Short Communication

Expression of Protein Tyrosine Kinase pp60$^{v\text{-}src}$ Variants in *Dictyostelium discoideum*

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

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Abstract. We achieved production of v-Src of the low-oncogenic PRC and its variant proviral structure H19 in *Dictyostelium discoideum*, an emerging host system suitable for synthesis of heterologous proteins. To accomplish their expression, the first six codons of the N-terminus of v-*src* had to be changed according to the *D. discoideum* codon preference. Alternatively, N-terminal fusions of 6xHis-tag or GFP were sufficient to overcome the incompatibility in codon usage. *D. discoideum*-expressed v-Src kinases of the expected molecular weight were recognized by Src-specific antibodies; GFP-PRC was distributed uniformly in the cytosol. In contrast to other lower eukaryotes, where the accumulation of v-Src leads to growth inhibition, *D. discoideum* cells silenced the kinase activity of PRC-derived v-Src and showed no developmental or growth defects.

Much effort has gone into understanding the physiological role and regulation of Src kinases. One approach to elucidate the function of vertebrate signalling proteins is to express them in simple eukaryotic organisms, such as yeasts or slime molds, where the networks of signalling partners are absent. The introduction of one desired partner protein then allows the study of "isolated" functional interactions, still *in vivo*. Such an approach was successfully used in research on trimeric G-proteins and their signalling partners (Voith et al., 1998). Using a similar rationale, lower eukaryotes, which lack canonical tyrosine kinase receptors, Src-family kinases, and the network of SH2 domain-mediated interactions, might be a heterologous host of choice for the study of tyrosine kinase signalling.

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

Here, we expressed v-Src in *D. discoideum*, an unicellular eukaryote, which diverged from the hypothetical ancestor before the onset of multicellularity (Kuma et al., 1995). This organism is nonetheless capable of primitive multicellular development and shares a number of characteristics with higher eukaryotes (Loomis and Smith, 1995). The amoebae resemble mammalian cell types in their chemotaxis and glycosylation, which is also why *D. discoideum* was used for the expression of secreted heterologous proteins (Voith and Dingermann, 1995; Voith et al., 1998). In *Dictyostelium*, canonical SH2 domains exist but are confined to a very limited set of proteins, e.g. the STAT factors, where they presumably serve as dimerization modules (Kawata et al., 1997). Several tyrosine kinases operate in *Dictyostelium*, albeit with unknown significance (Kawata et al., 1997; Kay, 1997). However, *Dictyostelium* lacks Src kinases, which apparently do not extend "below" the Porifera phylum (Ottilie et al., 1992). *Dictyostelium* amoebae seemed to be an attractive vehicle for the heterologous expression of v-Src isoforms, also because they lack cell walls and can be produced economically in ample amounts (Dingermann et al., 1991).

D. discoideum can fold mammalian proteins correctly, as shown by the production of membrane-spanning muscarinic receptors (Voith and Dingermann, 1995). Difficulties in heterologous protein expression were nevertheless encountered (Voith et al., 1998). This report documents the utility of *Dictyostelium* for the expression of vertebrate proteins, despite the significant differences in codon usage which exist between the two systems (Vervoort et al., 2000).

Material and Methods

Cloning and expression of v-src

The resources of the v-*src* genes used (v-Src derived from Schmidt-Ruppin A strain of RSV (SRA), kinase inactive mutant of v-Src derived from Schmidt-Ruppin A (SRM), v-Src derived from Prague C strain of RSV (PRC), and v-Src derived from the H-19 isolate of RSV (H19)), and the cloning of the original material were described previously (Brábek et al., 2001). For the expression of glutathione-S-transferase (GST)-tagged

