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Phil. Trans. R. Soc. Lond. B 1983 **302**, 151-156
doi: 10.1098/rstb.1983.0048

Phil. Trans. R. Soc. Lond. B **302**, 151–156 (1983) [151]
Printed in Great Britain

Avian sarcoma viruses, protein kinases and cell transformation

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Introduction

The first RNA tumour virus to be isolated and identified as such was the Rous sarcoma virus (RSV), which causes the transformation of cells in culture as well as fibrosarcomas when injected into suitable host animals (for reviews see Hanafusa (1977) and Bishop (1978)). The genome of RSV has been studied intensively for the past 10–12 years, and it has been shown that the virus itself carries a gene responsible for malignant transformation. This gene, denoted *src* for sarcoma, was identified genetically through the isolation of temperature-sensitive mutants that were conditional for cell transformation in culture. These mutants are able to transform cells at a temperature of 35 °C, the permissive temperature, but are unable to transform cells morphologically at 41 °C, the non-permissive temperature. The existence of such temperature-sensitive mutants implied that the product of the viral transforming gene, in RSV, was a protein (Kawai & Hanafusa 1971). In addition to temperature-sensitive mutants, non-conditional mutants were isolated that had deletions of the *src* gene. These mutants are unable to transform cells in culture or to cause fibrosarcomas under most conditions. About 4 years ago, the product of the *src* gene was identified as a phosphoprotein (M_r = 60000); this protein was denoted pp60^{src} (Purchio *et al.* 1978). The RSV genome and the expression of the *src* gene is illustrated in figure 1.

Very soon after the identification of pp60^{src} we and others ascribed to this molecule the function of protein phosphorylation (Collett & Erikson 1978; Levinson *et al.* 1978; for review see Erikson *et al.* (1980)). However, we were quite concerned that the enzymic activity observed might be due to contamination of preparations of the viral *src* gene product by one of the many protein kinases encoded by the cells used for infection and transformation by RSV, the starting material for our preparations of pp60^{src}. Thus, a great deal of our effort over the past several years has been devoted to attempting to determine unambiguously the source of the enzymic activity observed, because to understand cell transformation by RNA tumor viruses it is important to define the exact nature of the functions that are viral as opposed to cellular.

Expression of the RSV *src* gene in *Escherichia coli*

One approach to determining whether or not pp60^{src} is indeed a protein kinase was to obtain expression of the RSV *src* gene in a prokaryote such as *Escherichia coli*. These organisms are believed to produce few protein kinases that carry out reversible protein phosphorylation. Therefore, if the product of the *src* gene expressed in *E. coli* exhibited phosphotransferase

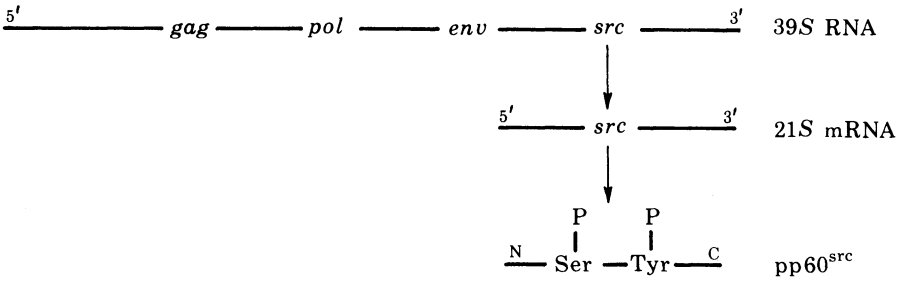

FIGURE 1

activity, it would be strong evidence that the enzymic activity observed was actually encoded by the *src* gene. Plasmids were constructed in which the RSV *src* gene was placed under the control of the *lac* promoter–operator in the hope of obtaining efficient transcription and, presumably, translation (Gilmer *et al.* 1982). Bacteria that expressed $p60^{src}$ were detected by immunoprecipitation of ^{35}S-labelled proteins and expressor cultures were used for purification of the *src* gene product. When these preparations were tested for protein kinase activity, we found that the protein produced in *E. coli* did indeed display protein kinase activity (Gilmer & Erikson 1981). It has many characteristics identical to the protein, produced in eukaryotic host cells, that we had characterized previously. It had the capacity to phosphorylate casein, α and β tubulin and anti-$pp60^{src}$ IgG; the amino acid phosphorylated in these protein substrates was tryosine (Gilmer & Erikson 1981; Hunter & Sefton 1980; Collett *et al.* 1980). Thus a number of characteristics suggest that the previously observed phosphotransferase activity closely associated with $pp60^{src}$ isolated from eukaryotic host cells is in fact an intrinsic property of the molecule itself.

