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Identification of Autophosphorylation Sites in c-Yes Purified from Rat Liver Plasma Membranes[1]

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c-Yes was purified 322-fold from a rat liver plasma membrane fraction to a single 60-kDa band on SDS-PAGE. The purified protein contained essentially no phosphotyrosine residues and was autophosphorylated with Mg^{2+}·ATP exclusively at tyrosine residues with a concomitant increase in the protein-tyrosine kinase activity. The autophosphorylated c-Yes was extensively digested by trypsin and the resultant two major phosphopeptides, peptides I and II, were purified by HPLC on a reversed-phase C-18 column. The amino acid sequence of peptide I was determined to be LIEDNEYTAR, which is identical with the sequence from Leu-418 through Arg-427 of mouse c-Yes, indicating that one of the autophosphorylation sites corresponds to Tyr-424 of the mouse c-Yes. After partial determination of the N-terminal sequence of 10 amino acid residues of peptide II, the 230 bp sequence of rat cDNA that encodes the N-terminal 76 amino acid residues of c-Yes covering peptide II, was determined. From the predicted amino acid sequence, the sequence of peptide II was assumed to be from Tyr-16 through Lys-46, YTPENPTEPVNTSAGHYGVE-HATAATTSSTK. The purified c-Yes phosphorylated the tyrosine residue of synthetic peptides covering Tyr-32 and its surrounding sequence but did not phosphorylate peptides covering Tyr-16 and its surrounding sequence, suggesting that the other autophosphorylation site is Tyr-32.

Key words: autophosphorylation sites, c-Yes, N-terminal sequence, purification, rat liver plasma membrane.

The protooncogene c-*yes* product, c-Yes, is a member of the *src*-family protein-tyrosine kinases which are encoded by closely related genes, lie in the size range of 505-543 amino acid residues, are highly conserved over a 460-residue contiguous region at the carboxyl terminus including SH2, SH3, and protein-tyrosine kinase domains, and differ greatly in an 80-residue contiguous region at the amino terminus. The kinase activity of c-Src is known to be negatively regulated by phosphorylation of its C-terminal Tyr-527 residue (*1-3*), whose surrounding sequence is well conserved in c-Yes (*4-9*). It is generally believed that the kinase activities are coordinately regulated by dephosphorylation and phosphorylation of this C-terminal tyrosine by protein-tyrosine phosphatases (*10*) and C-terminal Src kinase (*11, 12*) or autophosphorylation (*13*), respectively. The kinase activities are suggested to be also regulated by mechanisms other than C-terminal tyrosine phosphorylation, such as activation by autophosphorylation at Tyr-416 in c-Src (*3, 14-16*) and Ca^{2+}-dependent inactivation of c-Yes (*17*).

The sequences of c-Yes and c-Src, as well as other *src*-family tyrosine kinases, are divergent primarily in the N-terminal 80 amino acid residues, which begin with a myristoylated glycine. Although they lack a transmembrane domain, they are associated with the inner surface of plasma membranes *via* the myristoyl group (*18, 19*). The unique N-terminal regions of *src*-family tyrosine kinases may be involved in their interactions with distinct cellular proteins, such as membrane receptors, unique effectors or specific substrates, and may thus play a crucial role in the physiological actions of these kinases.

Our previous studies showed that c-Yes partially purified from rat liver plasma membranes and precipitated with a c-Yes specific monoclonal antibody, was mostly in a dephosphorylated form and was activated by the autophosphorylation of tyrosine residues, probably through intermolecular catalysis (*20*).

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Abbreviations: AMP-PNP, adenosine 5'-(β,γ-imido)triphosphate; dNTPs, deoxynucleoside triphosphates; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; PTH, phenylthiohydantoin; SH2, Src homology 2; SH3, Src homology 3; WGA, wheat germ agglutinin.

