A synthetic peptide containing the autophosphorylation site of the transforming protein of Harvey sarcoma virus is phosphorylated by the EGF-stimulated tyrosine kinase

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The transforming proteins (p21) of Harvey and Kirsten sarcoma viruses threonine kinase activity, which phosphorylates threonine 59 of the p21 proteins themselves. A tridecapeptide:

Arg–Arg–Leu[56]–Asp–Thr–Thr[59]–Gly–Gln–Glu–Tyr–Ser–Ala[66]

containing residues 56–66 of p21 is phosphorylated solely on tyrosine by the epidermal growth factor (EGF)-stimulated tyrosine kinase of A431 cell membranes. K_m-Values of 240 and 80 μM and V_{max} values of 1.7 and 0.1 nmol.min^{-1}.mg^{-1} were obtained in the presence and absence of EGF, respectively.

Epidermal growth factor receptor *Harvey virus oncogene* *Kirsten virus oncogene* *Protein p21* *Phosphothreonine* *Phosphotyrosine*

1. INTRODUCTION

The transforming proteins of many retroviruses possess the unusual ability to phosphorylate themselves and other proteins on tyrosine residues. Thus both the pp60 protein of Rous sarcoma virus [1] and the pp120 protein of Abelson leukemia virus [2] have associated tyrosine kinase activity. The observation that temperature sensitive mutations in both proteins affect kinase activity and transforming ability simultaneously suggests that tyrosine phosphorylation is intimately involved in the mechanism of transformation [3,4].

In contrast, the transforming proteins (p21) of Harvey and Kirsten sarcoma viruses possess threonine kinase activity [5]. The only reported acceptor site is threonine 59 of the p21 protein itself [5,6], and only GTP and dGTP can act as phosphate donors [5]. Although there is no direct evidence to connect threonine phosphorylation with transformation, binding of guanine nucleotides to the p21 of a mutant Kirsten virus temperature-sensitive for transformation is thermolabile [7]. The sequence surrounding threonine 59 in p21 from both Harvey and Kirsten virus [8,9] is:

Leu[56]–Asp–Thr–Thr[59]–Gly–Gln–Glu–Glu–Tyr–Ser–Ala[66]

The recognition site for tyrosine phosphorylation appears to be a sequence of acidic amino acids immediately N-terminal to the modified tyrosine [10–12]. To test whether the p21 Glu–Glu–Tyr sequence can be recognized by tyrosine kinases we have synthesized a peptide corresponding to residues 56–66 of p21, with 2 arginines attached to its N-terminus to permit separation from ATP and phosphate by binding to phosphocellulose paper [13]. We report here that this peptide is phosphorylated solely on tyrosine by the EGF-stimulated tyrosine kinase of A431 cell membranes.

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2. MATERIALS AND METHODS

A431 cell membranes were prepared and their protein concentrations determined as in [14] except that Ca^{2+} was omitted from the harvesting buffer to prevent Ca^{2+}-dependent proteolysis of the EGF-receptor [15]. EGF-α was prepared as in [16].

The tridecapeptide:

Arg–Arg–Leu–Asp–Thr–Thr–Gly–Gln–Glu–Glu–Tyr–Ser–Ala

was synthesized by the solid phase procedure in [17], except that the first amino acid was coupled to the chloromethyl polystyrene resin as in [18] and the peptide cleaved in HF. The free peptide (80 mg) was desalted in 0.1 M acetic acid on a Biogel P-2 column (1 × 85 cm) at 30 ml/h. The peak fractions were further purified by reverse-phase HPLC on a column of Ultrasphere octadecylsilica (15 cm × 4.6 mm i.d.) in 0.2% HFBA with a linear gradient of acetonitrile (0–50%) at 1 ml/min.

The peptide was phosphorylated for 5 min at 30°C in 30 μl of 20 mM Na + HEPES (pH 7.4), 2 mM $MnCl_2$, 0.2% Nonidet P40, 10 μM $Zn(acetate)_2$, 5 mM 2-mercaptoethanol, 50 μM [γ-^{32}P]ATP (20 Ci/μmol) and 5–10 μg A431 cell membranes, separated from the reaction mixture by binding to P81 phosphocellulose paper and counted as in [14]. Recovery of phosphorylated tridecapeptide (purified by anion exchange chromatography [19]) in this procedure was 99%.

To identify the phosphoamino acids present, samples of the anion exchange-purified peptide (2000 cpm) were hydrolysed and the phosphoamino acids separated by thin-layer electrophoresis on cellulose thin layers (Merck, Darmstadt) [20]. 2 μg each of phosphoserine (Sigma), phosphothreonine (Sigma) and phosphotyrosine (synthesized by IJS) were run as markers. Phosphoamino acids were detected by autoradiography (8 h) with a Dupont intensifying screen, and the marker amino acids were visualized with ninhydrin.