AUTOPHOSPHORYLATION OF $pp60^{src}$

As shown previously (Collett *et al.* 1980; Erikson *et al.* 1979*a*, *b*), $pp60^{src}$ purified from eukaryotic sources is able to undergo self-phosphorylation or autophosphorylation when [γ-^{32}p]ATP is added to the purified protein. There is so far no evidence however, that $p60^{src}$ produced in *E. coli*, the prokaryotic host carrying the recombinant *src*-containing plasmid, is able to self-phosphorylate. Since we believe that the phosphorylation of $pp60^{src}$ itself on a tyrosine residue is likely to be important in the regulation of its enzymic activity, we have attempted to elucidate the source of the enzyme responsible for the phosphorylation of this residue.

One approach to answering this question was to compare the thermolability of the autophosphorylation reaction of enzyme purified from temperature-sensitive transformation mutant-infected cells with that of enzyme purified from wild-type infected cells. It had been shown previously that the protein kinase activity of ts $pp60^{src}$ was considerably more thermolabile than that of wild-type $pp60^{src}$ (Erikson *et al.* 1979*a*, *b*). Similarly, we found that the autophosphorylation of the temperature-sensitive preparation was several times more thermolabile than that of the wild-type preparation (Erikson & Purchio 1982). These results could be interpreted to mean that $pp60^{src}$ is responsible for its own phosphorylation. Unfortunately, an alternative explanation is that the molecule, when denatured, is no longer a good substrate for a putative $pp60^{src}$-specific kinase. One way to resolve this issue would be to isolate a kinase that is specific for phosphorylating the correct tyrosine residue in $pp60^{src}$. Although we have identified

other tyrosine-specific protein kinases in eukaryotic host cells infected by RSV, we have been unable to show with certainty that any of these protein kinases are able to phosphorylate pp60^{src} and are, in fact, the pp60^{src}-specific kinase. Consequently this issue is still unresolved, although it appears, from the study of the eukaryotic enzyme, that pp60^{src} is most probably capable of self-phosphorylation.

Transforming gene products of other classes of avian sarcoma virus

A number of other avian sarcoma viruses (ASVs) have been now identified and characterized in the laboratories of Professor Hanafusa at the Rockefeller Institute and Professor Peter Vogt at the University of Southern California. There are at least three other classes of ASV, which have transforming genes distinct from that of RSV and which encode distinct transforming gene products. Although they are antigenically distinct from pp60^{src}, these transforming gene products apparently have a functional similarity to pp60^{src} in that they are able to phosphorylate protein substrates at tyrosine residues. As illustrated in table 1, all four classes of ASVs have an associated protein kinase activity that, in an immune complex with anti-*src* IgG from a

Table 1. Protein kinase activity associated with the transforming gene products of avian sarcoma viruses

class	avian sarcoma virus	transforming gene product	immunoprecipitated by		phosphorylates TBR IgG on tyrosine
I	Rous	pp60^{src}	TBR	+	+
			anti-gag	−	.
II	Fujinami	P140$^{gag\text{-}fps}$	TBR	−	+
	PRCII	P105$^{gag\text{-}fps}$	anti-gag	+	.
III	Y73	P90$^{gag\text{-}yes}$	TBR	−	+
	Esh	P80$^{gag\text{-}yes}$	anti-gag	+	.
IV	UR2	P68$^{gag\text{-}ros}$	TBR	−	+
			anti-gag	+	.

For references see Breitman *et al.* (1981), Feldman *et al.* (1980, 1982), Ghysdael *et al.* (1981*a*, *b*), Kawai *et al.* (1980), Lee *et al.* (1980), Neil *et al.* (1981) and Pawson *et al.* (1980).

rabbit bearing an RSV tumour, results in the efficient phosphorylation of the heavy chain of IgG; anti-pp60^{src} IgG is a good substrate for the protein kinases encoded by other classes of ASV. There is no clearcut explanation as to why this IgG is such a good substrate, but the result does demonstrate that it is likely that all classes of ASVs so far studied encode a protein kinase specific for tyrosine residues. In addition, the transforming proteins themselves become phosphorylated in the reaction and tyrosine appears to be the sole phosphorylated residue.

Epidermal growth factor (EGF)-stimulated phosphorylation and comparison with ASV-induced phosphorylation

One of the proteins that we and others have studied that appears to be directly phosphorylated by the activity of pp60^{src} in the infected cell or *in vitro* is a molecule of molecular mass 34 kDa (Radke & Martin 1979; Erikson & Erikson 1980). This protein is also phosphorylated with the same specificity in cells transformed by other classes of ASV (Erikson *et al.* 1981*b*).

