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A Cellular Protein Phosphorylated by the Avian Sarcoma Virus Transforming Gene Product

E. Erikson and R. L. Erikson

Cell transformation by ASVs is the consequence of the expression of a single viral gene, termed *src*, for sarcoma induction (Vogt 1977; Hanafusa 1977). The product of the *src* gene is a 60,000-dalton phosphoprotein termed pp60^{src} (Purchio et al. 1978). Extensively purified preparations of pp60^{src} exhibit phosphotransferase activity for various protein substrates (Erikson et al. 1979 a, b; Collett et al. 1980), implying that phosphorylation of cellular proteins may play a role in transformation. Evidence that pp60^{src} does mediate transformation via phosphorylation, however, requires the identification of a cellular protein(s) that is (are) phosphorylated directly as the result of pp60^{src} activity both in cells and in vitro and the correlation of this phosphorylation with a phenotype of transformation.

Earlier reports (Erikson et al. 1979b; Radke and Martin 1979) had described the detection of a newly phosphorylated protein in ASV-transformed chicken cells with a molecular weight of approximately 34,000 (34K) and a pI of about 7.5. In these reports no evidence was presented with regard to the possibility that this protein may be related to pp60^{src}, to its general distribution in ASV-transformed cells other than chicken, or whether it was a direct or indirect target of events initiated in cells by pp60^{src}. We describe here more direct experiments which show that this protein is a *src*-specific substrate found in normal cells and that upon transformation it is phosphorylated in ASV-infected cells of both avian and mammalian origin. The *src*-specific nature of the phosphorylation is demonstrated directly by comparative tryptic phosphopeptide analyses of this protein phosphorylated in transformed cells and in vitro.

The unphosphorylated form of 34K was purified from normal chicken embryo fibroblasts by conventional ion-exchange chromatography and tested as a substrate for pp60^{src}-specific phosphotransferase activity in vitro. Figure 1 demonstrates that 34K constituted at least 85% of the protein in the preparation and it was not detectably labeled by endogenous protein kinase activity (track 1). However, it could be phosphorylated by pp60^{src} (track 2). The *src*-specific nature of the phosphorylation is shown by the fact that anti-*src* IgG but not normal rabbit IgG (tracks 3 and 4) inhibited the phosphorylation of this protein.

However, pp60^{src} preparations are able to phosphorylate a number of proteins that may not be direct substrates of its activity in transformed cells (Erikson et al. 1979b; Collett et al. 1980), and thus the sites phosphorylated in both the protein isolated from radiolabeled transformed cells and that phosphorylated in vitro were compared by two-dimensional fingerprinting of tryptic digests. As shown in Fig. 2, there was a major and a minor phosphopeptide released by trypsin digestion of 34K prepared from transformed cells. Since the 34K preparation used for this digestion was homogeneous with respect to its migration during pH gradient gel electrophoresis (Fig. 3), the minor peptide may represent an incomplete digestion product, although there are other possible explanations for the unequal molarity consistently observed (see below). Phosphorylation of the 34K protein in vitro by pp60^{src} resulted in the phosphorylation of a peptide which comigrated with the major peptide identified in the protein from transformed cells.

Recently, it has been shown that pp60^{src} phosphorylates tyrosine residues in the immune-complex phosphotransferase reaction