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In this study, we purified c-Yes to electrophoretic homogeneity and identified the autophosphorylation sites. c-Yes was autophosphorylated not only at a tyrosine residue which corresponds to Tyr-424 in mouse c-Yes and to the *in vitro*-autophosphorylation site, Tyr-416, of chicken c-Src, but also at a tyrosine residue in the unique N-terminal region of c-Yes, possibly Tyr-32.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP was obtained from ICN Radiochemicals. Sephacryl S-300 superfine, Ampholine carrier ampholytes, and AutoRead sequence kit were obtained from Pharmacia LKB Biotechnology. Toyopearl HW-65 superfine and a packed TSK gel ODS-120T column (25 × 0.46 cm) were from Tosoh. Casein (Hammersten) was obtained from Merck. Casein-Toyopearl was prepared as described previously (*21*). WGA-agarose was purchased from Hohnen Oil. A hydroxylapatite KB column (15 × 0.78 cm, Type S) was obtained from Koken. Bovine serum albumin, human γ-globulin, human angiotensin II, PMSF, AMP-PNP, protein A-Sepharose CL-4B, and Tyr-Glu (1:4) copolymers (M_r = 47,000) were purchased from Sigma Chemical. *N*-Tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin was obtained from Worthington Biochemical. Taq Extender PCR Additive was from Stratagene. Anti-c-Yes monoclonal antibody, MAb 3H9 (*22*), was provided by Dr. Kumao Toyoshima. Anti-phosphotyrosine monoclonal antibody conjugated with Sepharose was obtained from Amersham. Chymostatin, pepstatin, leupeptin, and antipain were obtained from Peptide Institute. Human placenta RNase inhibitor was purchased from Toyobo. Taq polymerase and Moloney murine leukemia virus RNase H$^-$ reverse transcriptase were obtained from Life Technologies. Synthetic peptides, PAIKYTPEN (1Y16) and SAGHYGVEH (1Y32) were purchased from Bio-Synthesis, and KENKSPAIKYTPEN (2Y16) and EPVNTSAGHYGVEH (2Y32) were obtained from Tana Laboratories L.C. Other chemicals were purchased from various commercial sources.

Buffer Solution—All the buffer solutions contained 0.5 mM PMSF, 0.3 μg/ml chymostatin, 0.3 μg/ml pepstatin, 2 μg/ml antipain, 1 μg/ml leupeptin, and 1 mM benzamidine as protease inhibitors. HEPES-Triton buffer contained 20 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 0.5 mM DTT, 5% (w/v) sucrose, 0.2% (v/v) Triton X-100, and protease inhibitors.

Assay for Protein-Tyrosine Kinase—Protein-tyrosine kinase activity was determined by measuring the amount of ^{32}P incorporated into Tyr-Glu copolymers (*20*). The standard assay mixture (60 μl) contained 20 mM HEPES-NaOH, pH 7.4, 20 mM $MgCl_2$, 0.5 mM DTT, 50 μM sodium vanadate (Na_3VO_4), 0.2% Triton X-100, 0.5 mg/ml Tyr-Glu copolymers, 15 μM [γ-^{32}P]ATP (2-6 × 10^2 cpm/pmol), and enzyme preparation. Enzyme units were expressed by the square root of the initial rates (pmol/min) of the reaction under the standard assay conditions (*20*).

Purification of c-Yes—Twelve male Wistar albino rats (200-250 g) were starved overnight and killed by decapitation. All manipulations were carried out at 0-4°C. Membrane fractions (P_2 fraction) were prepared from 79 g of livers as described previously (*20*). c-Yes was solubilized (Triton X-100 fraction) from the membrane fractions, and partially purified by successive column chromatographies on Sephacryl S-300 (Sephacryl S-300 fraction) and WGA-agarose (WGA-agarose fraction) as described previously (*20*). After reducing the NaCl concentration in the WGA-agarose fraction to 30 mM, the enzyme solution was applied to a casein-Toyopearl column (19 × 1.6 cm) equilibrated with HEPES-Triton buffer at a flow rate of 40 ml/h. The column was washed with 40 ml of HEPES-Triton buffer, and the enzyme was eluted at a flow rate of 20 ml/h with a 360 ml linear (0-0.6 M) NaCl gradient in HEPES-Triton buffer. The active fractions indicated by a solid bar in Fig. 1 were pooled and stored at −80°C. These initial steps of enzyme preparation were repeated with livers from another 12 rats. The active fractions from casein-Toyopearl chromatography of both preparations obtained from 24 rats in total were pooled, concentrated to 24 ml by ultrafiltration through an Amicon YM-10 membrane (casein-Toyopearl fraction), and applied to a hydroxylapatite KB column (15 × 0.78 cm) equilibrated with HEPES-Triton buffer without EDTA. The enzyme was eluted at a flow rate of 24 ml/h with a 100 ml linear (0-0.15 M) gradient of sodium phosphate buffer, pH 7.4 in HEPES-Triton buffer without EDTA. The active fractions indicated by a solid bar in Fig. 2 were pooled, concentrated to 9 ml by ultrafiltration with an Amicon YM-10 membrane to reduce the phosphate concentration to below 10 mM (hydroxylapatite fraction), and subjected to isoelectrofocusing electrophoresis using an LKB Ampholine column (110 ml) as previously described (*20*) (Fig. 3). After having been neutralized with 0.1 ml of 1 M HEPES-NaOH, pH 7.4, active fractions were combined (Ampholine fraction) and could be stored at −80°C without any loss of activity for at least 6 months.

