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

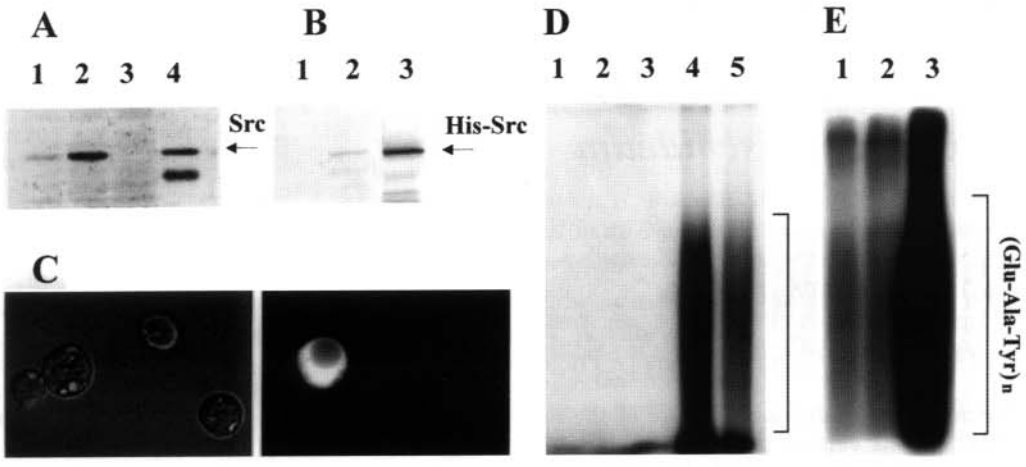

Fig. 1. The expression of untagged and His- or GFP-tagged Prague C v-Src in *Dictyostelium discoideum*
The expression of untagged H19 in *D. discoideum* cells (A). The v-Src protein was detected by mAb327. Lane 1: the expression of Src in the presence of 1 mM folate; lane 2: the expression of Src induced 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.