The Regulatory Region of Prague C v-Src Inhibits the Activity of the Schmidt-Ruppin A v-Src Kinase Domain

(v-*src* / Schmidt-Ruppin A strain / Prague C strain / protein tyrosine kinase / *Saccharomyces cerevisiae*)

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Abstract. Existing variants of the oncogene v-*src* differ in their transforming potential as well as in the range of their hosts. We compared the protein kinase activities of two Prague C v-Src variants (PRC and H19), reported to be of low oncogenic potential (Plachý et al., 1995), with the highly oncogenic Schmidt-Ruppin A v-Src (SRA). We employed *in vitro* kinase assays of affinity-purified proteins expressed in rabbit reticulocyte lysate and in *S. cerevisiae*. In both systems used, the specific kinase activity of the Prague C v-Src kinases amounted to only ca 20% of the activity of SRA. This positions the PRC Src close to activated c-Src, despite the lack of the regulatory C-terminal tail in PRC. We constructed chimeras between PRC and SRA v-Src and tested them for specific kinase activity in *S. cerevisiae*. Remarkably, the regulatory N-terminal part of PRC, when fused to the SRA-derived kinase domain, lowered the chimeras' PK activity to ca 20%, suggesting that it is the regulatory part of PRC that is responsible for its low phosphotransferase activity.

The gene *src* gave rise within the Rous sarcoma virus genome to an oncogene, which encodes a truncated form of the non-receptor tyrosine kinase c-Src. Remarkably, the truncation of the last 19 amino acids, ablating the SH2 domain binding site, is not the only difference between c- and v-Src. Additional mutations of the kinase domain and elsewhere in the molecule exist and there are several v-*src* genes, isolated from various viral strains, e.g. Prague C (PRC), H19, and Schmidt-Ruppin A (SRA) (Svoboda, 1958; Schmidt-Ruppin, 1964; Svoboda et al., 1983). These and other v-Src variants show remarkable host range dependency in their transforming potential in mammalian or avian cells (Deichman et al., 1989; Liebl et al., 1992), which may reflect the differences in their kinase activities as well as in the repertory of partners they bind.

The kinase activity of c-Src was found to be under complex control by a multitude of diverse cues such as mitotic or cell survival stimuli, growth factor and G-protein-mediated signalling, cell adherence, or oxidative stress (for review see Thomas and Brugge, 1997). The interactions within the Src molecule of SH2 and SH3 domains and at least two other regulatory regions (C-terminal tail and SH2-kinase linker (Moarefi et al., 1997)) are selectively displaced or modulated by various Src targets, including Cas, FAK, EGFR, Sam68, or TRAF6. This leads to changes in the activity and specificity of the kinase domain (Williams et al., 1998). The structure of c-Src (Xu et al., 1997) provided the explanation of some of the regulations of the kinase and biological activity of c-Src by ligands. The regulatory effects of the Src partners on the kinase and vice versa have not been, however, exhaustively explained.

In contrast to promoting proliferation in vertebrate cells, expression in *Saccharomyces cerevisiae* of v-Src as well as c-Src inhibits cell growth (Murphy et al., 1993). This effect depends on the integrity of both the kinase and SH2 domain of Src (Trager and Martin, 1997). The arrested cells display aberrant microtubule arrays and possess an elevated Cdc28 kinase activity, suggesting abnormalities in mitosis (Boschelli et al., 1993).

Here we report for the first time the comparison of kinase activities of H19 v-Src (H19) and PRC v-Src (PRC) with the v-Src of the commonly studied SRA strain (SRA). Chimeric proteins between PRC and SRA were prepared to find whether the regulatory region or the kinase domain itself was responsible for the remarkably low phosphotransferase activity in PRC.