3. RESULTS

The tridecapeptide:
Arg–Arg–Leu–Asp–Thr–Thr–Gly–Gln–Glu–Glu–Tyr–Ser–Ala

consisting of amino acids 56–66 of Harvey p21 with 2 added arginines at the N-terminus, was synthesized as in section 2, desalted on a Biogel P-2 column and further purified by reverse-phase HPLC (fig.1). Amino acid analysis [21] of the indicated peptide yielded the correct composition of Asp (1.0), Thr (2.0), Ser (1.0), Glu (3.1), Gly (1.0), Ala (1.1), Leu (1.1), Tyr (1.0) and Arg (1.7).

The tridecapeptide was phosphorylated by A431 cell membranes. Double reciprocal plots of the effect of peptide concentration on reaction rate in the presence and absence of EGF-α were linear (fig.2). EGF-α stimulated the reaction ~20-fold by increasing the maximum velocity of phosphorylation (table 1). A 2–3-fold increase in K_m was also observed.

The tridecapeptide contains 4 potential phosphorylation sites, namely one serine, two threonines and one tyrosine. When partial acid hydrolysates of the peptide, phosphorylated in the

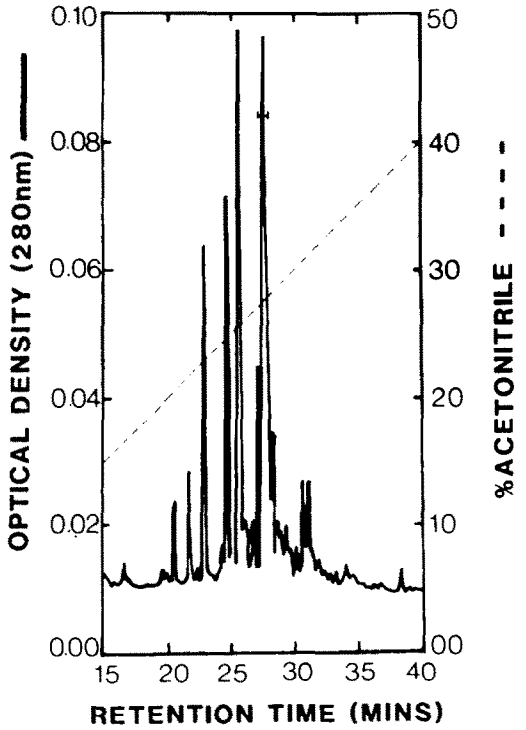

Fig.1. Purification of the Harvey p21 tridecapeptide. The crude tridecapeptide was desalted on a Biogel P-2 column, and further purified by reverse-phase HPLC on an Ultrasphere ODS column. The Harvey tridecapeptide eluted at 27.5 min. Experimental details are given in section 2.

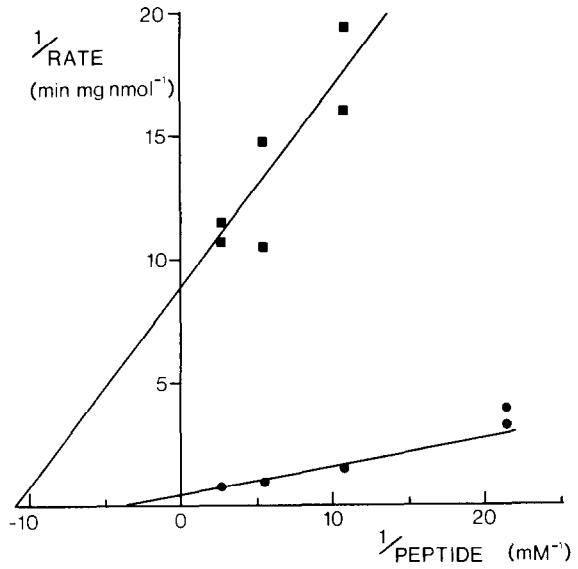

Fig.2. Double reciprocal plot for peptide phosphorylation. Peptide phosphorylation was assayed at 48–360 μM as in section 2 with (●) and without (■) 3 μg EGF-α/ml. Lines of best fit were constructed with the K_m and V_{max} values presented in table 1 expt 2.

