Differential Inhibition of Cellular and Viral pp60^{src} Kinase by P¹,P⁴-Di(Adenosine-5')Tetraphosphate

BARCEY T. LEVY, LAURIE K. SORGE, CYNTHIA C. DRUM, AND PATRICIA F. MANESS*

Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received 13 June 1983/Accepted 22 July 1983

We contrasted the protein kinase activities of pp60^{v-src}, the transforming protein of Rous sarcoma virus, and its normal cellular homolog pp60^{c-src} with respect to inhibition by P¹,P⁴-di(adenosine-5')tetraphosphate by using the immune complex protein kinase assay. The concentration of P¹,P⁴-di(adenosine-5')tetraphosphate required for 50% inhibition of pp60^{v-src} kinase (1 μ M) was found to be significantly lower than that required for inhibition of pp60^{c-src} kinase (46 μ M). Viral and cellular pp60^{src} kinases differed to a lesser extent with respect to inhibition by adenosine-5'-tetraphosphate, di(guanosine-5')tetraphosphate, and ADP. No significant differences were found in the ATP K_m values of pp60^{v-src} (0.108 ± 0.048 μ M) and pp60^{c-src} kinases (0.056 ± 0.012 μ M). These results demonstrate that the protein kinase activities of viral and cellular pp60^{src} are functionally distinguishable, particularly on the basis of enhanced sensitivity of the viral enzyme to inhibition by P¹,P⁴-di(adenosine-5')tetraphosphate. These functional differences are likely to be due to differences in the conformation of the active site and may be important for determining transformation potential.

Cell transformation by Rous sarcoma virus (RSV) is controlled by a single protein encoded by the *src* gene of the virus (reviewed in reference 1). The *src* gene product pp60^{v-src} has been identified as a 60,000-dalton phosphoprotein that acts as a protein kinase (4, 7, 13). A very similar homolog, pp60^{c-src}, is encoded by the genome of normal, uninfected cells in a wide variety of animal species (5, 6, 19, 22). pp60^{c-src} is also a 60,000-dalton phosphoprotein with associated protein kinase activity (5, 6, 12, 19, 22). Both protein kinases phosphorylate tyrosine in the heavy chain of immunoglobulin G (IgG) molecules from rabbits bearing RSV-induced tumors and in other protein substrates (2, 6-8, 10, 12, 14, 15, 19, 22, 23). The exact molecular details of how pp60^{v-src} induces transformation are not understood. Transformation could be the consequence of the 20- to 100-fold elevated levels of pp60^{v-src} in transformed cells compared with the levels of pp60^{c-src} in normal cells (5, 6, 12). Alternatively, differences in the activities of these proteins may lead to transformation in one case and normal cell growth in the other.

Functional differences in the protein kinase activities of pp60^{v-src} and pp60^{c-src} have not been reported, even though the viral and cellular forms of pp60^{src} can be structurally distinguished by several criteria. For example, many tumor-bearing rabbit (TBR) sera that recognize pp60^{v-src} do not recognize pp60^{c-src} (6). Addition-

ally, the peptide maps of the immunoprecipitated proteins show minor differences (5, 6, 12, 19, 22), and although both proteins are phosphorylated at serine and tyrosine residues *in vivo*, their phosphotyrosine residues are located in different tryptic peptides (11, 25). Finally, predicted amino acid sequences show differences in the carboxy-terminal domains that could be important for transformation (30). To directly contrast the enzymatic activities of pp60^{c-src} and pp60^{v-src}, it would be useful to develop inhibitors of tyrosine-specific protein phosphorylation that would discriminate between the two proteins. Recently we showed that P¹,P⁴-di(adenosine-5')tetraphosphate (Ap₄A) inhibits the protein kinase activity of pp60^{v-src} (17) that was purified 7,000-fold from the RSV-transformed rat tumor line RR1022 (16). Ap₄A is a nucleotide that is present in animal cells and has been proposed as a pleiotypic regulator of cell proliferation (21). In this paper we report that pp60^{c-src} and pp60^{v-src} can be functionally discriminated on the basis of the sensitivity of their protein kinase activities to inhibition by Ap₄A.