Material and Methods

Cloning and expression of v-src

The H19 variant of v-*src* was obtained from pUC19H19 (Svoboda et al., 1983), the kind gift of Prof. J. Svoboda (Inst. Molecular Genetics, Prague), the PRC variant was from pATV-8 (Katz et al., 1982; ATCC), and the SRA variant and the kinase-null mutant (SRM;

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Abbreviations: H19 v-Src – derived from the H-19 isolate of RSV, NP-40 – Nonidet P-40, PK – protein kinase, PRC v-Src – derived from the Prague C strain of RSV, RSV – Rous sarcoma virus, SRA v-Src – derived from the Schmidt-Ruppin A strain of RSV, SRM – kinase-inactive mutant of v-Src derived from Schmidt-Ruppin A.

K295M) were kindly provided by Prof. G. S. Martin (University of California, USA). The original v-*src* DNAs were PCR-amplified (5'-gtcggatccatgggtagtag-caagagcaagc-3' and 5'-gccgaattcttactcagcgacctccaacac-3'), inserted between *Bam*HI and *Eco*RI sites of pBluescript II KS(+) (Stratagene, La Jolla, CA), and verified by sequencing. The pBluescript constructs served as templates for the *in vitro* translation experiments. For the expression in *S. cerevisiae* the fragments were inserted into pYES2 (Invitrogen, Carlsbad, CA).

We constructed chimeras between the PRC variant and the SRA variant of v-*src*. Chimeric genes PRC-ch1 and SRA-ch1 were prepared by replacement of the *Bam*HI/*Stu*I fragment of PRC, corresponding to the N-terminal regulatory part of the molecule (amino acids 1–304), with a *Bam*HI/*Stu*I fragment of SRA and vice versa. Chimeras SRA-ch2 and PRC-ch2 were obtained by reciprocal exchange of *Sph*I/*Kpn*I fragments of the catalytic domain (amino acids 480–526).

The constructs were transformed into *S. cerevisiae* strain EGY48 by the standard Li-acetate method (Schiestl and Gietz, 1989), and into *Escherichia coli* by electroporation (Dower et al., 1988).

Preparation, immunoprecipitation, and in vitro PK assays of v-Src

Reticulocyte lysate: The TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI) was used to prepare Src proteins *in vitro*; labelling was performed by [^{35}S]methionine (Amersham Pharmacia Biotech, Piscataway, CA; 10 μCi/reaction). Reaction mixtures (1 μg DNA, 12.5 μl TNT lysate) were incubated for 60 min at 30°C and then diluted 10 times in LB1 (50 mM Hepes (pH 7.4), 5% glycerol, 100 mM sodium chloride, protease inhibitors (PI; 0.5 mg/ml Pefabloc, 5 μg/ml leupeptin, 5 μg/ml aprotinin), and phosphatase inhibitors (PhI; 1 mM sodium orthovanadate, 100 μM sodium molybdate, 1 mM NaF, 20 μM phenylarsinoxide) + 0.5% Nonidet P-40 (NP-40) for immunoprecipitation.

S. cerevisiae: Transformants were grown overnight in uracil-free SD medium (Difco, Detroit, MI) supplemented with 2% raffinose, pelleted by centrifugation, and transferred for an additional 4 h to a fresh medium containing 2% raffinose and 2% galactose. Cells were harvested by centrifugation, washed in LB1 with 0.5% NP-40, and vortexed for 5 min at 4°C with glass beads. The cell extracts were clarified by centrifugation at 15 000 *g* for 15 min.