Because of the tyrosine-specific nature of EGF-stimulated phosphorylation (Cohen *et al.* 1980) and the tyrosine-specific phosphorylation of proteins by ASV transforming gene products, we attempted to determine the specificity of these two protein kinase activities. We found that when EGF is added to growing cells, the 34 kDa protein shows increased phosphorylation (Erikson *et al.* 1981*a*). Further, to determine whether or not the sites of phosphorylation were similar in EGF-stimulation or ASV transformation, the 34 kDa protein was purified in the unphosphorylated form from the same cells that were used for the EGF studies, and then purified pp60^{src} or its normal cell homologue were used to phosphorylate the protein *in vitro* with [γ-^{32}P]ATP. Phosphopeptide maps were prepared and compared with the phosphopeptide maps of the protein that had been phosphorylated after the addition of EGF to growing cells. We found two major phosphopeptides after tryptic digestion and in all three cases these peptides were the same (Erikson *et al.* 1981*a*). Although this experiment does not identify the enzyme responsible for phosphorylation of the 34 kDa protein in EGF-stimulated cells, it does show that the kinase responsible for this phosphorylation has the same specificity as pp60^{src}. This illustrates the very similar nature of the two types of phosphorylation and suggests that the 34 kDa protein is perhaps a common substrate for the different types of kinases under study.

Clearly, in the identification of substrates for any of the growth-regulated phosphorylations that we are discussing here, one must show some functional change associated with the phosphorylation observed. To understand the functional significance of the phosphorylation, one must, of course, first understand the function of the substrate under study. We have as yet no clear understanding of the function of the 34 kDa substrate, and without an understanding of that function it is difficult to assign any significance to its phosphorylation. Our best evidence so far is that in fibroblasts the 34 kDa protein is a strong RNA-binding protein, seemingly associated with messenger RNA in the cytoplasm of growing fibroblasts. Another way of assessing the significance of a substrate for the protein kinase activities observed is to determine the distribution of the particular protein in various tissues of the host that would normally be infected by a virus such as RSV. We found that there is a vast difference in the expression of the 34 kDa protein in various tissues of the adult bird. For instance, it is undetectable in red blood cells, and there is only a low level of this protein in brain cells. On the other hand, there are intermediate levels of the protein in other tissues, and it is a fairly abundant protein in cultured fibroblasts. This fact raises an obvious point that should be made when considering the significance of transforming proteins and their substrates, which is that for viruses to be able to carry out cell transformation, not only must the viral transforming gene product be expressed but a suitable substrate must be present for the activity of that product in the susceptible host cell.

Phosphotyrosyl-protein phosphatases

The phosphorylation state and presumably the functional behaviour of a particular protein will be greatly influenced by the protein kinase activities and the protein phosphatase activities present in a particular cell (for review see Krebs & Beavo (1979)). To understand the circuits involved in phosphorylation–dephosphorylation of proteins on tyrosine residues, one must be concerned not only about the specific kinases responsible for the phosphorylation of these proteins but also about the phosphoprotein phosphatases that are involved in their dephosphorylation. Results recently obtained by Gordon Foulkes, working in Denver, who studied the distribution of tyrosine-specific phosphoprotein phosphatases taken from chicken brain,

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indicate that the phosphotyrosine-specific phosphatases are probably unique and quite different from the phosphoserine-specific phosphatases previously described (Foulkes *et al.* 1983).

Search for other substrates

The 34 kDa protein was identified by work in Denver, as well as by others, as a hyperphosphorylated normal cellular protein in RSV-transformed cells. However, when one compares phosphorylation patterns in transformed and untransformed cells, many quantitative changes are observed, and most of these occur not at tyrosine residues but at serine residues. One example of this is the work of Decker (1981), which shows that the ribosomal protein S6 is heavily phosphorylated on serine residues in RSV-transformed cells. Even under conditions of serum starvation the phosphorylation of S6 seems to be under the control of the *src* gene product. This result suggests other mechanisms for amplifying the activity of a viral transforming gene product. One might imagine in this case that pp60^{src} activates a protein kinase specific for serine and that the increased activity of this serine-specific protein kinase results in phosphorylation of S6 which, in turn, leads to an increased efficiency of protein synthesis. Alternatively, pp60^{src} could phosphorylate a phosphoprotein phosphatase specific for S6 dephosphorylation, inactivating it so that the protein kinases that are normally present are then able to cause increased phosphorylation of S6. Either of these two mechanisms is possible, but they suggest obvious approaches to study further the process of transformation by viral transforming gene products that encode protein kinases.

Clearly, one of the most important areas of investigation in the near future will be directed at a biochemical description of the pathways that lead to neoplasia initiated by these and related viruses.