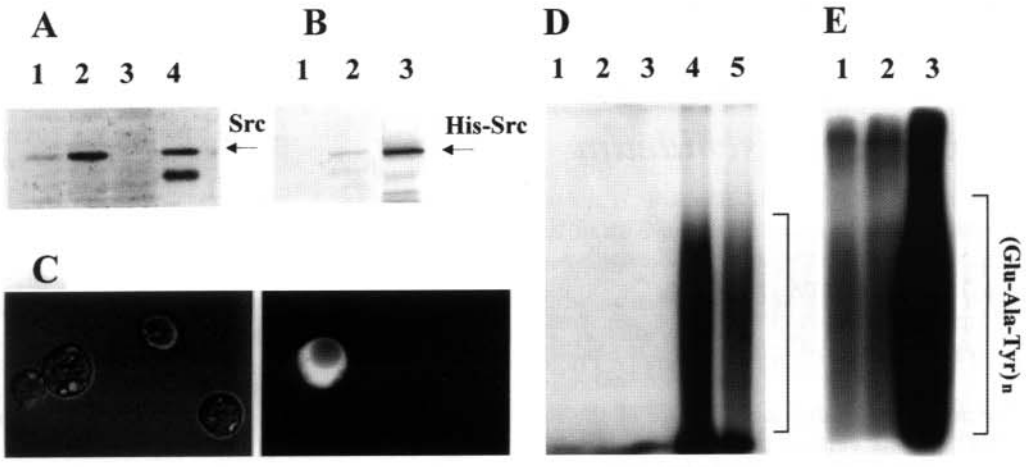

Fig. 1. The expression of untagged and His- or GFP-tagged Prague C v-Src in *Dictyostelium discoideum*
The expression of untagged H19 in *D. discoideum* cells (A). The v-Src protein was detected by mAb327. Lane 1: the expression of Src in the presence of 1 mM folate; lane 2: the expression of Src induced by the removal of folate; lane 3: wild-type cells; lane 4: Src protein isolated from Sf9 cells as a positive control. The expression of His-tagged PRC (B). The v-Src protein was purified by TALON affinity chromatography. Lane 1: wild-type cells; lane 2: His-tagged PRC in the lysate; lane 3: affinity-purified His-tagged PRC. The expression of GFP-tagged H19 (C). *Dictyostelium* cells expressing GFP-H19 were visualized by Nomarski differential interference contrast (left) and by GFP fluorescence (right). The pictures differ by a time frame of approximately 20 s. H19 (D) and PRC (E) expressed in *Dictyostelium* were found inactive in a protein tyrosine kinase assay. D – lane 1: negative control, wild-type cells; lanes 2, 3: immunopurified H19 Src has undetectable PK activity; lanes 4, 5: positive control, chicken c-Src expressed in insect Sf9 cells; lanes 1, 2, 4: assay incubation time 30 min; lanes 3, 5: assay incubation time 5 min. E – lane 1: PRC expressing *Dictyostelium* clone PN3; lane 2: wild-type cells; lane 3: positive control, SRA produced in *S. cerevisiae*. The gel was overexposed to compare the signal between PRC and negative control.

v-*src* in *E. coli*, the v-*src* fragments were excised from the cloning vector pBluescript II KS(+) by *Bam*HI and *Eco*RI and ligated into pGEX-2T (APB, Piscataway, NJ), for the expression in *S. cerevisiae* the fragments were inserted into pYES2 (Invitrogen, Carlsbad, CA).

We employed several strategies to express v-Src in *Dictyostelium discoideum*, three of which were successful. First, the N-terminally His-tagged PRC was expressed using the replicative *Dictyostelium* vector pDXA-HC (Manstein et al., 1995). Secondly, the GFP-tagged v-Src was expressed using the integrative vector pTX-GFP (Levi et al., 2000). Thirdly, to introduce the preferred codons according to the codon preference of the organism, the 5'-end of H19 v-*src* was PCR-modified by using the following primers: 5'- gcctctagaggttcatcaaagagcaagcctaaggac-3' and 5'-gttgacaatctgcaggcgttc-3' (the codons which have been altered and/or added to are underlined). The full-length open reading frame was inserted between the *Xba*I and *Kpn*I sites of the integrative pVEII vector (Blusch et al., 1992) under the control of the inducible discoidin-γ promoter of *Dictyostelium*.

The constructs were transformed into *D. discoideum* strains AX-2 by electroporation or calcium phosphate coprecipitation (Blusch et al., 1992), into *S. cerevisiae* EGY48 by the standard Li-acetate method (Schiestl and Gietz, 1989), and into *E. coli* by electroporation (Dower et al., 1988).

Preparation, immunoprecipitation, and in vitro protein kinase (PK) assays of v-Src

E. coli: The overnight culture in Luria broth containing 100 μg/ml ampicillin was diluted 1 : 10, grown at 30°C for 1.5 h, induced by adding IPTG (APB) to 0.1 mM, and shaken for an additional 3 h. Bacteria were lysed by sonication 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 (all from Serva, Heidelberg, Germany)), and phosphatase inhibitors (PhI; 1 mM sodium orthovanadate, 100 μM sodium molybdate, 1 mM NaF, 20 μM phenylarsinoxide). After 20 min of incubation in the presence of 1% TX-100, the lysate was centrifuged at 15 000 *g* for 20 min. For each mililiter of supernatant, 10 μl of glutathione-Sepharose beads (APB) equilibrated in LB1 were added, incubated for 3 h at 4°C, and washed 3 times thereafter.