MATERIALS AND METHODS

Nucleotides. Sodium Ap₂A, ammonium Ap₃A, ammonium Ap₄A, lithium Ap₅A, ammonium Ap₆A, and other nucleotides were purchased from Sigma Chemical Co. With the exception of Ap₆A, all compounds appeared pure at the 5% level when analyzed by thin-

layer chromatography (17). All diadenosine nucleotides were treated with alkaline phosphatase which was immobilized on polyacrylamide beads (Sigma) as described previously (17) to hydrolyze any ATP or ADP that might be present in the samples below the limit of detection. The concentrations of these and other nucleotide solutions were determined spectrophotometrically.

Cells, viruses, and extract preparation. Chick embryo fibroblasts (CEF) were prepared from virus-free, group specific (gs) antigen-negative embryos (SPAFAS, Norwich, Conn.) and were transformed with RSV-Schmidt-Ruppin subgroup D (RSV-CEF) obtained from H. Hanafusa, Rockefeller University, New York, N.Y. Cells were grown in Hams F10 medium with 5% calf serum. Cell extracts were prepared from 80 to 90% confluent cultures of CEF or confluent RSV-CEF grown on 100-mm tissue culture dishes. Each plate of cells was washed three times with 10 mM Tris-hydrochloride (pH 7.4)–150 mM NaCl–1 mM EDTA–100 Kallikrein inhibitor units of aprotinin per ml, and the cells were lysed at 4°C in 1 ml of lysis buffer which contained 20 mM potassium phosphate (pH 7.1), 1 mM EDTA, 0.5% (wt/vol) sodium deoxycholate, 1% (vol/vol) Nonidet P-40, 10 mM sodium fluoride, 2 mM dithiothreitol, 150 mM KCl, and 100 Kallikrein inhibitor units of aprotinin per ml. After being briefly vortexed, the lysate was clarified at $10,000 \times g$ for 30 min. The resulting cell extract was stored at -70°C .

Antisera. TBR serum was prepared from rabbits bearing tumors induced by the Schmidt-Ruppin strain of RSV by using the method of Brugge and Erikson (4). Of seven rabbit sera with positive titers for antibodies that recognized $pp60^{v-src}$, only one serum contained antibodies that also recognized $pp60^{c-src}$. The TBR serum that contained antibodies that recognized both proteins was used in all studies reported here. Enzymatic parameters for $pp60^{v-src}$ were confirmed by using one of the TBR sera that contained antibodies that only recognized $pp60^{v-src}$.

Protein kinase assays. $pp60^{v-src}$ and $pp60^{c-src}$ kinases were assayed in the immunoprecipitate with TBR IgG as the substrate by using a modification (15) of the method of Collett and Erikson (7). A 50- μl amount of CEF extract (111 μg of protein) or a 10- μl amount of RSV-CEF extract (22 μg of protein) brought to 50 μl with lysis buffer was incubated with 5 μl of TBR serum for 1 h at 4°C. Protein A-Sepharose was added for 30 min to adsorb the immune complexes formed, and the resulting immunoprecipitates were washed three times with 50 mM Tris-hydrochloride (pH 7.2)–0.15 M NaCl, once with 2.5 M KCl–60 mM Tris-hydrochloride (pH 7.2), and once with 40 mM Tris-hydrochloride (pH 7.2)–10 mM MgCl_2 . The reaction was initiated by the addition of 5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (New England Nuclear Corp.; 2,000 to 4,000 Ci/mmol) in 20 mM Tris-hydrochloride (pH 7.2)–5 mM MgCl_2 –5 mM dithiothreitol and nucleotide inhibitors, where indicated, in a total solution volume of 35 μl . The reaction was carried out at 37°C for 10 min. The immunoprecipitates were washed with 20 mM Tris-hydrochloride (pH 7.2)–5 mM MgCl_2 , and the reaction was terminated by heating in sodium dodecyl sulfate sample buffer for 2 min at 95°C. ^{32}P -labeled IgG heavy chains were separated on 8.5% sodium dodecyl sulfate-polyacrylamide gels that were then fixed, stained with Coomassie blue, and