Gel Electrophoresis and Immunoblotting—SDS-PAGE utilizing 10% gel and Western immunoblotting with MAb

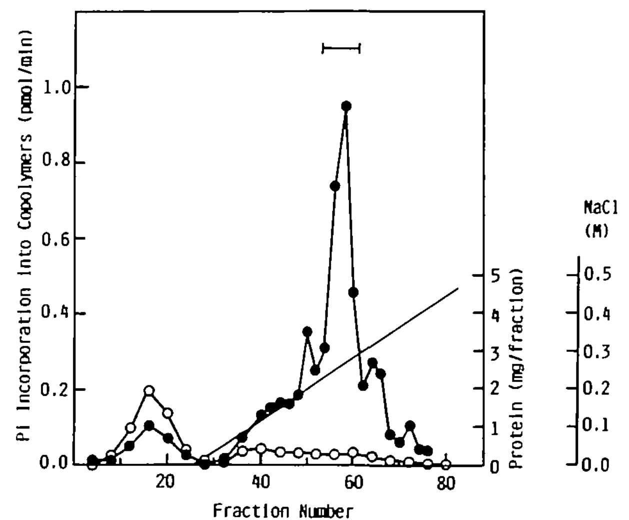

Fig. 1. **Casein-Toyopearl column chromatography of c-Yes.** WGA-agarose fraction (60 ml, 70 mg protein, 21,800 units) was applied to a casein-Toyopearl column (19 × 1.6 cm). Elution and assay for protein concentration (○), were performed as described under "EXPERIMENTAL PROCEDURES." Rates of P_i incorporation into Tyr-Glu copolymers (●) were measured with 10 μl of each fraction under the standard assay conditions. Fractions of 5 ml were collected. The active fractions (⊢⊣) were pooled.

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3H9 were performed as described previously (20). Protein bands were visualized by the use of Coomassie Blue R-250. Molecular mass standards were rabbit skeletal muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), and heavy chain (50 kDa) and light chain (23.5 kDa) of human γ-globulin.

Autophosphorylation of c-Yes—Autophosphorylation of the purified c-Yes (11,000 units, 136 μg protein) was carried out at 30°C for 30 min in six separate 900 μl reaction mixtures containing 30 μM [γ-^{32}P]ATP, 20 mM HEPES-NaOH, pH 7.4, 20 mM $MgCl_2$, 0.2% Triton X-100, 30 μM sodium vanadate, 0.5 mM DTT, then the reaction was stopped by adding 100 μl of 100% (w/v) trichloroacetic acid and the mixture was stored on ice for 30 min. The suspension was centrifuged at 15,000 $\times g$ for 10 min and the precipitate, which consisted of a protein upper layer and a Triton X-100 lower layer, was washed with 600 μl of ice-chilled 10% trichloroacetic acid. The trichloroacetic acid precipitate was finally washed with 400 μl of ice-chilled acetone to remove trichloroacetic acid as well as Triton X-100, and dried at room temperature. The precipitate was dissolved in 195 μl of SDS addition buffer containing 40 mM Tris-HCl, pH 7.8, 3% (w/v) SDS, 5% (v/v) glycerol, 0.017% (w/v) bromophenol blue, and 4 mM DTT. The mixture was heated at 40°C for 2 h and subjected to SDS-PAGE. The gel was stained with Coomassie Blue, dried, and autoradiographed at −80°C using an X-ray film (Fuji RX) with intensifying screens (Fuji Hi-Screen) to identify the radioactive 60-kDa bands of autophosphorylated c-Yes. The 60-kDa bands were excised from the gel, and the ^{32}P-radioactivity was measured in a vial with 10 ml of distilled water by counting Cerenkov radiation.

