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Fibroblasts Transformed with v-*src* Show Enhanced Formation of an Inositol Tetrakisphosphate

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The tyrosine kinase pp60$^{v\text{-}src}$, encoded by the v-*src* oncogene, seems to regulate phosphatidylinositol metabolism. The effect of pp60$^{v\text{-}src}$ on control points in inositol phosphate production was examined by measuring the amounts of inositol polyphosphates in Rat-1 cells expressing wild-type or mutant forms of the protein. Expression of v-*src* resulted in a five- to sevenfold elevation in the steady-state amount of an isomer of inositol tetrakisphosphate, whereas the concentrations of inositol trisphosphates or other inositol tetrakisphosphates were not affected. The activity of a key enzyme in the formation of inositol tetrakisphosphates, inositol (1,4,5)-trisphosphate 3-kinase, was increased six- to eightfold in cytosolic extracts prepared from the v-*src*–transformed cells, suggesting that this enzyme may be one target for the pp60$^{v\text{-}src}$ kinase and that it may participate in the synthesis of novel, higher order inositol phosphates.

ONCOGENES ARE ALTERED OR overexpressed forms of genes whose products participate in normal growth control, and oncogene products are presumed to induce malignancy by performing in an unregulated fashion activities that the proto-oncogene products control in normal cells (*1*). Thus, certain oncogene products are closely related to growth factors, mitogen receptors, and nuclear regulatory proteins (*1*). The protein encoded by the v-*src* oncogene, pp60$^{v\text{-}src}$ (*2*), may interact with inositol lipid signaling pathways because it regulates a membrane-bound phosphatidylinositol kinase (*3*) and stimulates phospholipid breakdown (*4*). However, control of the inositol signaling pathways is complex, with regulatory sites in both the membrane and cytoplasm (*5*).

To investigate the effect of expression of the v-*src* oncogene on inositol polyphosphate concentrations, we incubated growing, nearly confluent cultures of normal and *src*-transformed Rat-1 fibroblasts for 24 hours with [^{3}H]*myo*-inositol and examined the steady-state amounts of those isomers that are thought to serve as intracellular messengers, the inositol tris- and tetrakisphosphates (*5*). We added serum to the incubation medium so that the normal cells would continue to divide, thereby allowing us to compare normal growing cells with the constitutively proliferating v-*src*–transformed cells. The inositol polyphosphates present in extracts of normal and v-*src*–transformed cells were resolved on a strong anion-exchange column by high-performance liquid chromatography (HPLC) (*6*). When the results from five such experiments were averaged and corrected for sample protein content, the steady-state amount of inositol tetrakisphosphate (IP_4) was 6.2 (±1.9) times as great in v-*src*–transformed cells as in normal fibroblasts, whereas the amounts of inositol (1,4,5)-trisphosphate [$I(1,4,5)P_3$] and inositol (1,3,4)-trisphosphate [$I(1,3,4)P_3$] were similar in the two cell types (Fig. 1). The amount of IP_4 was not increased in Rat-1 cells containing the psV-*neo* vector alone. Our studies suggest that the high concentration of IP_4 in v-*src*–transformed cells is not due to an agonist