dried. The heavy chain regions were excised from the dried gel, and gel slices were counted for ^{32}P in Aquasol (Brinkmann Instruments, Inc.). The results of duplicate determinations were averaged. In some cases the data were confirmed by using $pp60^{v-src}$ purified 7,000-fold from the rat tumor cell line RR1022 (16).

Determinations of apparent K_m . The apparent K_m values for ATP were determined by using the assay described above with the following modifications. Immunoprecipitates were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.005 to 1.8 μM , 2,900 Ci/mmol) at 37°C for 1 min. Time course experiments indicated that the reaction velocity was linear at this time. The reaction was terminated by heating the samples for 1 min at 95°C. K_m values were obtained from plots of duplicate samples by application of the weighted least-squares analysis of Wilkinson (28).

RESULTS

To compare $pp60^{v-src}$ and $pp60^{c-src}$ kinase activities for inhibition by Ap_4A under identical conditions, the kinases were immunoprecipitated from extracts of uninfected CEF and RSV-CEF by using a TBR serum that recognized both proteins. Phosphorylation of TBR IgG heavy chains in the immunoprecipitates was carried out in the presence of various amounts of Ap_4A . Conditions of antibody excess were employed in all experiments. Although the amounts of $pp60^{c-src}$ and $pp60^{v-src}$ protein immunoprecipitated were not identical, their sensitivity to inhibition by Ap_4A could be compared by plotting the percent inhibition of activity as a function of Ap_4A concentration. $pp60^{v-src}$ kinase was found to be significantly more sensitive to inhibition by Ap_4A than $pp60^{c-src}$ kinase (Fig. 1). The concentration of Ap_4A required for 50% inhibition of $pp60^{v-src}$ kinase activity (IC_{50} , 1 μM) was almost 50-fold lower than the concentration required to inhibit $pp60^{c-src}$ (IC_{50} , 46 μM) at 50 nM ATP. The extent of inhibition of $pp60^{v-src}$ kinase by Ap_4A was not significantly affected by using a TBR serum that recognized only $pp60^{v-src}$ (IC_{50} , 4 μM).

To rule out the possibility that Ap_4A was preferentially degraded by contaminants such as phosphodiesterases in immunoprecipitates of CEF extracts, Ap_4A at various concentrations was preincubated with immunoprecipitates from CEF extracts containing $pp60^{c-src}$ for 10 min at 37°C and transferred to immunoprecipitates from RSV-CEF extracts containing $pp60^{v-src}$. Kinase activity was measured in the immunoprecipitates. Ap_4A that had been preincubated with the CEF immunoprecipitates inhibited $pp60^{v-src}$ kinase activity with the same effectiveness (IC_{50} , 1.2 μM) as fresh Ap_4A (IC_{50} , 1.6 μM) or Ap_4A that had been preincubated with immunoprecipitates of $pp60^{v-src}$ prepared from RSV-CEF extracts (IC_{50} , 1.2 μM). As an additional test of Ap_4A degradation, $pp60^{c-src}$ and $pp60^{v-src}$ were immunoprecipitated from an

FIG. 1. Inhibition of pp60^{c-src} and pp60^{v-src} kinase activities by Ap₄A. The phosphorylation of TBR IgG by pp60^{c-src} (●) and pp60^{v-src} (○) was measured in immunoprecipitates in the presence of the indicated concentrations of Ap₄A as described in the text, and the percent of inhibition of activity was calculated. The uninhibited activity of pp60^{v-src} was 85,100 cpm of ³²P incorporated into IgG heavy chains under the conditions of the assay, and for pp60^{c-src} it was 2,700 cpm of ³²P.