D. discoideum: The cells were grown in shaking suspension to a density of 1–5 x 10^6 cells/ml (axenic medium; Blusch et al. (1992)), washed repeatedly, and lysed in LB1 containing 0.5% NP-40 (final cell density 1 x 10^8/ml). The lysate was clarified by centrifugation at 15 000 *g* for 20 min. 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. The binding of antigen antibody complexes to anti-mouse-IgG1-agarose (A3665; Sigma, St. Louis, MN) was performed for 1 h at 4°C with gentle mixing. 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). Alternatively, His-tagged Src was purified using the TALON™ metal affinity resin as described (Clontech, Palo Alto, CA). The Src protein levels were detected immunochemically with mAb327 and measured by densitometry.

The production of v-Src in *S. cerevisiae* and in the reticulocyte lysate *in vitro* translation system was performed as described (Brábek et al., 2002).

PK assays were performed with purified Src kinases using the incorporation of [γ-^{32}P]ATP into a synthetic substrate (poly(Glu-Ala-Tyr); Sigma, St. Louis, MN) as described in Brábek et al. (2002). Briefly, the immunoprecipitates were incubated for 20 min at 30°C in a kinase buffer consisting of 50 mM Hepes (pH 7.4), 8 μM $MgCl_2$, 2 mM $MnCl_2$, 100 μM Na_3VO_4, 10 μM ATP, PI, 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; APB, Piscataway, NJ), and 10 μg of substrate. Kinase activities were normalized with respect to the amount of the Src protein. Autophosphorylation assays were carried out in the kinase buffer containing 0.5 mM ATP. Phosphorylation of the activation loop-tyrosine was detected on immunoblots using the anti-Src phosphospecific antibody (pY418, Biosource International, Camarillo, CA).

Fluorescence microscopy

Dictyostelium cells were grown to a density of 4 x 10^6 cells/ml, washed in 17 mM phosphate buffer (pH 6.0), and starved at the density of 1 x 10^7 cells/ml for 3–4 h with shaking. Cells were transferred onto a glass cover slip and allowed to settle for 3 min. To better visualize the cell bodies, the cells were overlaid with a 0.3 mm-thick slice of agarose and the excess of buffer was removed. The cells were observed using a fluorescence microscope Olympus-Provis equipped with a unit for Nomarski differential interference contrast. GFP fluorescence was observed using the FITC filter set. Pictures were captured with an integrating monochromatic camera and adjusted with the image analysis Lucia G/F software (Laboratory Imaging, Prague, Czech Republic).

Results and Discussion

We expressed the H19 v-Src in *D. discoideum* using the integrative vector pVEII, which allowed expression regulation by folate. The first six codons of v-*src* were changed according to the *D. discoideum* codon usage table to avoid likely problems due to the unavailability of rare tRNAs (Sharp and Devine, 1989; Vervoort et al., 2000). His-tagged PRC was expressed using the replicative tagging-expression vector pDXA-HC (Manstein et al., 1995), which has a strong constitutive actin15 promotor. The expression of N-terminally GFP-tagged H19 was achieved in the integrative vector pTX-GFP with the same promotor. These N-terminal fusions were apparently adequate to overcome the problems with C/G-rich templates, reported previously (Heikoop et al., 1998).

The expression of H19 and PRC in *Dictyostelium* produced proteins of the expected molecular weight (Fig. 1A, B), but in contrast to the other expression systems used the proteins had undetectable kinase activity (Fig. 1D, E). The transformation with the pDXA-HC vector carrying PRC yielded over 80% of geneticin-resistant clones with the detectable PRC protein of the correct size. Using the same vector with SRA v-*src*, we were not able to detect the SRA protein in any clone. This phenomenon was reported previously in *Dictyostelium* expressions (K. Weijer, personal communication); its mechanism is, however, unknown. The cells expressing either H19 or PRC showed no increase in the tyrosine phosphorylation of proteins (not shown), no defects in growth, and developed normally. We reisolated