Table 1

Kinetic constants for phosphorylation of the Harvey peptide

Peptide	$K_m \pm$ SE (μM)		$V_{max} \pm$ SE (nmol.min^{-1}.mg^{-1})	
	+EGF	−EGF	+EGF	−EGF
R.R.L.D.T.T.G.Q.E.E.Y.S.A. (Harvey peptide)				
Expt 1	243 ± 36	79 ± 39	1.65 ± 0.13	0.09 ± 0.01
Expt 2	242 ± 43	91 ± 49	2.25 ± 0.25	0.11 ± 0.02
R.L.I.E.D.N.E.Y.T.A.R.Q.E.amide (Rous peptide) [14]	4300	3500	6.4	1.4
R.R.L.E.E.E.E.E.A.Y.G (Gastrin peptide) [14]	150	300	1.7	0.4

Peptide phosphorylation was assayed with 2 separate membrane preparations as in section 2. K_m and V_{max} values were obtained by fitting the data to the Michaelis–Menten equation with a weighted least squares program [24]

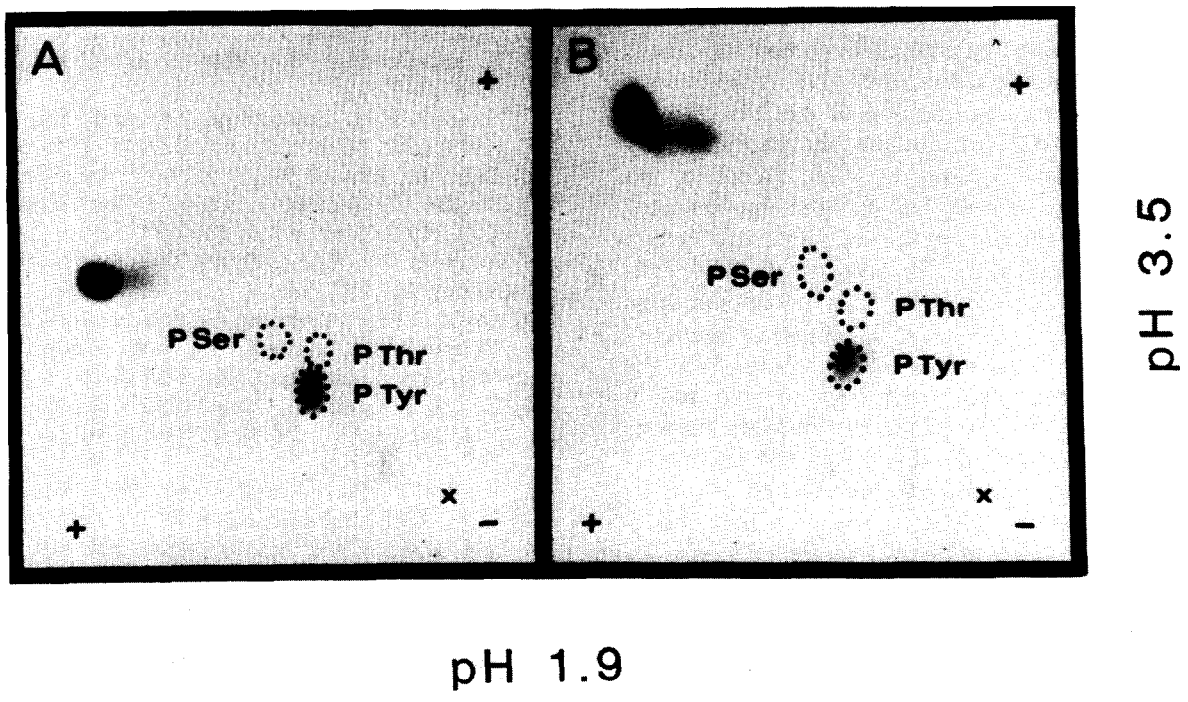

Fig.3. Phosphoamino acid composition of the Harvey p21 tridecapeptide. An amino acid hydrolysate of the phosphorylated peptide, labelled in the absence (A) and presence (B) of EGF-α, was analyzed by two-dimensional electrophoresis [20]: Markers were visualized with ninhydrin; (×) origin.

presence and absence of EGF-α, were subjected to two-dimensional electrophoresis, autoradiography revealed that radioactivity in both cases was incorporated only into phosphotyrosine (fig.3).

4. DISCUSSION

A peptide containing residues 56–66 of the p21 transforming proteins of Harvey and Kirsten murine sarcoma viruses was phosphorylated by the EGF-stimulated tyrosine kinase of A431 cell membranes. Values of both K_m and maximum velocity in the presence of EGF-α were ~20-fold less than the values obtained for a peptide comprising residues 412–424 of the p60 transforming protein of Rous avian sarcoma virus, but very similar to the values obtained for a peptide containing residues 5–13 of human gastrin 17 (table 1). In the absence of EGF-α, K_m and maximum velocity were consistently lower than the values obtained for the Rous and gastrin peptides (table 1).