amount of CEF (111 μg) or RSV-CEF extract (22 μg) usually used in the assay and also from 10-fold excess amounts. The washed immunoprecipitates were incubated with Ap₄A (6 mM) for 10 min at 37°C. Samples were chromatographed on polyethyleneimine cellulose thin-layer sheets with fluorescent indicator in 0.7 M LiCl-0.02% formic acid (vol/vol) and examined under UV light. No degradation of Ap₄A to ATP, ADP, AMP, or adenosine was observed in any sample. A comparison with various amounts of ADP as standards indicated that any degradation of Ap₄A in the incubation mixture would be less than 9% of the input Ap₄A concentration.

A mixing experiment was carried out to determine whether the relaxed sensitivity of pp60^{c-src} to Ap₄A inhibition was due to a free substance in normal uninfected cells that could bind to either pp60^{c-src} or pp60^{v-src} and render it less sensitive to Ap₄A (Table 1). The same antiserum was used to immunoprecipitate pp60^{v-src} and pp60^{c-src} from RSV-CEF extracts, CEF extracts, and a mixture of RSV-CEF and CEF extracts. Kinase activity was measured in the immunoprecipitates in the presence of Ap₄A at various concentrations. pp60^{v-src} immunoprecipitated from the mixed extracts was as sensitive to Ap₄A inhibition as pp60^{v-src} from the RSV-CEF extract, as seen from the IC₅₀ values. Thus, the reduced sensitivity of pp60^{c-src} to inhibition by Ap₄A appears to be an intrinsic property of the Ap₄A binding site of pp60^{c-src} molecules.

Related diadenosine nucleotides of general

structure Ap_nA (*n* = 2 to 6) were evaluated as inhibitors of the protein kinase activities of pp60^{c-src} and pp60^{v-src} to gain information concerning the molecular dimensions of their Ap₄A binding sites (Fig. 2). The compounds differ only in the length of the phosphoryl group bridge linking the two adenosine moieties at the 5' position. Ap₄A and Ap₅A inhibited both kinase activities maximally when present at a single concentration (100 μM). Ap₂A, Ap₃A, and Ap₆A were less effective. These results imply that the Ap₄A binding sites of both enzymes are similar and can best accommodate diadenosine nucleotides with greater than three phosphoryl groups.

Other nucleotides structurally related to Ap₄A did not discriminate between the enzymatic activities of pp60^{v-src} and pp60^{c-src} to the extent that Ap₄A did (Table 2). Adenosine tetraphosphate (Ap₄) was nearly as effective as Ap₄A as an inhibitor of pp60^{v-src}. However, there was only a 3- to 4-fold difference in the IC₅₀ values of Ap₄ when the viral enzyme was compared with the cellular enzyme, in contrast to the 50-fold difference in IC₅₀ values of Ap₄A. Surprisingly, di(guanosine-5')tetraphosphate was not found to be an effective inhibitor of either enzyme, indicating a high degree of specificity for the adenine moiety of Ap₄A. Di(guanosine-5')tetraphosphate (100 μM) inhibited pp60^{v-src} kinase activity by only 23% and pp60^{c-src} kinase activity by 24%. ADP, which is a product of the kinase reaction, inhibited both enzymes at low concentrations. However, ADP failed to discriminate between pp60^{v-src} and pp60^{c-src}. The threefold difference of observed inhibition by ADP does not appear to be significant, because an intermediate IC₅₀ value (0.22 μM) was obtained for

TABLE 1. Effect of mixing extracts on Ap₄A inhibition^a

Immuno-precipitate from:	Kinase activity in immunoprecipitates (cpm of ³² P incorporated into IgG) ^b at Ap ₄ A concn:				IC ₅₀ (μM)
	0 μM	1 μM	4 μM	40 μM	
RSV-CEF	28,800	12,900	6,090	1,320	1
CEF	1,530	1,330	970	860	40
RSV-CEF + CEF	23,200	11,160	6,530	1,220	2

^a pp60^{v-src} and pp60^{c-src} were immunoprecipitated with the same TBR serum from extracts of RSV-CEF (17 μg of protein), CEF (17 μg of protein), and a mixture of both extracts (17 μg of protein each).

