodies prepared against srPLA2: the complex between v-Src and srPLA2 proteins could be immunoprecipitated from cell lysates. srPLA2 was detected as a 17-kDa precursor in the cells and as a mature 14-kDa form secreted into the culture medium. According to the molecular weight of srPLA2 in the complex, it is suggested that the target of the Src protein could be the cellular 17-kDa precursor form of srPLA2. We also showed that both the 17- and...
the 14-kDa proteins, identified by Ab srPLA2, respectively in RSV-transformed cells lysates and in culture medium, were phosphorylated on tyrosine.

Although v-srcHM and v-srcLM present structural changes in their C-terminal region, we observed no difference in their interaction with srPLA2, which appears, therefore, to recognize both isoforms irrespective of their metastatic potential.

According to the NetPhos 2.0 protein phosphorylation prediction program (28), tyrosine 91 in the srPLA2 protein is the potential site for phosphorylation. There is no direct evidence, as yet, that this protein is phosphorylated as a result of Src tyrosine kinase activity because none of the tyrosines in srPLA2 (including Tyr-91) are in a known preferred environment for phosphorylation by the Src tyrosine kinase (29). Structure preferences for tyrosine kinases, however, are weakly pronounced (30). Considering that a 17-kDa srPLA2 was observed in complexes with the v-Src product, we believe that Src could be involved in srPLA2 phosphorylation.

According to our preliminary indirect immunofluorescence imaging, srPLA2 is localized in the endoplasmic reticulum (ER) and is presented on the surface of transformed cells. We speculate that in the process of maturation, the srPLA2 precursor interacts with the Src protein at the cell membrane; thereafter, the mature form of tyrosine phosphorylated srPLA2 is secreted. How srPLA2, which is stored in either granules or ER, can interact with v-Src at the plasma membrane, and how it affects the enzyme secretion is questionable. It was shown that induction of growth factor independence and granulocyte-macrophage colony-stimulating factor secretion by the v-Src oncogene does not require membrane association of pp60v-src (31). Therefore, v-Src functions may not be dependent on membrane localization. Whether the interaction of srPLA2 with Src protein is specific only for RSV-transformed v-Src-producing cells or plays a general role in the regulation of group IIA PLA2 remains to be determined.

The initial aim of the present research was to use a unique cell system model to detect cellular genes that could possibly play a role in the acquisition (or loss) of the metastatic activity of transformed cells. The identification of srPLA2, related to the phospholipase A2 family, interacting with Src as a potential target protein, was an inadvertent finding. Phospholipases A2 (phosphatidylcholine 2-acylhydrolases; EC 3.1.1.4) catalyze the hydrolysis of the sn-2 ester bond in phospholipids, generating free fatty acids, such as arachidonic acid and lysophospholipids (32, 33). Arachidonic acid is the key substrate for the synthesis of potent lipid mediators of inflammation (e.g. prostaglandin, leukotrienes, etc.). Group IIA PLA2 has been proposed to play a role in many pathological conditions, such as rheumatoid arthritis (34), septic shock (35), pancreatitis (36), and psoriasis (37) that are mediated by arachidonic acid release.

Several reports demonstrate that PLA2 are probably also involved in the processes of malignant progression of the transformed cell. (i) The basal PLA2 activity was found to be increased in rodent fibroblasts transformed by membrane-associated oncogenes (38). (ii) The Pla2 g2a gene, encoding group IIA PLA2, has been implicated in colon cancer. The “murine intestinal neoplasia” or min1 gene is the murine homologue of the APC gene, mutated in human familial adenomatosis polyposis, a hereditary form of colon cancer. It is worth noting that, recently, it was shown that c-Src-activating mutations in the regulatory C-terminal end region of the protein are observed in 12% of advanced human colon cancer cases. This change in c-Src is activating, transforming, tumorigenic, and promotes metastasis (39). (iii) specific antisense oligonucleotide ASGII blocked the appearance of a heparin-releasable group IIA PLA2 in the culture supernatants of macrophage-like P388D1 cells. The disappearance of this protein correlated with reduced prostaglandin E2 production by activated cells, indicating that an extracellular pool of group IIA PLA2 is involved in prostaglandin production by P388D1 cells (40). In the treat-
ment of NIH3T3, fibrosarcoma, and sarcoma, but not lymphoma or mastocytoma cells, with both porcine pancreatic and Naja naja phospholipases A₂ causes these cells to dramatically invade the extracellular matrix in vitro in a dose-dependent manner (41).

As it was mentioned above, RSV-transformed hamster cells are secreting PGF₂α, which inactivates the cytotoxic activity of NK cells (1). Among normal cells, the same phenotype is expressed in activated macrophages. The significant decrease of v-src gene expression correlates, in turn, with the complete suppression of PGF₂α secretion as well as with other properties related to resistance of cells to NK lymphocytes and macrophages. According to preliminary data, the production of sr-PLA₂ was lower in cells not expressing active Src and PGF₂α.

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