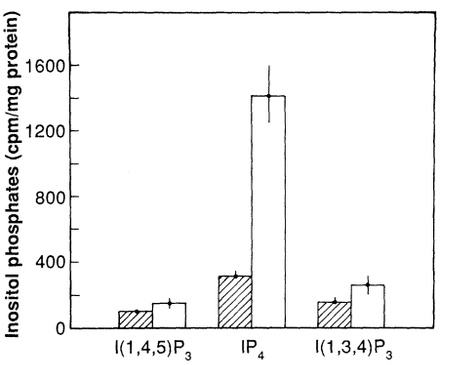

Fig. 1. Effect of expression of v-*src* on the steady-state amounts of inositol polyphosphates in Rat-1 cells. Normal (hatched bars) and v-*src*–transformed (open bars) Rat-1 fibroblasts were labeled with [^{3}H]*myo*-inositol for 24 hours, and ^{3}H-labeled inositol polyphosphates were extracted from the cells (*6*). The $I(1,3,4)P_3$, $I(1,4,5)P_3$, and IP_4 in the sample were resolved by HPLC on a strong anion-exchange column, and the counts in each compound were corrected for the protein corresponding to the sample applied to the column. Data represent the mean ± SEM from five experiments performed on two to three dishes of cells per experiment. The effect of v-*src* on IP_4 concentrations was significant at the $P < 0.05$ confidence interval using a one-tailed, paired *t* test.

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Table 1. Amounts of inositol phosphates in fibroblasts transfected with the v-*src* oncogene. Rat-1 cells were grown and labeled with [^{3}H]*myo*-inositol and the water-soluble inositol phosphates prepared for HPLC analysis (*6*). Cell lines were transfected with the *src* and *ras* oncogene constructs and selected as described (*8*). The data are expressed as the mean ± SEM of the fold increase over the amounts of inositol phosphates found in paired experiments with the appropriate control cells. Each experiment (*n*) represents one plating of cells in which triplicate dishes were analyzed by separate HPLC runs.

Cell type	Amount (fold increase over control)			*n*
	I(1,4,5)P_3	IP_4	I(1,3,4)P_3	
Normal Rat-1 (control)	1.0	1.0	1.0	
Rat-1 c-*src*	1.2 ± 0.1	1.3 ± 0.7	1.1 ± 0.3	3
Rat-1 v-*src*	0.9 ± 0.2	6.2 ± 1.9*	1.1 ± 0.3	5
Rat-1 c-*src* 527F	1.3 ± 0.1	4.3 ± 1.6*	1.3 ± 0.2	3
Rat-1 tsLA29 at 39.5°C (control)	1.0	1.0	1.0	
Rat-1 tsLA29 at 35.5°C	1.1 ± 0.3	2.3 ± 0.9*	1.1 ± 0.2	3
Swiss 3T3 (control)	1.0	1.0	1.0	
Swiss 3T3 *ras*	1.3 ± 0.2	0.9 ± 0.1	2.1 ± 0.4*	2
Swiss 3T3 v-*src*	0.9 ± 0.1	2.0 ± 0.1*	1.6 ± 0.2*	2

*$P < 0.05$ by one-tailed, paired *t* test.

effect of serum, as this phenomenon was also observed in v-*src*–transformed cells that were labeled with [^{3}H]*myo*-inositol in serum-free medium. Furthermore, the large increase in the amount of IP_4 was not seen in normal cells treated with bombesin, endothelin, epidermal growth factor, or serum for 3 to 5 min, even when the cells were incubated with LiCl before stimulation (*7*).

To determine if the tyrosine kinase activity of pp60$^{v\text{-}src}$ was required for the effect on cellular IP_4 content, we measured the amounts of inositol polyphosphates in cells expressing either a temperature-sensitive pp60$^{v\text{-}src}$ kinase (tsLA29 *src*) or an activated c-*src* kinase (Table 1). Rat-1 cells expressing the tsLA29 *src* contained greater amounts of IP_4 when grown at the permissive temperature for the activity of the pp60$^{v\text{-}src}$ kinase (35.5°C), as compared to the nonpermissive temperature (39.5°C) (*8*). The c-*src* mutant, in which the codon for tyrosine-527 was altered to encode phenylalanine, specifies a protein with an activated tyrosine kinase and is able to transform cells (*9, 10*). Rat-1 cells expressing this mutant c-*src* had IP_4 concentrations that approached those found in Rat-1 cells transformed by wild-type v-*src* (Table 1). A tenfold overexpression of the nontransforming pp60$^{c\text{-}src}$ proto-oncogene product did not significantly change the amounts of the three inositol polyphosphates in these cells. Our results show a correlation between the *src* product tyrosine kinase activity and greater amounts of IP_4 in rodent fibroblasts. The effect of *src* on IP_4 concentration was not restricted to Rat-1 cells, as an increased amount of IP_4, albeit of lesser magnitude, was also observed in Swiss 3T3 cells. The amount of IP_4 was not measurably elevated in Swiss 3T3 cells transformed by H-*ras*, suggesting that the effect is not a general property of transformed cells but may be specific to transformation by v-*src* (Table 1).