^b Kinase activity was measured in immunoprecipitates at 50 nM ATP with various Ap₄A concentrations, as described in the text. Ap₄A IC₅₀ values were determined by plotting the percent of inhibition as a function of Ap₄A concentration, as in Fig. 1.

FIG. 2. Effect of diadenosine nucleotides on inhibition of $pp60^{C-src}$ and $pp60^{V-src}$ kinase activities. The phosphorylation of TBR IgG by $pp60^{C-src}$ (●) and $pp60^{V-src}$ (○) was measured in immunoprecipitates in the presence of diadenosine nucleotides (100 μM) as described in the text, and the percent inhibition of activity was calculated. The uninhibited activity of $pp60^{V-src}$ was 11,100 cpm of ^{32}P incorporated into IgG heavy chains under the conditions of the assay, and for $pp60^{C-src}$ it was 450 cpm of ^{32}P .

$pp60^{V-src}$, using different TBR serum. Finally, we found that neither enzyme was inhibited by AMP or adenosine (100 μM).

ATP and Ap_4A compete for binding to purified $pp60^{V-src}$ probably at the enzyme phosphodonor site (17). Thus, it was possible that the different affinities of $pp60^{C-src}$ and $pp60^{V-src}$ for Ap_4A reflected different affinities for its ATP moiety. To address this we determined their apparent K_m values for ATP in the immune complex protein kinase assay. No significant differences in the apparent ATP K_m values for the viral and cellular enzyme were found, using the same antiserum to measure the activity of both enzymes (Table 2). A different TBR serum, which contained antibodies that recognized only $pp60^{V-src}$, gave a K_m of $0.040 \pm 0.017 \mu M$ for the viral enzyme, a value similar to the K_m with the antiserum that recognized both proteins. K_m values for ATP approximated a K_m value deter-

mined by using purified $pp60^{V-src}$ ($0.016 \pm 0.003 \mu M$) assayed with TBR IgG in solution rather than in immunoprecipitates. To the extent that K_m determinations in the immune complex assay reflect binding of ATP, the differential sensitivity of $pp60^{C-src}$ to inhibition by Ap_4A cannot be accounted for by differential affinities of the enzymes for ATP. Thus, the second adenosine moiety of Ap_4A appears to provide a unique binding determinant that discriminates between the active sites of $pp60^{C-src}$ and $pp60^{V-src}$.

DISCUSSION

In this paper we report evidence for functional differences between the active sites of $pp60^{C-src}$ and $pp60^{V-src}$, as revealed by a great difference in sensitivity of these two enzymes to inhibition by a small molecule, Ap_4A . These findings are in accord with our previous studies which showed that Ap_4A and ATP compete for a common binding site on the $pp60^{V-src}$ molecule that is most likely to be the phosphodonor site (17). Similar results have also been recently reported by Barnekow (A. Barnekow, Biosci. Rep., in press).

The implications of the results reported here are limited by the nature of the immune complex protein kinase assay. It is possible that certain subpopulations of TBR antibody molecules that recognize only the viral *src* protein are responsible for the difference in sensitivity to Ap_4A . On the other hand, a slight difference in the antibody substrates may not significantly affect the degree of inhibition by Ap_4A . For example, similar concentrations of Ap_4A effectively inhibit the phosphorylation by $pp60^{V-src}$ of the unrelated phosphoacceptor substrates tubulin, microtubule-associated proteins, vinculin, and TBR IgG (17). These substrates presumably share a unique tyrosine-containing peptide sequence (20) that is recognized by both proteins. Moreover, two different antibodies used as the substrate for the viral enzyme gave similar IC_{50} values for Ap_4A and ADP and a similar K_m value for ATP. Nonetheless, it will be important to confirm the results reported here with purified $pp60^{C-src}$ when it becomes available or with monoclonal antibodies as the substrate. At present, however, the immune complex kinase assay is