Tryptic Digestion of ^{32}P-Labeled c-Yes—^{32}P-labeled c-Yes (74 μg) in hydrated gel pieces (less than 1 mm in size) was placed in 500 μl of 50 mM NH_4HCO_3, pH 8.2, and digested with shaking for 24 h at 37°C by adding 1:200 (w/w) trypsin five times. During the incubation, the buffer was changed three times. The supernatant taken from the gel suspension was further incubated at 37°C with newly added 1:400 (w/w) trypsin in parallel with the digestion of ^{32}P-labeled c-Yes in gel suspension. Totally, 1:30 (w/w) trypsin was used for the digestion of ^{32}P-labeled c-Yes. All supernatants were combined and lyophilized.

HPLC of Tryptic Phosphopeptides—The lyophilized tryptic phosphopeptides derived from ^{32}P-labeled c-Yes were dissolved in 0.8 ml of 5% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (Solvent A) and filtered with a Millipore filter (0.45 μm)-attached syringe. The filtrate was applied to a C-18 column (25 $\times$ 0.46 cm, TSK gel ODS-120T) equilibrated with Solvent A. The column was developed with an increasing gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.4 ml/min. The gradient was 5% acetonitrile for 10 min, 5-12.5% acetonitrile over 10 min, 12.5-31.3% acetonitrile over 60 min, 31.3-72.5% acetonitrile over 5 min, and 72.5% acetonitrile for 5 min. The peptide peaks were monitored at 220 nm. The ^{32}P-radioactivity in fractions was evaluated by counting Cerenkov radiation.

Amino Acid Sequence Analysis—The N-terminal amino acid sequences of the tryptic phosphopeptides were determined by Edman degradation on an Applied Biosystems model 470A sequencer equipped with a model 120 PTH-amino acid analyzer. About 100 pmol of phosphopeptides was subjected to analysis, and 15 cycles and 20 cycles of degradation and analysis were performed on peptides I and II, respectively.

Preparation of cDNA from Rat Liver Total RNA—Total RNA was extracted from rat liver by the guanidinium

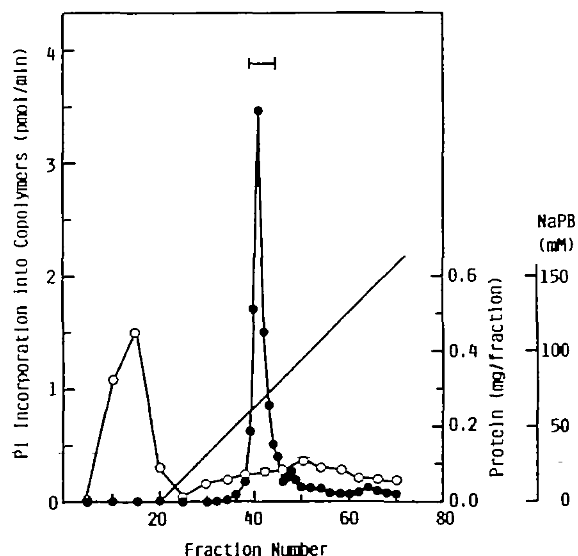

Fig. 2. **Hydroxylapatite column chromatography of c-Yes.** Casein-Toyopearl fraction (24 ml, 3.16 mg protein, 7,970 units) was applied to a hydroxylapatite KB column (15 $\times$ 0.78 cm). Elution and assay for protein concentration (○) were performed as described under "EXPERIMENTAL PROCEDURES." Rates of P_i incorporation into Tyr-Glu copolymers (●) were measured with 10 μl of each fraction under the standard assay conditions. Fractions of 2 ml were collected. The active fractions (⊢⊣) were pooled.