Because multiple isomers of IP_4 exist in cells (*11*), we modified the HPLC elution conditions (*12*) to determine which IP_4 isomers were affected by expression of pp60$^{v\text{-}src}$. Comparison of the elution positions of the IP_3 and IP_4 standards (Fig. 2A) with the pattern obtained from normal and transformed cells (Fig. 2B) revealed that v-*src*–transformed Rat-1 cells contained large amounts of an IP_4 isomer that is distinct from inositol (1,3,4,5)-tetrakisphosphate [I(1,3,4,5)P_4] and inositol (1,3,4,6)-tetrakisphosphate [I(1,3,4,6)P_4]. In *src*-transformed Rat-1 cells, including the tsLA29 and c-*src* 527 mutants described in Table 1, >95% of the labeled IP_4 was the novel isomer. On the basis of its elution position relative to that of I(1,3,4,5)P_4 and I(1,3,4,6)P_4 in similar gradients (*12*), we propose that this isomer is inositol (3,4,5,6,)-tetrakisphosphate [I(3,4,5,6)P_4]; indeed, it coelutes with an authentic I(3,4,5,6)P_4 standard on a weak anion-exchange column (*13*). However, until chemical analysis of its structure is performed, this assignment must remain tentative. The finding that the IP_4 isomer accumulating in the v-*src*–transformed cells is probably I(3,4,5,6)P_4 may explain why the concentrations of the inositol trisphosphates are not markedly altered by *src* transformation, in that this IP_4 isomer is not formed directly from I(1,4,5)P_3 (*11*).

Although the details of the pathways used to synthesize I(3,4,5,6)P_4 and higher forms of the inositol polyphosphates are still uncertain, it appears that the first step is the conversion of I(1,4,5)P_3 to I(1,3,4,5)P_4 (*11, 14*). The enzyme that catalyses this reaction is inositol (1,4,5)-trisphosphate 3-kinase [I(1,4,5)P_3 3-kinase] (*5, 11*). The I(1,4,5)P_3 3-kinase activity in cytosolic and particulate fractions from normal and v-*src*–transformed Rat-1 cells was assayed by measuring the rate of conversion of ^{3}H-labeled I(1,4,5)P_3 to I(1,3,4,5)P_4. About 85% of the 3-kinase activity in both cell types was found in the cytoplasm, in agreement with results from other cells (*15–17*). However, the amount of 3-kinase activity was significantly greater in v-*src*–transformed cells than in normal cells (Fig. 3A). The IP_4 isomer produced in this assay was I(1,3,4,5)P_4 on the basis of its elution position in the modified HPLC gradient and the quantitative conversion of the ^{3}H-labeled I(1,4,5)P_3 to IP_4 in the assay (Fig. 3B). To determine if activation of the 3-kinase was an early step in the *src* transformation process, we assayed the enzyme in cytosolic fractions prepared from tsLA29 *src*–infected Rat-1 cells at intervals between 1.5 and 24 hours after a