References

Bishop, J. M. 1978 *A. Rev. Biochem.* **47**, 35–88.

Breitman, M. L., Neil, J. C., Moscovici, C. & Vogt, P. K. 1981 *Virology* **108**, 1–12.

Cohen, S., Carpenter, G. & King, L. Jr 1980 *J. biol. Chem.* **255**, 4834–4842.

Collett, M. S. & Erikson, R. L. 1978 *Proc. natn. Acad. Sci. U.S.A.* **75**, 2021–2024.

Collett, M. S., Purchio, A. F. & Erikson, R. L. 1980 *Nature, Lond.* **285**, 167–169.

Decker, S. 1981 *Proc. natn. Acad. Sci. U.S.A.* **78**, 4112–4115.

Erikson, E., Cook, R., Miller, G. J. & Erikson, R. L. 1981*b* *Molec. cell. Biol.* **1**, 43–50.

Erikson, E. & Erikson, R. L. 1980 *Cell* **21**, 829–836.

Erikson, E., Shealy, D. J. & Erikson, R. L. 1981*a* *J. biol. Chem.* **256**, 11381–11384.

Erikson, R. L., Collett, M. S., Erikson, E. & Purchio, A. F. 1979*a* *Proc. natn. Acad. Sci. U.S.A.* **76**, 6260–6264.

Erikson, R. L., Collett, M. S., Erikson, E., Purchio, A. F. & Brugge, J. S. 1979*b* *Cold Spring Harb. Symp. quant. Biol.* **44**, 907–917.

Erikson, R. L. & Purchio, A. F. 1982 In *Advances in virology oncology*, (ed. G. Klein), vol. 1, pp. 43–47. New York: Raven Press.

Erikson, R. L., Purchio, A. F., Erikson, E., Collett, M. S. & Brugge, J. S. 1980 *J. Cell Biol.* **87**, 319–325.

Feldman, R. A., Hanafusa, T. & Hanafusa, H. 1980 *Cell* **22**, 757–765.

Feldman, R. A., Wang, L. H., Hanafusa, H. & Balduzzi, P. C. 1982 *J. Virol.* **42**, 228–236.

Foulkes, J. G., Erikson, E. & Erikson, R. L. 1983 *J. biol. Chem.* **258**, 431–438.

Ghysdael, J., Neil, J. C. & Vogt, P. K. 1981*a* *Proc. natn. Acad. Sci. U.S.A.* **78**, 2611–2615.

Ghysdael, J., Neil, J. C., Wallbank, A. M. & Vogt, P. K. 1981*b* *Virology* **111**, 386–400.

Gilmer, T. M. & Erikson, R. L. 1981 *Nature, Lond.* **294**, 771–773.

Gilmer, T. M., Parsons, J. T. & Erikson, R. L. 1982 *Proc. natn. Acad. Sci. U.S.A.* **79**, 2152–2156.

Hanafusa, H. 1977 In *Comprehensive virology* (ed. H. Fraenkel Conrat & R. P. Wagner), pp. 401–483. New York: Plenum Publishing Corporation.

Hunter, T. & Sefton, B. M. 1980 *Proc. natn. Acad. Sci. U.S.A.* **77**, 1311–1315.

Kawai, S. & Hanafusa, H. 1971 *Virology* **46**, 470–479.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Kawai, S., Yoshida, M., Segawa, K., Sugiyama, H., Ishizaki, R. & Toyoshima, K. 1980 *Proc. natn. Acad. Sci U.S.A.* **77**, 6199–6203.
Krebs, E. G. & Beavo, J. A. 1979 *A. Rev. Biochem.* **48**, 923–959.
Lee, W. H., Bister, K., Pawson, A., Robins, T., Moscovici, C. & Duesberg, P. H. 1980 *Proc. natn. Acad. Sci. U.S.A.* **77**, 2018–2022.
Levinson, A. D., Oppermann, H., Levintow, L., Varmus, H. E. & Bishop, J. M. 1978 *Cell* **15**, 561–572.
Neil, J. C., Ghysdael, J. & Vogt, P. K. 1981 *Virology* **109**, 223–228.
Pawson, T., Guyden, J., Kung, T. H., Radke, K., Gilmore, T. & Martin, G. S. 1980 *Cell* **22**, 767–775.
Purchio, A. F., Erikson, E., Brugge, J. S. & Erikson, R. L. 1978 *Proc. natn. Acad. Sci. U.S.A.* **75**, 1567–1571.
Radke, K. & Martin, G. S. 1979 *Proc. natn. Acad. Sci. U.S.A.* **76**, 5212–5216.

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