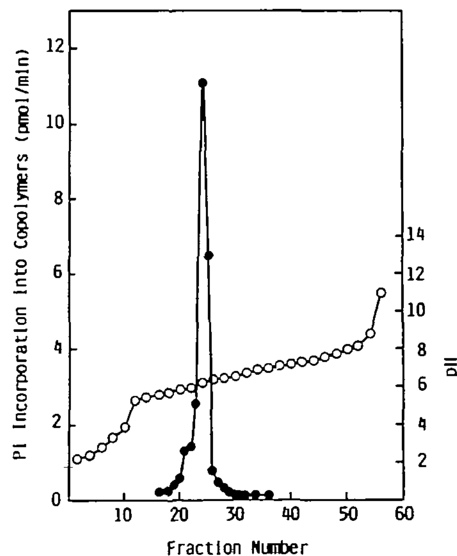

Fig. 3. **Isoelectrofocusing electrophoresis of c-Yes.** Hydroxylapatite fraction (9 ml, 0.88 mg protein, 2,350 units) was applied to an Ampholine 8100-1 electrofocusing column (110 ml). Electrofocusing was performed with carrier ampholytes (pH 5-7) as described under "EXPERIMENTAL PROCEDURES." Fractions of 1.7 ml were collected. Rates of P_i incorporation into Tyr-Glu copolymers (●) were measured with 5 μl of each fraction under the standard assay conditions. pH (○) of each fraction was measured.

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thiocyanate/cesium chloride method (*23*). The RNA sample (5 μg) was mixed with 50 pmol of random hexanucleotides, heated at 70°C for 10 min, and then subjected to reverse transcription in a final volume of 50 μl containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 50 units of RNase inhibitor from human placenta, and 1,000 units of Moloney murine leukemia virus RNase H^- reverse transcriptase. The reaction mixture was incubated at 37°C for 60 min, heated at 95°C for 10 min, and then cooled on ice.

Preparation of Rat c-yes cDNA—Amplification of rat c-*yes* cDNA was achieved by PCR, using synthetic oligonucleotide primers derived from the nucleotide sequence of mouse c-*yes* cDNA (*9*). The sequences of the primers were 5′-AGTAAAGAAAACAAAAGTCCAGC-3′ (primer S1, residues from 618 through 640, sense strand), 5′-CGTGG-AGCGAGCGGATTTGA-3′ (primer S2, residues from 581 through 600, sense strand), and 5′-AGGATATGAACTTG-ACACCACTGA-3′ (primer A, residues from 831 through 854, antisense strand). Primers S1 and A, and primers S2 and A would yield 237 and 274 bp products corresponding to the amino acid residues from Ser-6 through Pro-84 and from Met-1 through Pro-84, respectively. One-tenth of the cDNA was subjected to amplification in a volume of 50 μl containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 0.2 mM dNTPs, 0.25 μM sense and antisense primers, 2 units of Taq polymerase, and 2 units of Taq Extender PCR Additive. After denaturation for 2 min at 94°C, the PCR was performed in a programmable thermal controller for 45 cycles, each consisting of 40-s denaturation at 92°C, 30-s annealing at 50°C, and 1-min extension at 72°C. The amplified fragment was analyzed in ethidium bromide-stained 1.5% agarose gel.

Determination of Nucleotide Sequence—Rat liver c-*yes* cDNA was ligated to the *Sma*I site of pUC19 vector and sequenced by the dideoxy chain-termination method (*24*) using an AutoRead sequence kit and an A.L.F. DNA sequencer (Pharmacia). The nucleotide sequences corresponding to the amino acid residues from Met-1 through Pro-13 and from Ile-14 through Phe-76 were confirmed with three and eight independent clones, respectively.

Other Procedures—Phosphoamino acid analyses were performed as described before (*20*). Protein was determined by the method of Bradford (*25*) with bovine serum albumin as the standard.

RESULTS

Purification of c-Yes from Rat Liver Plasma Membranes—c-Yes was solubilized from the rat liver plasma membrane fraction with a buffer containing 2% Triton X-100 and 0.5 M NaCl, and purified by successive column chromatographies on Sephacryl S-300, WGA-agarose, casein-Toyopearl (Fig. 1), and hydroxylapatite (Fig. 2), followed by isoelectric focusing on an Ampholine column (Fig. 3). A main activity peak with pI 6.2 gave a single Coomassie Blue-stained band with an estimated molecular mass of 60 kDa on SDS-PAGE (Fig. 4). The purified 60-kDa protein was identified as c-Yes by immunoprecipitation and Western blotting with c-Yes specific monoclonal antibody, MAb 3H9 (data not shown). c-Yes was purified 322-fold from the rat liver plasma membrane fraction with an overall yield of 3%, and had a specific activity of 87,600 units/mg (Table I).