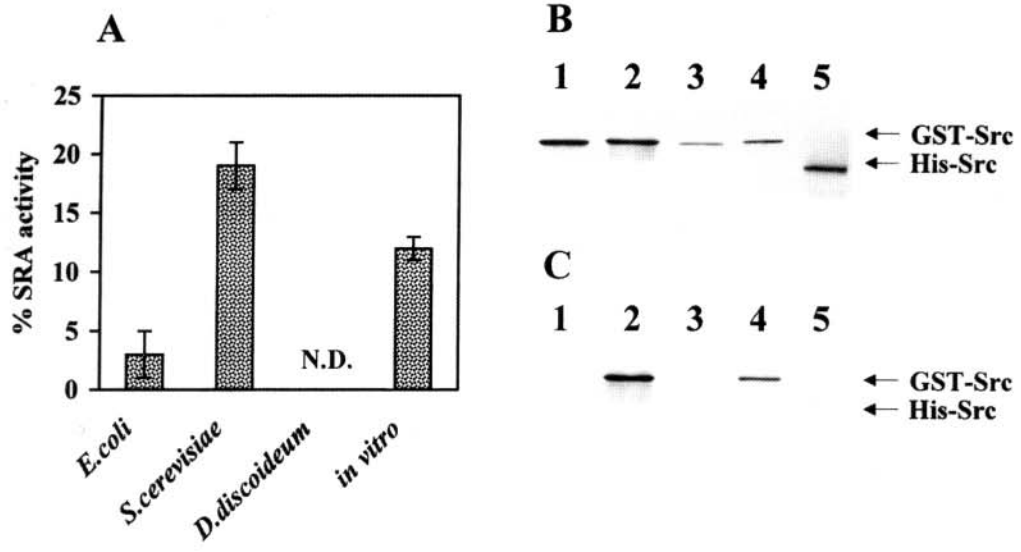

Fig. 2. A comparison of kinase activities and tyrosine 416 phosphorylation of v-Src obtained in various expression systems

PRC v-Src was expressed in four different expression systems and assayed for the kinase activity using SRA, expressed in the same system, as a standard (A). Translation *in vitro* was done as described previously (Brábek et al., 2002). The bars denote S.D. from three independent experiments. The kinase activity of *D. discoideum*-expressed PRC was below the detection limit of the assay; production of SRA could not be achieved (N.D.).

PRC v-Src expressed in *D. discoideum* is not phosphorylated on tyrosine 416 (B and C). GST-PRC and GST-SRM v-Src variants were expressed in *E. coli* and purified by glutathione-Sepharose affinity chromatography. His-PRC v-Src was expressed in *D. discoideum* and purified by TALON affinity chromatography. Purified proteins were immunoblotted with mAb327 (B) and with anti pY-416 antibody (C). Lane 1: GST-SRM; lane 2: GST-PRC; lane 3: GST-SRM (dilution 1 : 5); lane 4: GST-PRC (dilution 1 : 5); lane 5: His-tagged PRC.

the pDXA-HC vector from *D. discoideum* cells, recloned the v-*src* fragment into pBluescript II KS(+) and sequenced it. The sequence was identical to PRC, excluding the possibility that the PK-inactivity in *Dictyostelium* is the result of inactivating mutation occurring after transformation. We tested the distribution of the v-Src protein in *D. discoideum* cells using recombinant GFP-H19 (Fig. 1C). The protein was distributed uniformly within the cytosol, suggesting that it is not sequestered in Golgi or other compartments.

In parallel, the same DNA fragments of v-*src* that were employed for the expression in *D. discoideum* were used for the expression in *E. coli*. In contrast to *D. discoideum*, *E. coli* produced active H19 and PRC, despite the lack of certain eukaryotic chaperones such as BiP or GRP94 (Zapun et al., 1999). We expressed the Src variants as GST-fusion proteins and performed *in vitro* kinase activity tests using glutathione-Sepharose precipitation (Fig. 2A). In addition, PRC as well as H19 were kinase-active when expressed in *S. cerevisiae* or produced *in vitro* (Fig. 2A and Brábek et al., 2002).

To further characterize the v-Src proteins expressed in *Dictyostelium*, we analysed the phosphorylation of Y416 in the kinase-activation loop using the Y416 phosphospecific antibody. *E.coli*-expressed PRC was recognized by the Y416-phosphospecific antibody (Fig. 2C, lanes 2, 4), whereas the SRM variant was not (Fig. 2C, lanes 1, 3). In contrast to PRC isolated from *E. coli*, the PRC isolated from *D. discoideum* was not phosphorylated at Y416 (Fig. 2C, lane 5). Affinity-purified His-tagged PRC did not undergo autophosphorylation at Y416 in the kinase reaction mix (8 mM Mg^{2+}, 0.5 mM ATP; Osusky et al. (1995)) and it was not phosphorylated, even after an active v-Src kinase (SRA expressed in *S. cerevisiae*) was added to the incubation (data not shown). Notably, no dephosphorylation of the added active kinase was observed during the incubations. We hypothesize that in *D. discoideum*, a posttranslational modification or a tightly binding protein factor can efficiently inactivate v-Src of the PRC type.