Two-dimensional electrophoresis of acid hydrolysates of phosphorylated peptide revealed that phosphotyrosine was the only labelled amino acid. This observation confirms the finding with the SRC peptide that the EGF–receptor kinase is absolutely specific for tyrosine [11]. In addition it raises the intriguing question of whether tyrosine 64 of intact p21 can also be phosphorylated by ATP-dependent kinases in vivo. However, no tyrosine kinase activity has yet been demonstrated in cells transformed by Harvey virus, and in fact previous studies with $^{32}P_i$ labelling of mouse cells transformed by Harvey or Kirsten sarcoma virus [6], or with [γ-^{32}P]GTP labelling of partially purified p21 [5] have identified threonine 59 as the major phosphorylation site both in vivo and in vitro. The proximity of the two residues suggests that phosphorylation at tyrosine 64 might influence phosphorylation at threonine 59. If the latter modification indeed represents a phosphoenzyme intermediate in the p21 threonine kinase activity [5], then tyrosine phosphorylation could modulate that activity in the same way that phosphorylation of both middle T antigen and the SRC protein increases their tyrosine kinase activity [22,23]. Thus the results presented here suggest the possibility of a direct connection between two of the possible mechanisms of viral transformation, namely tyrosine and threonine phosphorylation.

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REFERENCES

[1] Czernilofsky, A.P., Levinson, A.D., Varmus, H.E., Bishop, J.M., Tischer, E. and Goodman, H.M. (1980) Nature 287, 198–203.
[2] Witte, O.N., Rosenberg, N., Paskind, M., Shields, A. and Baltimore, D. (1978) Proc. Natl. Acad. Sci. USA 75, 2488–2492.
[3] Collett, M.S. and Erikson, R.L. (1978) Proc. Natl. Acad. Sci. USA 75, 2021–2024.
[4] Witte, O.N., Goff, S., Rosenberg, N. and Baltimore, D. (1980) Proc. Natl. Acad. Sci. USA 77, 4993–4997.
[5] Shih, T.Y., Papageorge, A.G., Stokes, P.E., Weeks, M.O. and Scolnick, E.M. (1980) Nature 287, 686–691.
[6] Shih, T.Y., Stokes, P.E., Smythers, G.W., Dhar, R. and Oroszlan, S. (1982) J. Biol. Chem. 257, 11767–11773.
[7] Scolnick, E.M., Papageorge, A.G. and Shih, T.Y. (1979) Proc. Natl. Acad. Sci. USA 76, 5355–5359.
[8] Dhar, R., Ellis, R.W., Shih, T.Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D. and Scolnick, E.M. (1982) Science 217, 934–937.
[9] Tsuchida, N., Ryder, T. and Ohtsubo, E. (1982) Science 217, 937–939.
[10] Smart, J.E., Oppermann, H., Czernilofsky, A.P., Purchio, A.F., Erikson, R.L. and Bishop, J.M. (1981) Proc. Natl. Acad. Sci. USA 78, 6013–6017.
[11] Pike, L.J., Gallis, B., Casnellie, J.E., Bornstein, P. and Krebs, E.G. (1982) Proc. Natl. Acad. Sci. USA 79, 1443–1447.
[12] Hunter, T. (1982) J. Biol. Chem. 257, 4843–4848.
[13] Glass, D.B., Masaracchia, R.A., Feramisco, J.R. and Kemp, B.E. (1978) Anal. Biochem. 87, 566–575.
[14] Baldwin, G.S., Burgess, A.W. and Kemp, B.E. (1982) Biochem. Biophys. Res. Commun. 109, 656–663.
[15] Cassel, D. and Glaser, L. (1982) J. Biol. Chem. 257, 9845–9848.
[16] Burgess, A.W., Knesel, J., Sparrow, L.G., Nicola, N.A. and Nice, E.C. (1982) Proc. Natl. Acad. Sci. USA 79, 5753–5757.

[17] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[18] Gisin, B.F. (1973) Helv. Chim. Acta 56, 1476–1482.
[19] Kemp, B.E., Benjamini, E. and Krebs, E.G. (1976) Proc. Natl. Acad. Sci. USA 73, 1038–1042.
[20] Cooper, J.A. and Hunter, T. (1981) Mol. Cell. Biol. 1, 165–178.
[21] Simpson, R.J., Begg, G.S., Dorow, D.S. and Morgan, F.J. (1980) Biochemistry 19, 1814–1819.
[22] Courtneidge, S.A. and Bishop. J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 7117–7121.
[23] Segawa, K. and Ito, Y. (1982) Proc. Natl. Acad. Sci. USA 79, 6812–6816.
[24] Kemp, B.E. (1979) J. Biol. Chem. 254, 2638–2642.