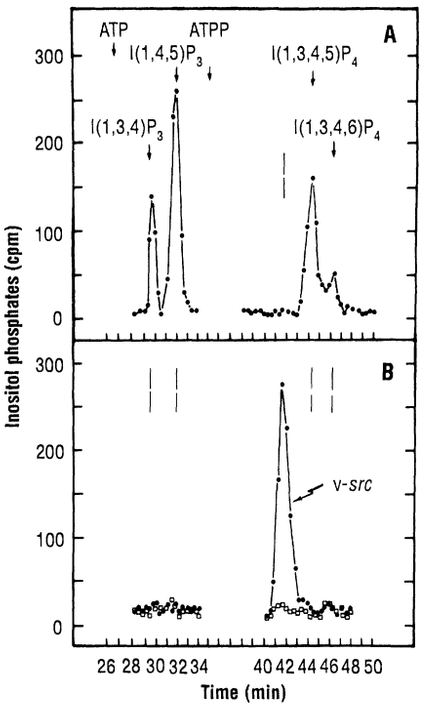

Fig. 2. Resolution of IP_4 isomers. (**A**) HPLC elution profile of a mixture of ATP, adenosine tetraphosphate (ATPP), and ^{3}H-labeled I(1,4,5)P_3, I(1,3,4)P_3, I(1,3,4,5)P_4, and I(1,3,4,6)P_4 standards. The conditions used to elute the isomers from the strong anion-exchange (SAX) column were modified to optimize the separation of the tetrakisphosphate isomers as described (*12*). Collection of 0.4-ml fractions at 20-s intervals began 28 min after injection of the sample and stopped 6 min later; collection restarted at 38 min and stopped at 50 min. This schedule minimized the number of fractions per sample. (**B**) Representative elution profile of ^{3}H-labeled inositol polyphosphates from one dish of normal (open boxes) or v-*src*–transformed (closed circles) Rat-1 fibroblasts. Elution conditions were as described in (A). Equal numbers of cells were seeded onto each dish, but data are not corrected for protein. Data are representative of ten experiments. The vertical dashed lines mark the elution positions of each inositol polyphosphate.

shift to the permissive temperature for expression of the mutant pp60$^{v\text{-}src}$ kinase activity (35.5°C). The 3-kinase was activated five- to sixfold within 1.5 to 3 hours of the temperature shift, suggesting that activation of this enzyme may be an early event in *src* transformation.

To characterize the stimulated 3-kinase activity in extracts of *src*-transformed cells, we partially purified the enzyme from normal and v-*src*–transformed cells by a modification of the phosphocellulose cation-exchange chromatography described by Johanson *et al.* (*17*). The increased activity expressed in crude cytosolic extracts from v-*src*–transformed cells was still apparent after column chromatography, with a peak of 3-kinase activity eluting at 0.05*M* K_2HPO_4 (Table 2). The activity of the 3-kinase in the partially purified extracts from both cell types was not significantly affected by the addition of 5 μ*M* calmodulin to the assay mixture (*7*). The fact that 3-kinase activity remains elevated in extracts of v-*src*–transformed cells after elution from a phosphocellulose column suggests that the activation of this enzyme is not mediated by a small molecule or cofactor.

Our findings suggest that cells expressing an active pp60$^{v\text{-}src}$ tyrosine kinase have at least two alterations in their inositol lipid signaling pathways, a markedly elevated I(1,4,5)P_3 3-kinase activity and an increased concentration of a particular IP_4 isomer. Currently, the roles of all inositol tetrakisphosphates are unclear. It has been proposed that I(1,3,4,5)P_4 regulates Ca^{2+} influx after acute hormonal stimulation (*18*), whereas the more recently discovered I(1,3,4,6)P_4 and I(3,4,5,6)P_4 have been observed to accumulate in chick red blood cells and under prolonged hormonal stimulation of other cell types (*12, 19*). The source of the novel IP_4 isomer is not established, but it seems unlikely to be the unique inositol phospholipid produced by the phosphatidylinositol kinase activated by v-*src*, as this inositol phospholipid is reported to have phosphate in the *d*-1 position (*3*). The finding that the activity of the I(1,4,5)P_3 3-kinase is markedly elevated in cells transformed with v-*src* is more germane, as this enzyme may be a control point for the subsequent synthesis of the novel inositol tetrakisphosphate isomers (*11, 14*). This protein has been purified or partially purified from several sources and found to be an adenosine 5′-triphosphate (ATP)–requiring enzyme that is sensitive to Ca^{2+} and calmodulin (*15–17, 20*). In addition, the enzyme in lymphocytes and hepatocytes may be activated by phosphorylation (*21*). It is possible that the activation of the 3-kinase in *src*-transformed cells is the result of posttranslational modification, such as phosphorylation. Alternatively, it could be due to an increase in the amount of the 3-kinase protein. Regardless of the mechanism, the marked effects of *src* transformation on inositol polyphosphate metabolism provide another link between the *src* oncogene and the inositol lipid signaling pathway.