Autophosphorylation and Activation of c-Yes—When the purified c-Yes was incubated with 30 μM [γ-^{32}P]ATP at 30°C for 30 min, it was phosphorylated exclusively at tyrosine residues (data not shown) with a stoichiometry of 0.7 mol of phosphate incorporated per mol of c-Yes. The protein-tyrosine kinase activity of the purified c-Yes was stimulated 3.9-fold by preincubation with 30 μM ATP, but was not stimulated by preincubation with 30 μM AMP-PNP (Table II). When the purified c-Yes was preincubated with ATP, and then incubated with a Sepharose-conjugated anti-phosphotyrosine monoclonal antibody, about 60% of the total protein-tyrosine kinase activity recovered after the treatment was immunoprecipitated (Table II). On the other hand, when c-Yes was preincubated without ATP, only 2% of the protein-tyrosine kinase activity was immunoprecipitated with the anti-phosphotyrosine antibody (Table II). The same results were obtained without preincubation, indicating that dephosphorylation of tyrosine in the enzyme did not take place during the preincubation. These results indicate that the purified c-Yes contained essentially no phosphotyrosine residue, and was stimulated by autophosphorylation at tyrosine residues.

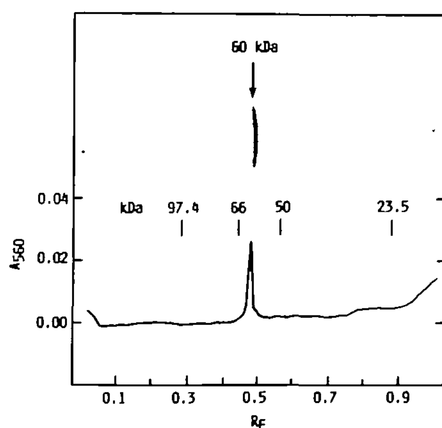

Fig. 4. **SDS-PAGE of the purified c-Yes.** The purified c-Yes (Ampholine fraction, 39 units, 0.38 μg protein) was subjected to SDS-PAGE and stained with Coomassie Blue as described under "EXPERIMENTAL PROCEDURES." The stained gel was scanned at 560 nm with a Gelman DCD-16 digital computing densitometer. The protein band of c-Yes is indicated by an arrow. For reference, the positions of protein markers (kDa) electrophoresed in parallel are shown.

TABLE I. **Purification of c-Yes from rat liver membrane fractions.** The enzyme was prepared from 158 g of rat liver. Purification and assay procedures were described under "EXPERIMENTAL PROCEDURES."

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Plasma membrane	278	75,500	272	1.0	100
Triton X-100	270	73,500	272	1.0	97
Sephacryl S-300	139	44,500	320	1.2	59
WGA-agarose	136	45,600	335	1.2	60
Casein-Toyopearl	3.16	7,970	2,520	9.3	11
Hydroxylapatite	0.88	2,350	2,670	9.8	3
Ampholine	0.029	2,540	87,600	322	3

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Identification of Autophosphorylation Sites of c-Yes—The autophosphorylated c-Yes was extensively digested with trypsin, and the resultant tryptic phosphopeptides were analyzed by HPLC on a reversed-phase C-18 column (Fig. 5). Two radioactive phosphopeptides, denoted by peptide I and peptide II, were eluted at acetonitrile concentrations of 17 and 20%, respectively. To eliminate the possibility that contaminating tyrosine kinases phosphorylate c-Yes, the purified c-Yes was immunoprecipitated with c-Yes-specific monoclonal antibody, then autophosphorylated, and the tryptic phosphopeptides were analyzed. The immunoprecipitated c-Yes produced an elution profile of the tryptic phosphopeptides similar to that of the purified c-Yes (data not shown), indicating that peptide I and peptide II indeed represent autophosphorylation sites of c-Yes.