Protein kinase assays were performed with purified Src kinases. Cell lysates were incubated with 1 μg/ml of anti-v-Src mAb327 monoclonal antibody (Calbiochem, San Diego, CA) for 3 h at 4°C. Antigen-antibody complexes were allowed to bind for 1 h at 4°C to anti-mouse-IgG1 agarose with gentle mixing (A3665; Sigma, St. Louis, MN). The affinity matrix was washed 3 times with >50 volumes of the LB1 + 0.5 % NP-40 and once with 50 mM Hepes (pH 7.4). Src protein levels were quantified using mAb327 and densitometry scanning. Kinase activities were measured as incorporation of [γ-^{32}P]ATP into a synthetic substrate (poly(Glu-Ala-Tyr); Sigma, St. Louis, MN) according to the method of Brown (Brown and Gordon, 1984). Immunoprecipitates were incubated for 20 min at 30°C in a kinase buffer consisting of 50 mM Hepes (pH 7.4), 8 mM $MgCl_2$, 2 mM $MnCl_2$, 100 μM Na_3VO_4, 10 μM ATP, PI, 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech, USA), and 10 μg of the substrate. The reaction mixtures were analysed on SDS-PAGE, and the levels of the phosphorylated substrate were quantified by determining the ^{32}P-radioactivity in excised gel bands or by densitometry scanning of the autoradiograms. Kinase activities were normalized with respect to the amount of the Src protein. Total cellular phosphotyrosine levels were determined by using the anti-phosphotyrosine antibody (PY20; Calbiochem).

Viability assays in S. cerevisiae

The overnight cultures grown in raffinose-supplemented SD medium lacking uracil were diluted to $O.D._{600nm} = 0.05$ in a fresh medium containing 2% raffinose and 2% galactose, and shaken at 200 rpm at 30°C. Aliquots were taken at various times, optical density was determined, and cells were plated in two dilutions onto glucose plates in triplicates.

Results

Comparison of the kinase activities of Prague C and Schmidt-Ruppin A v-Src variants

We aimed to compare within one assay the phosphotransferase activities of SRA and PRC variants of v-Src, which differ as regards the efficiency with which they induce oncogenic transformation (see Discussion). The rabbit reticulocyte lysate-coupled transcription-translation system was employed to produce the kinases, and the assays were performed with immunopurified proteins and the $(GluAlaTyr)_n$ polypeptide as a substrate. The translation in the rabbit reticulocyte lysate provided folding and posttranslational conditions close to the situation in mammalian or avian cells (Bachand and Autexier, 2001). Normalized kinase activities of *in vitro* translated H19 and PRC were found to be significantly less, 14% and 11%, respectively, than the activity of SRA (Fig. 1). Nevertheless, the H19 and PRC clones used in this work (LTR containing pUC19H19 and pATV-8) were tested positive for the induction of growth of chicken embryo fibroblasts in soft agar (data not shown). These values make the Prague C variants of v-Src similar to the activated (or partially activated) c-Src (Kornbluth et al., 1987) and would explain the earlier observations of Plachý (Plachý et al., 1995).

Expression of v-Src variants in S. cerevisiae

S. cerevisiae was used successfully for inducible production of Src, although the v-Src kinases interfered with cell-cycle progression once they have accumulated in cells. The levels of background activity are very low in the preparations of *S. cerevisiae*-expressed v-Src as the yeast lacks canonical tyrosine kinases and SH2-domain-mediated signalling of its own (Superti-Furga et al., 1996). The *S. cerevisiae* system was used here to produce v-Src kinases for comparison with the *in vitro* translated proteins. Galactose-inducible expression of the variants was achieved from the plasmid pYES2 in the strain EGY48 to obtain high and comparable levels of the Src protein (Fig. 2A). Similarly to the *in vitro* translated kinases, the normalized activities of H19 and PRC were 21% and 19% of the activity of SRA, respectively (the average from 4 independent experiments). Strains carrying the *src* inserts, with the exception of kinase-inactive SRM, ceased to grow when repeatedly streaked out on a solid medium containing the inductor (Fig. 2B). To quantify the retardation effects, the growth rates were measured (Fig. 2C) and aliquots were taken every 3 h for determination of cell viability. H19, PRC, and SRA all retarded growth to a similar extent and reduced the cell viability to ca 50%. Reinoculation of the cells from the stationary phase of the experiment in Fig. 2C (time 29 h) into a fresh inducing medium resulted in the growth arrest of Src-expressing cells (not shown). Consistent with this observation is the finding that there was no significant difference in the amount and pattern of tyrosine-phosphorylated proteins in the lysates from H19, PRC, or SRA transformants (Fig. 3).