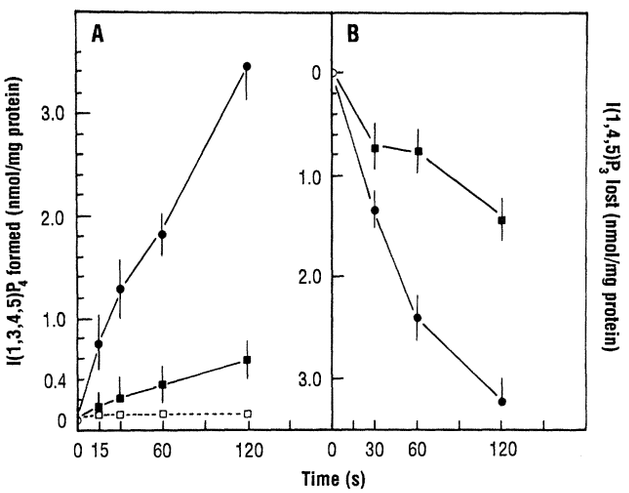

Fig. 3. (**A**) Production of ^{3}H-labeled I(1,3,4,5)P_4 from ^{3}H-labeled I(1,4,5)P_3 by cytosolic extracts prepared from normal (■) and v-*src*–transformed cells (●). (**B**) Loss of ^{3}H-labeled I(1,4,5)P_3 in the same assay. Cells were homogenized and I(1,4,5)P_3 3-kinase activity measured as described (*22*). No measurable amounts of I(1,3,4)P_3 were produced in the assay. The assay was terminated after 2 min because the rate of I(1,3,4,5)P_4 production fell below the initial rate, apparently because of reconversion to I(1,4,5)P_3, a phenomenon that has been described during the assay of the 3-kinase from human erythrocytes (*24*) and porcine brain cytosol (*25*). The results are expressed as nanomoles of I(1,3,4,5)P_4 formed per milligram of protein and are the mean ± SEM for assays from three cytosolic preparations. In (A) results are shown from a control experiment in which no protein was present in the assay (□).

Table 2. Partial purification of I(1,4,5)P_3 3-kinase from normal and v-*src*–transformed Rat-1 cells. Cytosolic fractions were prepared from normal or v-*src*–transformed Rat-1 cells as described (*22*), except that the cells were homogenized in 25 m*M* Hepes, 45 m*M* tris (*p*H 8.1), 1 m*M* $MgCl_2$, 2 m*M* EGTA, 1 m*M* dithiothreitol, and leupeptin (2 mg/ml). The cell extract was prepared for purification as described (*23*). The specific activity of the 3-kinase activity in the 100,000*g* supernatant fraction applied to the phosphocellulose was 0.4 nmol min^{-1} mg^{-1} in normal cells and 1.9 nmol min^{-1} mg^{-1} in v-*src* cells. The values for purification are multiples of these activities. The fold increase in specific activity of the v-*src*–transformed cells over the normal cells is shown in parentheses for each fraction. The results are representative of three separate experiments.