The N-terminal amino acid sequences were determined by an amino acid sequencer to be LIEDNEYTAR for peptide I and YXPENPXEPV for peptide II, where X is an amino acid residue whose assignment was ambiguous.

The amino acid sequence of peptide I is identical with the sequence from Leu-420 through Arg-429, LIEDNEYTAR, of human c-Yes (*4*), and with the sequence from Leu-418 through Arg-427 of mouse c-Yes (*9*). The same sequence was also found in chicken c-Src from Leu-410 through Arg-419 (*26*), human Fyn from Leu-414 through Arg-423 (*27, 28*), and murine Lck from Leu-388 through Arg-397 (*29*). Since only one tyrosine residue exists in peptide I, one of the *in vitro*-autophosphorylation sites in rat c-Yes was determined to be the tyrosine residue corresponding to Tyr-424 in mouse c-Yes. In chicken c-Src, Tyr-416 has been determined to be the major *in vitro*-autophosphorylation site (*30*).

The N-terminal amino acid sequence of peptide II corresponds to the sequence from Tyr-16 through Val-25 in c-Yes from mouse (*9*) and human (*4*). To facilitate the localization of phosphorylated tyrosine residues, the 230 bp sequence of rat cDNA that encodes the N-terminal unique region of c-Yes, including peptide II, was determined (Fig. 6). The amino acid sequence predicted from the rat cDNA sequence shows 91 and 76% identity to the corresponding amino acid sequence of c-Yes from mouse and human, respectively (Fig. 6). From the predicted amino acid

TABLE II. **Immunoreaction of c-Yes with an antibody against phosphotyrosine.** The purified c-Yes (6.4 units, 78 ng protein) was preincubated with or without 30 μM ATP or AMP-PNP at 30°C for 30 min in 60 μl of a standard assay mixture. The reaction was stopped by adding 10 μl of 200 mM EDTA and then 30 μl of HEPES-Triton buffer. Sixty microliters of the mixture was incubated at 0°C for 2 h with a Sepharose-conjugated monoclonal antibody against phosphotyrosine (7.5 μl gel, 106 μg of antibody), with resuspension by gentle mixing with a glass rod every 15 min, and then centrifuged for 3 min at 16,000×g. The supernatant was used as the enzyme sample (Supernatant). The precipitate was washed once with 200 μl of HEPES-Triton buffer, and associated c-Yes was extracted three times with 60 μl of 27 mM p-nitrophenyl phosphate in HEPES-Triton buffer at 0°C by gentle mixing four times with a glass rod every 5 min. The combined extracts were used as the enzyme sample (Immunoprecipitate). P_i incorporation into Tyr-Glu copolymers was measured under the standard assay conditions with 10 μl of enzyme samples.

Enzyme sample	P_i incorporation (pmol/min) into copolymers by enzyme sample		
	c-Yes preincubated with		
	None	AMP-PNP	ATP
Untreated	0.406	0.413	1.60
Treated with antibody			
Supernatant	0.261	–	0.249
Immunoprecipitate	0.006	–	0.421

Fig. 5. **Preparation of tryptic phosphopeptides from autophosphorylated c-Yes.** The purified c-Yes (11,000 units, 136 μg protein) was autophosphorylated to a stoichiometry of 0.72. After having been isolated by SDS-PAGE, the ^{32}P-labeled c-Yes (74 μg) was digested extensively with 1:30 (w/w) trypsin. The tryptic phosphopeptides were purified by reversed-phase HPLC on a C-18 column. Fraction volumes were as follows: 0.27 ml from fraction 1 to 45, 0.17 ml from fraction 46 to 135, and 1.00 ml, fractions over 135. The details of the experiments were described under "EXPERIMENTAL PROCEDURES."