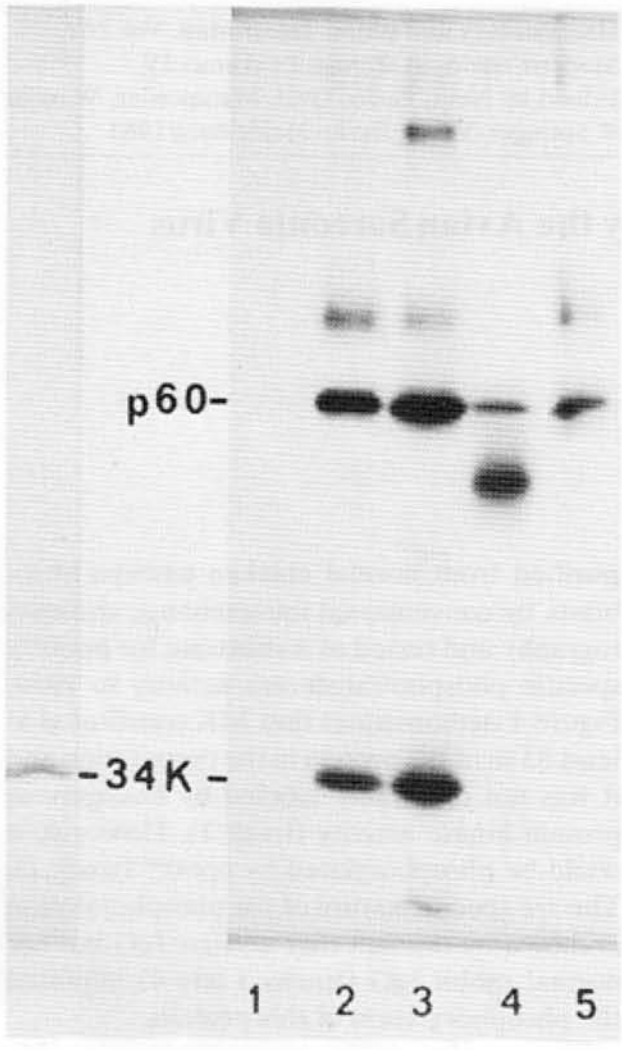

Fig. 1. In vitro phosphorylation of 34K by pp60^{src}. A preparation of the 34K protein purified from normal chicken embryo fibroblasts was incubated in the protein kinase reaction mixture with pp60^{src}. Reactions were carried out at 22°C for 15 min in a total volume of 35 μl as previously described (Erikson et al. 1979). Each reaction mixture contained 5 μl of partially purified pp60^{src} prepared by immunoaffinity chromatography (Erikson et al. 1979), 1.5 μg bovine serum albumin, and approximately 0.5 μg 34K protein. Reactions were initiated by the addition of $MnCl_2$ and [γ-^{32}P]ATP (400–600 Ci/mmol) to a final concentration of 2.5 m*M* and 1 μ*M*, respectively. Reactions were terminated by the addition of ¼ volume of five times concentrated electrophoresis sample buffer and by heating at 95°C for 1 min. Reaction products were then analyzed by polyacrylamide gel electrophoresis and autoradiography. *Left panel* A Coomassie blue stained SDS-polyacrylamide gel after electrophoresis of the 34K preparation. *Right panel,* autoradiogram of SDS-polyacrylamide gel analysis of protein kinase reaction products: track *1,* 34K preparation alone, and with *(2)* pp60^{src}, *(3)* pp60^{src} and normal rabbit IgG, and *(4)* pp60^{src} and rabbit anti-pp60^{src} IgG. Track *5* pp60^{src} preparation alone. Serum containing anti-pp60^{src} antibody was obtained from tumor-bearing rabbits (Brugge and Erikson 1977). The IgG fraction was obtained from the sera by ammonium sulfate precipitation. The IgGs were present in the reaction mixture at 600 μg/ml