Fraction	Total activity (nmol min^{-1})	Total protein (mg)	Specific activity (nmol min^{-1} mg^{-1})	Purification (fold)
Void volume				
Rat-1	2.0	1.1	1.8	4.3
Rat-1 v-*src*	13.3	2.8	4.9 (2.7×)	2.6
Eluates				
0.05*M* phosphate				
Rat-1	1.0	0.6	1.8	4.4
Rat-1 v-*src*	17.7	1.4	12.4 (6.7×)	6.5
0.2*M* phosphate				
Rat-1	0.9	0.3	2.9	7.2
Rat-1 v-*src*	3.2	0.5	6.2 (2.1×)	3.2
0.4*M* phosphate				
Rat-1	0.2	0.1	1.6	3.7
Rat-1 v-*src*	1.0	0.4	2.3 (1.5×)	1.2

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Shawver, M. J. Weber, *J. Cell Physiol.* **131**, 450 (1987)]. The v-*src*–transformed Rat-1 cells were derived from Rat-1 cells transfected with plasmids carrying the v-*src* gene under the control of the long terminal repeat of Moloney murine leukemia virus (*9*). The cells were cotransfected with psV-*neo* to allow for selection based on resistance to the antibiotic G418. Colonies of cells expressing the $pp60^{v\text{-}src}$ protein were selected based on their morphological transformation and production of the $pp60^{v\text{-}src}$ protein as assayed by protein blotting and an in vitro kinase assay. Cells were maintained in humidified 7.5% CO_2 and 92.5% air at 37°C unless otherwise specified. Before confluency was achieved, the medium was changed, and the cells were incubated for 24 hours with [^{3}H]*myo*-inositol (10 μCi/ml) supplemented with 10 μ*M* unlabeled *myo*-inositol. The medium was then aspirated and the cells were rinsed three times with cold phosphate-buffered saline (PBS). The ^{3}H-labeled inositol polyphosphates were extracted from the cells by adding 0.75 ml of ice-cold 0.5 M $HClO_4$ containing 0.5 m*M* EDTA and 0.1 m*M* diethlenetriamine pentaacetic acid and incubating on ice for 30 min. The *p*H of the samples was adjusted to neutral and the samples were then centrifuged, filtered, and subjected to HPLC analysis as described [R. M. Johnson and J. C. Garrison, *J. Biol. Chem.* **262**, 17285 (1987)] except that the solvent used was $NH_4H_2PO_4$ (*p*H 3.7) instead of NH_4COOH. Protein was assayed as described [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)] after removal of the perchloric acid extracts and solubilization of protein in 1 ml of 0.1*M* NaOH and 1% SDS.