The regulatory region of PRC v-Src confers low kinase activity when fused to the SRA kinase domain

We prepared a set of chimeras to assess the regions of v-Src responsible for the differences in the kinase activity between PRC and SRA (Fig. 4). We took advantage of the *StuI* site at position 914 of *src* (see Fig. 4) to swap their regulatory regions (containing SH2 and SH3 domains and the unique and amino-terminal regions). All chimeric open reading frames were cloned into pYES2 and expressed in the *S. cerevisiae* EGY48 strain at comparable levels. Immunoprecipitated chimeric proteins were tested for kinase activity and compared to wild-type (wt) controls. Notably, SRA-ch1, which contains the regulatory part of PRC and the kinase domain of SRA, has almost as low a kinase activity as PRC (23% of SRA in Fig. 4). Apparently, the regulatory regions of PRC can down-regulate the kinase activity of the SRA kinase domain. In addition, the activity of the PRC kinase domain was raised considerably (60% of SRA) when it was part of the chimera PRC-ch1. The chimera PRC-ch2, containing the C-terminal 46 amino acids from SRA, had nearly the same activity as PRC.

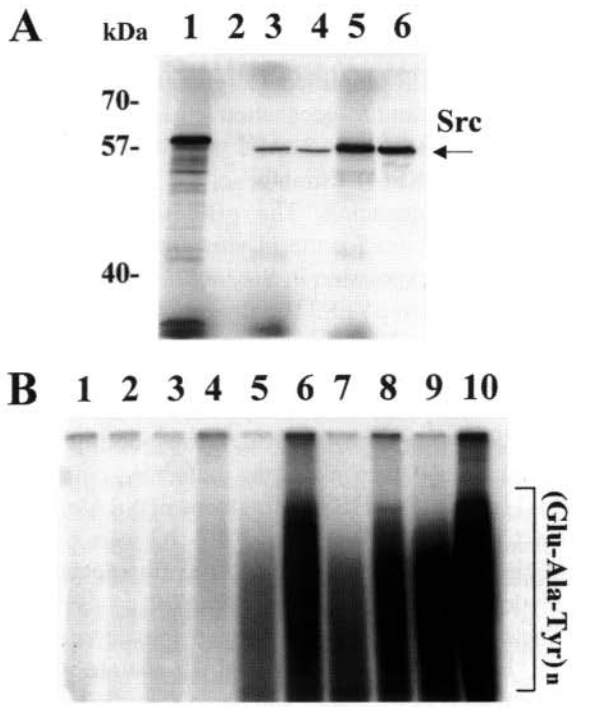

Fig. 1. The comparison of the kinase activities of purified Prague C and Schmidt Ruppin A v-Src variants expressed in the reticulocyte lysate-coupled transcription-translation system

The autoradiograph shows the production and immunoprecipitation of [^{35}S]-methionine-labelled Src proteins using the rabbit reticulocyte lysate system (A). Lanes 1, 2: luciferase T7 control DNA; lanes 3, 4: SRA in pBluescript II KS(+); lanes 5, 6: H19 in pBluescript II KS(+); lanes 1, 3, 5: reaction mixtures; lanes 2, 4, 6: the reaction mixtures were diluted 1 : 10 and the Src proteins were immunoprecipitated with mAb327.

The protein tyrosine kinase assay of *in vitro* translated Src variants (B) was carried out as described in Methods. Lanes 1, 2: luciferase as a negative control; lanes 3, 4: SRM; lanes 5, 6: H19; lanes 7, 8: PRC; lanes 9, 10: SRA. After incubation, the reaction mixtures were subdivided into supernatants (odd-numbered lanes) and Sepharose pellets (even-numbered lanes).