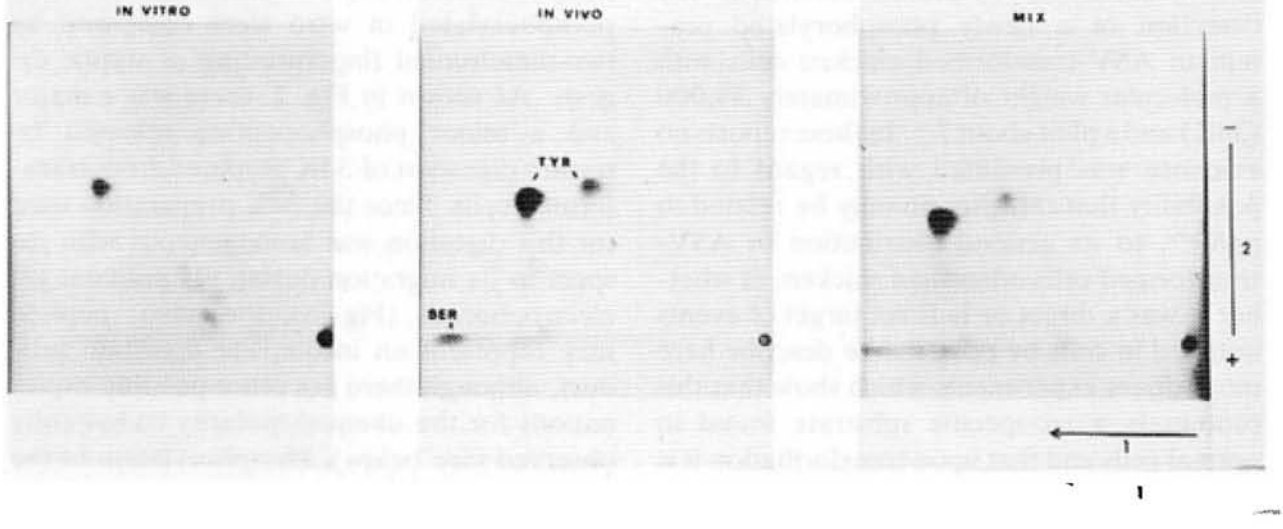

Fig. 2. Phosphopeptide analysis of 34K. Two-dimensional tryptic fingerprints of [^{32}P]-labeled 34K isolated from transformed cells *(center),* phosphorylated in vitro by pp60^{src} as shown in Fig. 1, track *2 (left),* and a mixture of the two preparations *(right).* Radiolabeled 34K was localized in preparative gels by autoradiography, excised, eluted from the gels, precipitated, and digested with trypsin. A portion of the digest was analyzed by ascending chromatography in n-propanol:s-butanol:isoamyl alcohol:pyridine:water (1:1:1:3:3) in the first dimension followed by electrophoresis at pH 3.5 [pyridine:acetic acid:water (1:10:189)] in the second dimension