We succeeded in producing untagged as well as tagged versions of PRC and H19 v-Src. The results indicate interesting differences between the *Dictyostelium* model and *S. cerevisiae*. H19 and PRC, while proved active in *S. cerevisiae* as well as in *E. coli* (Fig. 2A), were kinase-inactive when expressed in *Dictyostelium*. This feature might be of use when producing inactive kinase(s) for structural analyses, but it could also be exploited as a more general tool.

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References

Blusch, J., Morandini, P., Nellen, W. (1992) Transcriptional regulation by folate – inducible gene expression in Dictyostelium transformants during growth and early development. *Nucleic Acids Res.* **20**, 6235-6238.

Brábek, J., Mojžita, D., Hamplová, L., Folk, P. (2002) The regulatory region of Prague C v-Src inhibits the activity of the Schmidt-Ruppin A v-Src kinase domain. *Folia Biol. (Praha)* **48**, 28-33.

Dingermann, T., Troidl, E.-M., Broeker, M., Nerke, K. (1991) Expression of human antithrombin III in the cellular slime mold Dictyostelium discoideum. *Appl. Microbiol. Biotechnol.* **35**, 496-503.

Dower, W. J., Miller, J. F., Ragsdale, C. W. (1988) High efficiency transformation of E. coli by high voltage electroporation. *Nucleic Acids Res.* **16**, 6127-6145.

Heikoop, J. C., Grootenhuis, P. D. J., Blaauw, M., Veldema, J. S., Van Haastert, P. J. M., Linskens, M. H. K. (1998) Expression of a bioactive, single-chain choriogonadotropin in Dictyostelium discoideum. *Eur. J. Biochem.* **256**, 359-363.

Kawata, T., Shevchenko, A., Fukuzawa, M., Jermyn, K. A., Totty, N. F., Zhukovskaya, N. V., Sterling, A. E., Mann, M., Williams, J. G. (1997) SH2 signaling in a lower eukaryote: a STAT protein that regulates stalk cell differentiation in Dictyostelium. *Cell* **89**, 909-916.

Kay, R. R. (1997) Dictyostelium development: lower STATs. *Curr. Biol.* **7**, R723-R725.

Kuma, K., Nikoh, N., Iwabe, N., Miyata, T. (1995) Phylogenetic position of Dictyostelium inferred from multiple protein data sets. *J. Mol. Evol.* **41**, 238-246.

Levi, S., Polyakov, M., Egelhoff, T. T. (2000) Green fluorescent protein and epitope tag fusion vectors for Dictyostelium discoideum. *Plasmid* **44**, 231-238.

Loomis, W. F., Smith, D. W. (1995) Consensus phylogeny of Dictyostelium. *Experientia* **51**, 1110-1115.

Manstein, D. J., Schuster, H. P., Morandini, P., Hunt, D. M. (1995) Cloning vectors for the production of proteins in Dictyostelium discoideum. *Gene* **162**, 129-134.

Osusky, M., Taylor, S. J., Shalloway, D. (1995) Autophosphorylation of purified c-Src at its primary negative regulation site. *J. Biol. Chem.* **270**, 25729-25732.

Ottilie, S., Raulf, F., Barnekow, A., Hanning, G., and Schartl, M. (1992) Multiple src-related kinase genes, srk1-4, in the fresh water sponge Spongilla lacustris. *Oncogene* **7**, 1267-1630.

Schiestl, R. H., Gietz, R. D. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**, 339-346.

Sharp, P. M., Devine, K. M. (1989) Codon usage and gene expression level in Dictyostelium discoideum: highly expressed genes do "prefer" optimal codons. *Nucleic Acids Res.* **17**, 5029-5039.

Vervoort, E. B., Ravestein, A., Peij, N. N., Heikoop, J. C., Haastert, P. J., Verheijden, F., Linskens, M. H. (2000). Optimizing heterologous expression in *Dictyostelium*: importance of 5' codon adaptation. *Nucleic Acids Res.* **28**, 2069-2074.

Voith, G., Dingermann, T. (1995) Expression of CSA-hm2 fusion in Dictyostelium discoideum under the control of the Dictyostelium ras promoter reveals functional muscarinic receptors. *Pharmazie* **50**, 758-762.

Voith, G., Kramm, H., Zundorf, I., Winkler, T., Dingermann, T. (1998) Expression of the rat muscarinic receptor gene m3 in Dictyostelium discoideum. *Pharmazie* **53**, 707-710.

Zapun, A., Jakob, C. A., Thomas, D. Y., Bergeron, J. J. (1999) Protein folding in a specialized compartment: the endoplasmic reticulum. *Structure Fold. Des.* **7**, R173-R182.