TABLE 2. Effect of nucleotides related to Ap_4A on kinase activity of $pp60^{V-src}$ and $pp60^{C-src}$

Kinase ^a	Ap_4A IC_{50} (μM)	Ap_4A IC_{50} (μM)	Gp_4G^b IC_{50} (μM)	ADP IC_{50} (μM)	ATP K_m (μM)
$pp60^{V-src}$	1	2.5	>100	0.34	0.108 ± 0.048
$pp60^{C-src}$	46	9	>100	0.13	0.056 ± 0.012

^a $pp60^{V-src}$ and $pp60^{C-src}$ were immunoprecipitated with the same TBR serum from extracts of RSV-CEF and CEF, and assays were carried out as described in the text.

^b Gp_4G , Di(guanosine-5') tetraphosphate.

the only means of comparing the activities of pp60^{v-src} and pp60^{c-src} under the same conditions.

One model for the inhibition of pp60^{c-src} kinases by Ap₄A is that Ap₄A binds in the active site owing to its resemblance to ATP during catalytic transfer to a tyrosine residue on the substrate protein. Presumably the γ -phosphoryl group of ATP moves out to a position on the enzyme molecule equivalent to the fourth position of Ap₄A during transfer to tyrosine. In support of this model, Ap₄A and only those diadenosine nucleotides with greater than three phosphoryl residues were found to be effective inhibitors. The reduced sensitivity of the cellular enzyme to inhibition by Ap₄A appears to be due to a reduced affinity for the second adenosine moiety, because pp60^{c-src} and pp60^{v-src} were similar in their affinities for Ap₄, ATP, and ADP. Differences in the primary structures of the two enzymes have been identified at their carboxyl termini (26) that could alter the conformation of the active site in such a way as to affect the binding of the Ap₄A molecule. Alternatively these conformational changes could result from differences in phosphorylation (11, 25) or palmitoylation (9, 24).

The possibility that Ap₄A is a regulator of pp60^{v-src} activity *in vivo* should be considered. Clearly Ap₄A is present in animal cells at micromolar concentrations and increases during cell proliferation (21). Assuming that pp60^{c-src} plays a role in growth control of normal cells, Ap₄A may participate in the regulation of pp60^{c-src} activity during cell cycle progression, possibly as a feedback inhibitor. In transformed cells, Ap₄A may not reach an inhibitory concentration. In this regard it is interesting that an enzyme that specifically degrades Ap₄A, a diadenosine tetraphosphatase, has recently been isolated from human leukemia cells in which it is present in high amount (18).

ACKNOWLEDGMENTS

This work was supported by grant PCM-8203857 from the National Science Foundation, grants 2-P01-CA19014 and 5-T32-CA09156 from the National Cancer Institute, and grant BC-416 from the American Cancer Society.