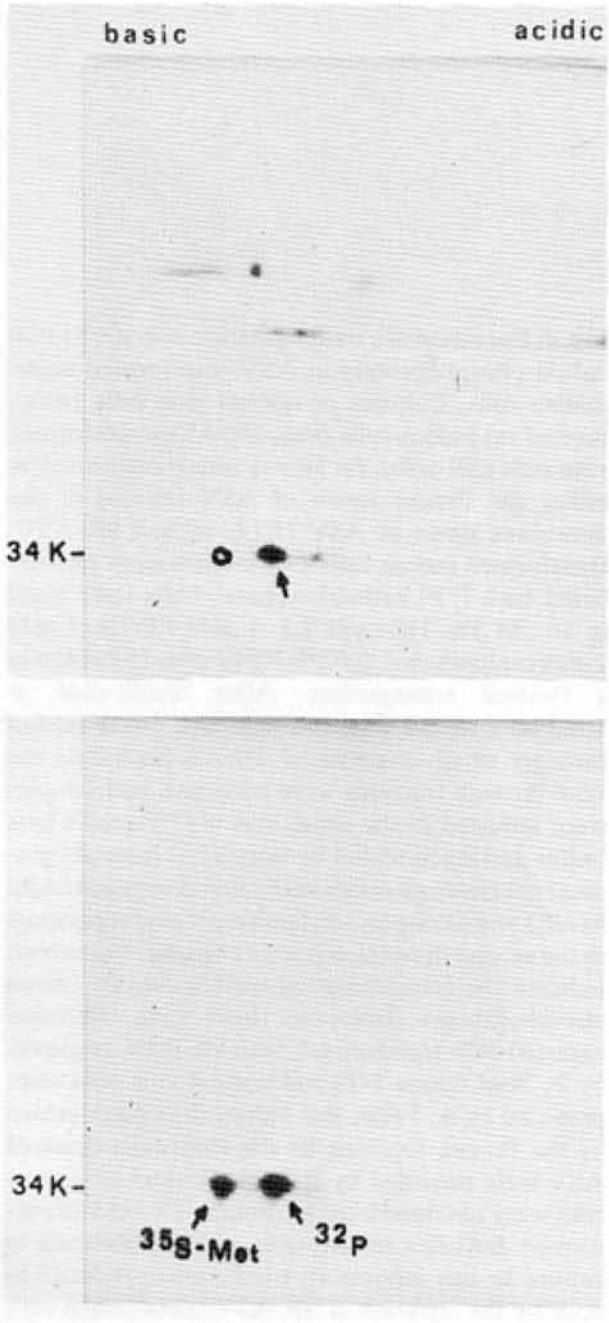

Fig. 3. Two-dimensional fractionation of 34K protein phosphorylated in vitro by pp60^{src}. *Upper panel* The 34K protein was phosphorylated in vitro as shown in Fig. 1, track 2. The reaction mixture was adjusted to the conditions of O'Farrell's lysis buffer (O'Farrell 1975) (9.5M urea, 5% 2-mercaptoethanol, 2% NP40, 2% ampholines) and analyzed by nonequilibrium pH gradient gel electrophoresis in the first dimension (right to left) and SDS-polyacrylamide gel electrophoresis in the second dimension (top to bottom) (O'Farrel et al. 1977). The location of Coomassie blue stained 34K is indicated by the *open circle*. The *arrow* indicates the position of [^{32}P]-labeled 34K. *Lower panel* Preparations of purified 34,000-dalton protein from [^{35}S]methionine-labeled normal chicken embryo fibroblasts (^{35}S-met) and [^{32}P]-labeled SR-ASV-transformed chicken embryo fibroblasts (^{32}P) were mixed and fractionated as described above

(Hunter and Sefton 1980) and in a number of protein substrates during more conventional soluble reactions as well (Collett et al. 1980). To determine whether this amino acid specificity holds for the 34K protein, phosphoamino acid analyses were carried out on 34K phosphorylated in vitro and isolated from radiolabeled transformed cells. The in vitro reaction resulted in phosphorylation of tyrosine residues exclusively, whereas the phosphoprotein from transformed cells yielded phosphotyrosine and some phosphoresine as well. In Fig. 2 the peptides labeled TYR have been shown to contain phosphotyrosine, whereas the one labeled SER contains phosphoserine (data not shown).

The 34K protein phosphorylated in vitro was also subjected to two-dimensional electrophoresis in order to gain additional information concerning the number of sites phosphorylated. The result, illustrated in Fig. 3, shows that the in vitro phosphorylation resulted in a shift in migration of the phosphate-containing polypeptide with respect to the phosphate-free polypeptide identical to that observed in vivo and indicates that each 34K molecule contains the same number of phosphate groups (probably one). Therefore, the major and minor tryptic phosphopeptides shown in Fig. 2 may be from separate 34K molecules.

The significance of such an ASV-specific phosphorylation would be clearer if it occurred in other ASV-transformed cells as well. Consequently, various mammalian cells transformed by ASV were examined for a similar phosphorylated protein and the results as shown in Fig. 4 reveal that mouse, vole, and rat cells all contained a similar phosphoprotein. Normal fibroblasts from all of these species showed either greatly reduced or undetectable levels of a phosphoprotein with a similar pI and molecular weight; two such examples are shown in Fig. 4. These 34K proteins also contain phosphotyrosine and a trace of phosphoserine (data not shown).

In this communication we have described the isolation of a protein from normal chicken embryo fibroblasts that appears to be a substrate for the phosphotransferase activity associated with pp60^{src}. This protein, or an analogous protein, is found to be partially phosphorylated in all ASV-transformed cells examined to date. The apparently similar nature of the protein in both avian and mammalian cells suggests that it is highly conserved. In view of