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8. Rat-1 cells containing a temperature-sensitive v-*src* mutant (LA29) were developed by infection with Rous sarcoma virus (tsLA29) of a subgroup D pseudotype as described [A. W. Stoker, P. J. Enrietto, J. A. Wyke, *Mol. Cell. Biol.* **4**, 1508 (1984); W. J. Wasilenko, L. K. Shawver, M. J. Weber, *J. Cell. Physiol.* **131**, 450 (1987)]. To express the active $pp60^{v\text{-}src}$ mutant protein, we incubated the infected cells at the permissive temperature (35.5°C) for 24 hours before labeling with [^{3}H]*myo*-inositol. Rat-1 cells overexpressing the normal c-*src* protein or the c-*src* 527 mutant protein (*9*) were derived by cotransfection of these genes and pSV-*neo* by the calcium phosphate technique. The colonies of cells were screened for morphological transformation and $pp60^{src}$ expression by protein blotting with antisera to $pp60^{src}$. The Swiss 3T3 cells were infected with an amphotrophic recombinant murine retrovirus containing either the v-*src* or the H-*ras* oncogene [S. M. Anderson and E. M. Scolnick, *J. Virol.* **46**, 594 (1983); R. C. Schwartz, L. W. Stanton, S. C. Riley, K. B. Marcu, O. N. Witte, *Mol. Cell. Biol.* 6, 3221 (1986)]. Colonies of Swiss 3T3 cells transformed with either oncogene were chosen by virtue of their ability to form colonies in soft agar.
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22. Normal or v-*src*–transformed Rat-1 cells were grown in 100-mm culture dishes (*6*). The medium was removed from five plates, and the cells were rinsed twice with 5 ml of PBS containing 0.1 m*M* EDTA. A further 1 ml of this solution was added per dish, and the cells were incubated another 5 to 10 min at 37°C to detach the cells from the dish. The cells from five dishes were pooled and centrifuged for 5 min. The supernatant was removed, and the cells were resuspended in 400 μl of homogenization buffer (*15*). Cells were homogenized at 4°C with 40 strokes of a Teflon pestle rotating at ~1500 rpm in a glass homogenizer. The homogenate was centrifuged at 200*g* for 10 min at 4°C to remove unbroken cells, and the supernatant was then centrifuged at 100,000*g* for 1 hour at 4°C. Assays for I(1,4,5)P_3 3-kinase were performed as described (*15*) except that the incubations were started with the addition of 60 μg of protein (~5 μl of 100,000*g* supernatant) to give a final volume of 100 μl containing 2.5 μ*M* IP_3. Free Ca^{2+} concentration of the assay medium was ~5 μ*M* as checked with a Ca^{2+}-sensitive electrode. The reaction was terminated by the addition of 10 μl of cold 5*M* $HClO_4$, 5 m*M* EDTA, and 1.0 m*M* diethylenetriamine pentaacetic acid and placing the tubes on ice. The *p*H of the samples was neutralized, and the formation of ^{3}H-labeled I(1,3,4,5)P_4 was measured by HPLC (*6*).
23. The homogenate was centrifuged at 100,000*g* for 1 hour at 4°C, the supernatant fraction was stirred on ice with 0.5 g of phosphocellulose for 1 hour, and the mixture was then packed into a 2-cm diameter column as described (*17*). Protein was eluted from the column at 4°C with the indicated concentrations of potassium phosphate buffer (*p*H 6.9) containing 1 m*M* dithiothreitol. Samples of each fraction were diluted to 0.1 mg of protein per milliliter and assayed for 3-kinase activity by monitoring the formation of I(1,3,4,5)P_4 after 60 s of incubation as described (*23*).
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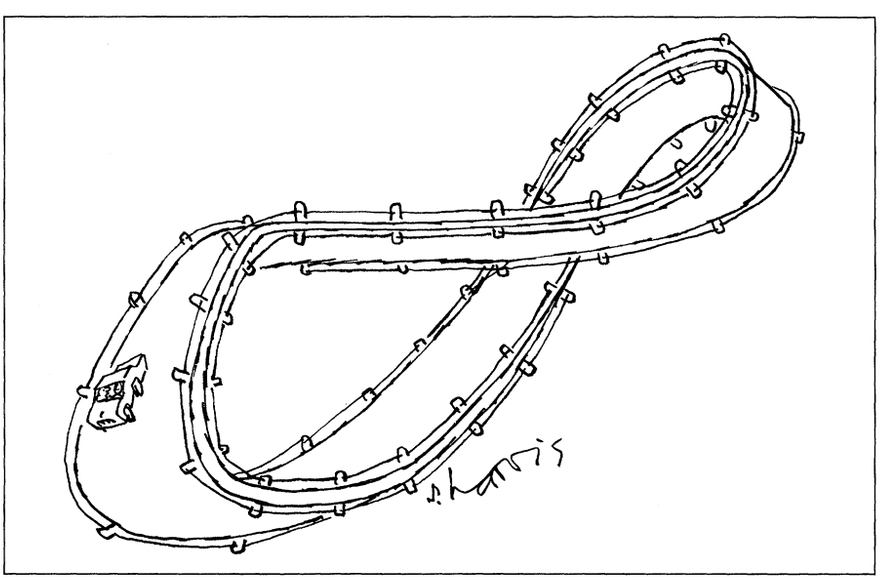

"Mobius? Who's Mobius?"