Discussion

The v-*src* gene sequences in the Rous sarcoma virus isolates diverged from c-*src* and from each other. The v-*src* products of the strains SRA and PRC differ in 22 amino acids (16 substitutions are non-conserved), mostly located in the regulatory parts of the molecule (Czernilofsky et al., 1983). The "standard" SRA strain is highly oncogenic, whereas the PRC strain had a much lower oncogenic ability when tested in chickens (Plachý et al., 1995). Interestingly, the PRC was used to immunize chickens against infection with the highly oncogenic Bryan variant of Rous sarcoma virus. The H19, which is a derivative of PRC, was isolated from a cryptovirogenic hamster cell line from renal metastasis 6 months after inoculation into an animal in which the

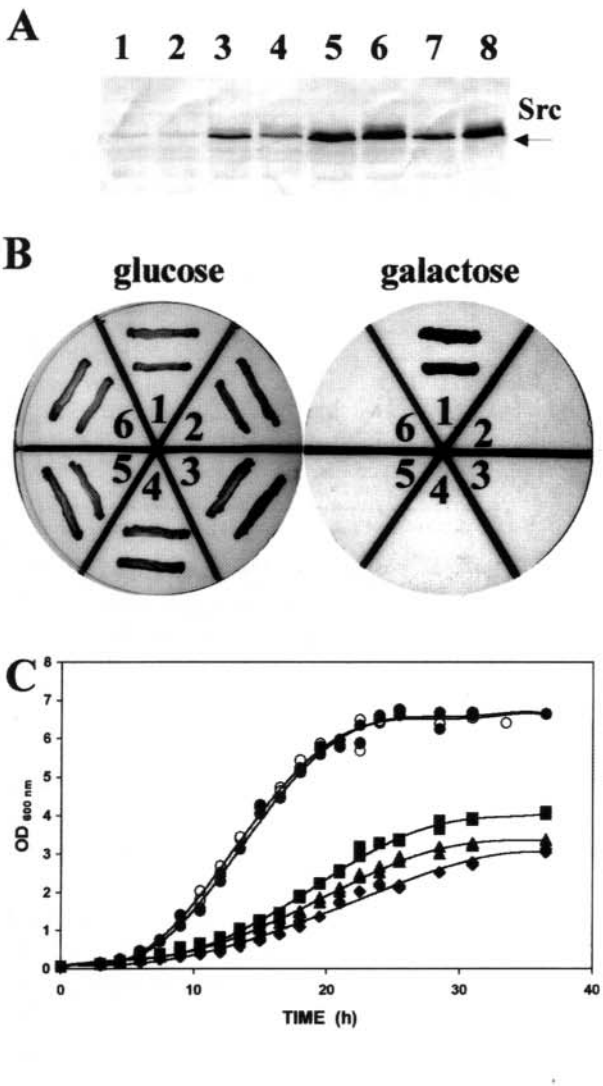

Fig. 2. The expression and growth-inhibitory effects of v-Src variants expressed in *S. cerevisiae*

Comparable levels of Src variants (ca 200 ng/ml cell culture) were produced in *S. cerevisiae* (A). Lanes 1, 2: uninduced cells; lanes 3 to 8: induction for 90 min (3, 4), 180 min (5, 6), and 330 min (7, 8). Lysates in all lanes correspond to equal amounts of cells (3×10^7 cells per lane) and contain either SRM (odd-numbered lanes) or SRA (even-numbered lanes).

The strains carrying *src* inserts in pYES2 were streaked out on a solid medium containing either glucose or galactose + raffinose and were allowed to grow for 3 days at 30°C. To show the effects of the indicated Src variants on cell viability under non-inducing or inducing conditions, the cells were restreaked onto new plates with glucose or galactose + raffinose, respectively, and photographed after an additional 3 days at 30°C (B). Inserts contain SRM (1), H19 (2), PRC (3), SRA-ch1 chimera (4), PRC-ch1 chimera (5), and SRA (6).

