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

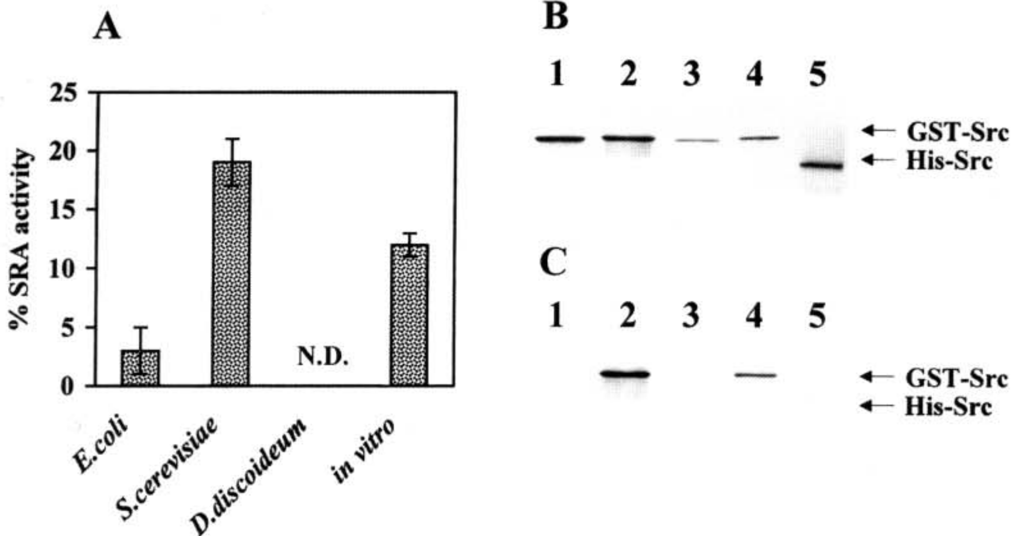

Fig. 2. A comparison of kinase activities and tyrosine 416 phosphorylation of v-Src obtained in various expression systems

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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