LITERATURE CITED

- Bishop, J. M., and H. Varmus. 1982. Functions and origins of retroviral transforming genes, p. 999-1108. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Blithe, D. L., N. D. Richert, and I. H. Pastan. 1982. Purification of a tyrosine-specific protein kinase from Rous sarcoma virus-induced rat tumor. *J. Biol. Chem.* 257:7135-7142.
- Brugge, J., E. Erikson, M. S. Collett, and R. L. Erikson. 1978. Peptide analysis of the transformation-specific antigen from avian sarcoma virus-transformed cells. *J. Virol.* 26:773-782.
- Brugge, J. S., and R. L. Erikson. 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature (London)* 269:346-348.
- Collett, M. S., J. S. Brugge, and R. L. Erikson. 1978. Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product. *Cell* 15:1363-1369.
- Collett, M. S., E. Erikson, A. F. Purchio, J. S. Brugge, and R. L. Erikson. 1979. A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. *Proc. Natl. Acad. Sci. U.S.A.* 76:3159-3163.
- Collett, M. S., and R. L. Erikson. 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. *Proc. Natl. Acad. Sci. U.S.A.* 75:2021-2024.
- Erikson, R. L., M. S. Collett, E. Erikson, and A. F. Purchio. 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic AMP-independent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* 76:6260-6264.
- Garber, E. A., J. G. Krueger, H. Hanafusa, and A. R. Goldberg. 1983. Only membrane-associated RSV *src* proteins have amino-terminally bound lipid. *Nature (London)* 302:161-163.
- Hunter, T., and B. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. U.S.A.* 77:1311-1315.
- Karess, R. E., and H. Hanafusa. 1981. Viral and cellular *src* genes contribute to the structure of recovered viruses. *Cell* 24:155-164.
- Karess, R. E., W. S. Hayward, and H. Hanafusa. 1979. Cellular information in the genome of recovered avian sarcoma virus directs the synthesis of transforming protein. *Proc. Natl. Acad. Sci. U.S.A.* 76:3154-3158.
- Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell* 15:561-572.
- Levinson, A. D., H. Oppermann, H. E. Varmus, and J. M. Bishop. 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. *J. Biol. Chem.* 255:11973-11980.
- Maness, P. F., H. Engeser, M. E. Greenberg, M. O'Farrell, W. E. Gall, and G. M. Edelman. 1979. Characterization of the protein kinase activity of avian sarcoma virus *src* gene product. *Proc. Natl. Acad. Sci. U.S.A.* 76:5028-5032.
- Maness, P. F., and B. T. Levy. 1983. Highly purified pp60^{src} induces the actin transformation in microinjected cells and phosphorylates selected cytoskeletal proteins *in vitro*. *Mol. Cell. Biol.* 3:102-112.
- Maness, P. F., M. E. Perry, and B. T. Levy. 1983. P¹, P⁴-Di(adenosine-5')tetraphosphate inhibits phosphorylation of immunoglobulin G by Rous sarcoma virus pp60^{src}. *J. Biol. Chem.* 258:4055-4058.
- Ogilvie, A., and W. Antl. 1983. Diadenosine tetraphosphatase from human leukemia cells. *J. Biol. Chem.* 258:4105-4109.
- Oppermann, H., A. D. Levinson, H. E. Varmus, L. Levintow, and J. M. Bishop. 1979. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (*src*). *Proc. Natl. Acad. Sci. U.S.A.* 76:1804-1808.
- Patschinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton. 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 79:973-977.
- Rapaport, E., and P. C. Zamecnik. 1976. Presence of diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap₄A) in mammalian cells in levels varying widely with proliferative activity of the tissue: a possible positive "pleiotypic activator". *Proc. Natl. Acad. Sci. U.S.A.* 73:3984-3988.
- Rohrschneider, L. R., R. N. Eisenman, and C. R. Leitch. 1979. Identification of a Rous sarcoma virus transformation-related protein in normal avian and mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 76:4479-4483.

23. Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart. 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. *Cell* 20:807-816.
24. Sefton, B. M., I. S. Trowbridge, J. A. Cooper, and E. M. Scolnick. 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. *Cell* 31:465-474.
25. Smart, J. E., H. Oppermann, A. P. Czernilofsky, A. F. Purchio, R. L. Erikson, and J. M. Bishop. 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60^{v-src}) and its normal cellular homologue (pp60^{c-src}). *Proc. Natl. Acad. Sci. U.S.A.* 78:6013-6017.
26. Takeya, T., and H. Hanafusa. 1982. DNA sequence of the viral and cellular *src* gene of chickens. II. Comparison of the *src* genes of two strains of avian sarcoma virus and of the cellular homolog. *J. Virol.* 44:12-18.
27. Takeya T., and H. Hanafusa. 1983. Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. *Cell* 32:881-890.
28. Wilkinson, G. N. 1961. Statistical estimates in enzyme kinetics. *Biochem. J.* 80:324-332.