The growth retardation effects of H19 and PRC were similar to SRA in *S. cerevisiae* (C). The strains carrying *src* inserts in pYES2 were grown in liquid culture and aliquots were collected in duplicate every 1.5 h for determination of $O.D_{600nm}$. The control cells carrying vector only (○) and *src* transformants SRM (●), SRA (■), H19 (▲), and PRC (♦) were measured.

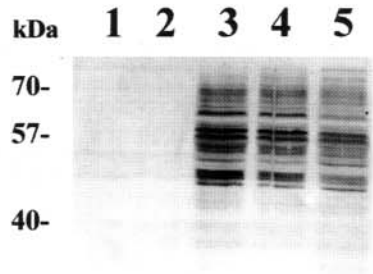

Fig. 3. The effect of v-Src expression on the level of tyrosine phosphorylation in *S. cerevisiae* cells

Cells were extracted in the presence of the phosphatase and protease inhibitors and equal amounts of the total protein were blotted with PY20 monoclonal antibody. Lane 1: wild-type cells – EGY48; lane 2: SRM; lane 3: H19; lane 4: PRC; lane 5: SRA.

primary tumour had necrotized (Svoboda et al., 1983). The *in vitro* kinase measurements reported here complement the biological characteristics of Prague C RSV-derived v-Src variants.

The assays of both *in vitro* translated and *S. cerevisiae*-expressed PRC kinases yielded comparable values ranging from 11% to 21% of the SRA standard. The kinase activities of PRC and H19 were thus close to the values of activated c-Src, which was shown to have one sixth of the SRA activity when expressed in *S. cerevisiae* (data taken from Kornbluth et al., 1987). When c-Src was phosphorylated at tyrosine 527, e.g. fully inhibited, like in animal cells, it had only 4% activity of SRA (Kato et al., 1986). The comparatively low kinase activity of PRC can still be sufficient to induce anchorage-independent growth under permissive conditions (Hložánek et al., 1993 and data not shown), similarly to what was proved for overexpressed c-Src (Zhou and Duesberg, 1990). Quantitative differences in Src kinase activities were indeed reported to distinguish between transforming and non-transforming events (Dezelee et al., 1992).

The expression of all tested kinase-active v-Src variants inhibited growth and reduced viability in *S. cerevisiae*. We directly compared the effects of SRA, PRC, and H19 and found an almost identical inhibition of growth. The transformants harboured high levels of the v-Src protein, which may explain why the inhibition was not proportional to the differences in specific kinase activities. Similar or even higher toxicities were reported previously, albeit in unrelated experiments, for PRC and SRA (Kornbluth et al., 1987; Boschelli et al., 1993). Boschelli reported that 95% of yeast cells were inviable after 4 h of PRC induction (Boschelli et al., 1993) but Kornbluth found that, in accordance with our data, SRA induction only prolonged the cell doubling time from 3 to 5.5 h (Kornbluth et al., 1987).

The measurements of the chimeric v-Src kinases produced in *S. cerevisiae* illustrate the dependence of the kinase domain on the regulatory parts of the molecule.