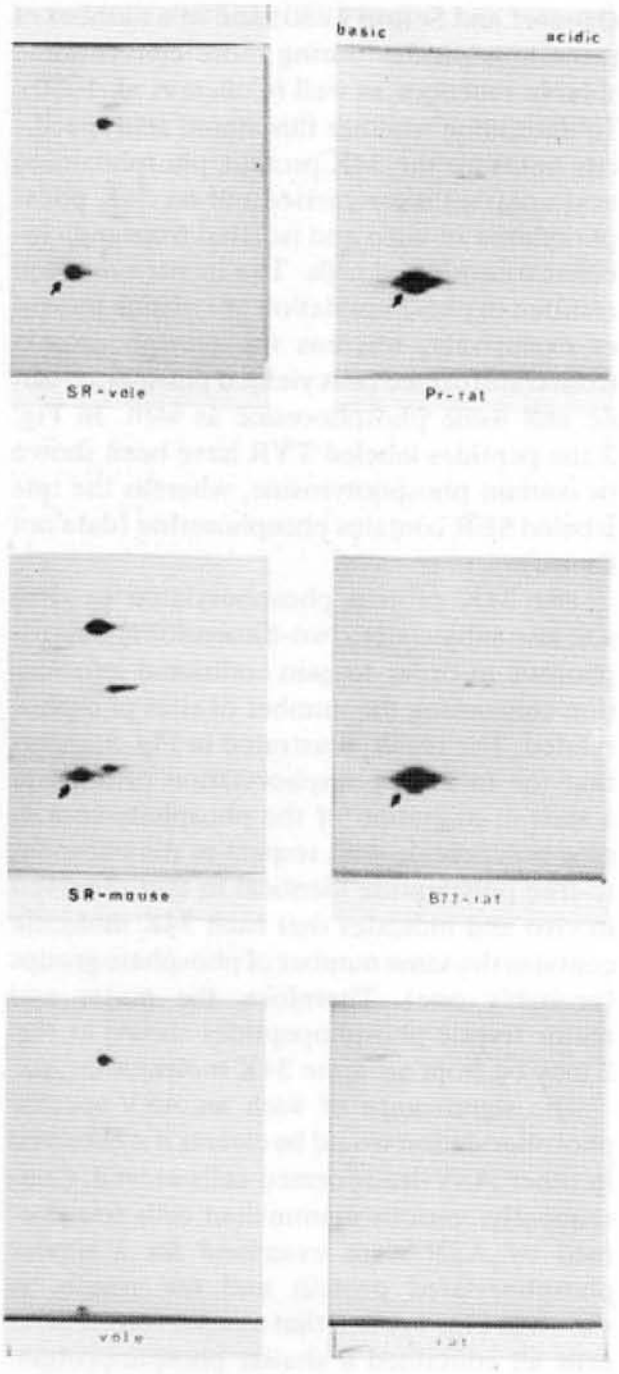

Fig. 4. Detection of a transformation-specific 34,000 dalton phosphoprotein in ASV-transformed mammalian cells. Cultures of normal vole cells *(vole)*, normal rat kidney cells *(rat)*, SR-ASV-transformed vole cells *(SR-vole)*, rat kidney cells transformed by either the Prague strain of ASV *(Pr-rat)* or the Bratislava strain of ASV *(B77-rat)* and SR-ASV-transformed mouse cells *(SR-mouse)* were radiolabeled with [^{32}P] orthophosphate. Cells were lysed in 10^{-2}M Tris-HCl pH 7.2, 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol, 0.05% NP40 with 25 strokes in a Dounce homogenizer. After clarification at 100,000 g for 30 min, the supernates were passed through small columns of DEAE-Sephacel, the flow-through fractions were collected, and samples were adjusted to the conditions of O'Farrell's lysis buffer and fractionated by nonequilibrium pH gradient gel electrophoresis in the first dimension (right to left) and SDS-polyacrylamide gel electrophoresis in the second dimension (top to bottom). The *arrows* indicate the transformation-specific 34,000 dalton phosphoprotein. European field vole (Microtus agrestis) cells transformed with SR-ASV originally by P. Vogt (clone 1-T) and normal vole cells were provided by A. Faras. Rat kidney cells transformed by the Prague strain or by the Bratislava strain of ASV were provided by P. Vogt. Normal rat kidney cells were obtained from M. Imada. SR-ASV-transformed BALB/c mouse cells were established in culture in this laboratory from tumors induced in mice by the injection of SR-ASV-transformed cells originally obtained from J. T. Parsons

the generally similar outcome of ASV transformation of fibroblasts from different species, one might expect similar targets to exist in any normal cell able to be transformed by ASV.

The pp60^{src}-specific nature of the phosphorylation is shown by the phosphorylation of 34K by pp60^{src} in vitro at a site identical to that phosphorylated in transformed cells. This result strongly suggests that 34K is a direct rather than indirect substrate of pp60^{src} in the cell. Such a result also suggests that pp60^{src} acts as a protein kinase in the transformed cell as well as in vitro and that it mediates transformation, at least in part, by phosphorylation of 34K and perhaps of other normal cell proteins. However, this conclusion does not eliminate the possibility that pp60^{src} may have other functions that influence the transformation process.

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