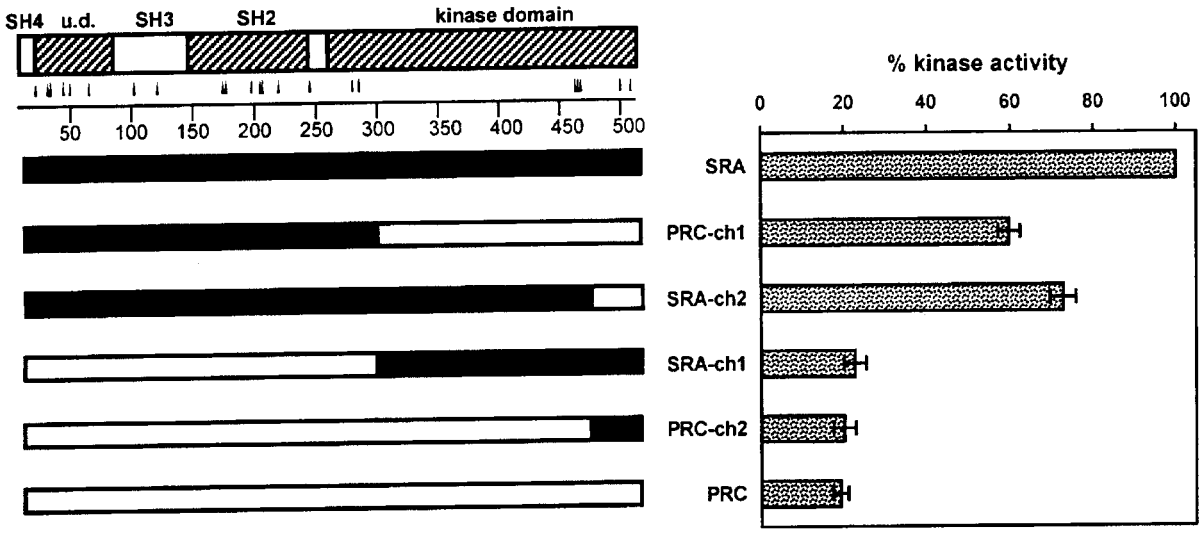

Fig. 4. The construction and kinase activity assay of chimeras between PRC and SRA v-Src. Left: structures of v-Src chimeras. The solid and open boxes denote the regions derived from SRA and PRC, respectively. The diagrammatic domain structure of v-Src is shown at the top (u.d. – unique domain) with the amino acid differences between SRA and PRC indicated by arrows. Right: kinase activities associated with chimeric v-Src proteins. The results were obtained in three independent experiments and normalized with respect to SRA (100%).

The swapping experiments in Fig. 4 show that the low kinase activity of PRC is due, at least in part, to the inhibitory effect of its N-terminal regulatory region. Accordingly, the regulatory region of PRC can inhibit the activity of the SRA kinase domain to the levels observed for PRC. As shown in Fig. 4, the variants SRA and PRC differ in 22 amino acids, with most of the differences located in the regulatory regions. We assume that the important point mutations are located in the SH3 and unique domains. Several mutations in the SH3 domain are known to influence the activity of c-Src (Kato et al., 1986). Among these, the replacement of D117 of c-Src (present in PRC) with asparagine (present in SRA) is known to relax the inhibitory interaction between the SH3 domain and the kinase domain when present together with mutation R95W (Miyazaki et al., 1999). Single mutation D117N had no effect on the kinase activity (Miyazaki et al., 1999). However, the simultaneous presence of mutation T96I in SRA makes it likely that the changes D117N and T96I, which distinguish PRC (and c-Src) from SRA, would influence the strength of SH3 domain inhibitory interaction. We assume that the differences in the unique domain (positions 16, 36, 37, 43, 46, and 62) should be of equal importance. Recent information suggests that the unique domain may affect the kinase activity because the binding of SIVmac Nef to the unique domain increased the phoshotransferase activity of Src (Dr. S. Lang; www.viro.med.uni-erlangen.de/selang/lan.htm). The catalytic domain was previously mapped with respect to mutations influencing its activity (Parsons and Weber, 1989) and includes the differences between SRA and PRC at positions 467, 469, and 474.

At least nine v-*src* variants were being used in the literature, often without accounting for the differences in their biochemical properties. Most of them derive from the original Rous isolate, such as the Prague C strain described by Svoboda (Svoboda, 1958). We found the kinase activity of PRC v-Src to be at the levels observed for activated c-Src. The low activity of PRC can be ascribed to the effects of its regulatory domain, which can also effectively inhibit the SRA-kinase domain. The difference between PRC and the more active SRA may represent an important step in the deregulation of the Src kinase and should be useful for the understanding of Src physiology.

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