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      TAATGGGCTGCATTAAAAGTAAAGAAAACAAAAGTCCAGCCATTAAA
Rat    M  G  C  I  K  S  K  E  N  K  S  P  A  I  K
Mouse  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·
Human  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·
       1           5              10             15
       TATACACCGGAAAATCCTACAGAGCCTGTAAACACAAGTGCCGGT
Rat    Y  T  P  E  N  P  T  E  P  V  N  T  S  A  G
Mouse  ·  ·  ·  ·  ·  L  ·  ·  ·  ·  S  P  ·  ·  S
Human  ·  R  ·  ·  ·  T  P  ·  ·  ·  S  ·  ·  V  S
       16          20             25             30
       CATTATGGAGTGGAACATGCTACAGCTGCCACGACCTCTTCCACA
Rat    H  Y  G  V  E  H  A  T  A  A  T  T  S  S  T
Mouse  ·  ·  ·  ·  ·  ·  ·  ·  V  ·  P  ·  ·  ·  ·
Human  ·  ·  ·  A  ·  P  T  ·  V SPCP S  ·  ·  A
       31          35             40             45
       AAGGGAGCATCAGCTAATTTTAACAGTCTTTCCATGACACCCTTT
Rat    K  G  A  S  A  N  F  N  S  L  S  M  T  P  F
Mouse  ·  ·  ·  ·  V  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·
Human  ·  ·  T  A  V  ·  ·  S  ·  ·  ·  ·  ·  ·  ·
       46          50             55             60
       GGAGGGTCCTCTGGGGTGACTCCTTTTGGAGGAGCATCTTCCTCATTC
Rat    G  G  S  S  G  V  T  P  F  G  G  A  S  S  S  F
Mouse  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·
Human  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·
       61          65             70             75
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Fig. 6. **Partial nucleotide and deduced amino acid sequences of rat c-*yes* cDNA, and comparison with the predicted amino acid sequences from mouse and human c-*yes* cDNAs.** The deduced amino acid sequences of rat, mouse (*9*), and human (*4*) c-*yes* cDNAs are aligned in accordance with the sequence homology between them. Amino acids of the mouse and human sequences are shown only where they are different from those of the rat sequence. Conserved amino acids are marked with (·). Amino acids were numbered according to the predicted amino acid sequences of rat and mouse c-*yes* cDNAs.

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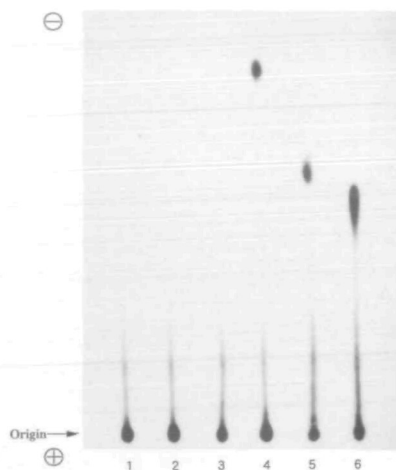

Fig. 7. **Phosphorylation of peptides covering the sequence surrounding Tyr-16 or Tyr-32 by c-Yes.** Phosphorylation was carried out at 30°C for 60 min in a 30 μl mixture containing 10 mM HEPES-NaOH, pH 7.4, 20 mM $MgCl_2$, 0.5 mM DTT, 0.1% Triton X-100, the purified c-Yes (1.72 units, 20 ng protein), and 30 μM [γ-^{32}P]ATP (3.5×10^3 cpm/pmol) in the absence (lane 1) or presence of 600 μM peptide 1Y16 (lane 2), 2Y16 (lane 3), 1Y32 (lane 4), 2Y32 (lane 5), or angiotensin II (lane 6). The reaction was terminated by heating the mixture for 5 min at 60°C. One microliter of sample was spotted on Whatman 3MM paper, and electrophoresed at pH 3.5 for 120 min at 3 kV in pyridine/acetic acid/H_2O, 1 : 10 : 89 (v/v). After electrophoresis, the paper was dried and exposed for 4 days to an X-ray film (Fuji RX) with an intensifying screen (Fuji Hi-Screen) at −80°C. P_i incorporation into 1Y32, 2Y32, and angiotensin II was 1.2, 1.3, and 3.4 pmol, respectively.

sequence, the amino acid sequence of peptide II was assumed to be from Tyr-16 through Lys-46, in which two tyrosine residues, Tyr-16 and Tyr-32, exist (Fig. 6).

Since the autophosphorylation of c-Yes was assumed to be intermolecular (*20*), the phosphorylation of synthetic peptides covering the sequences around Tyr-16 or Tyr-32 by c-Yes was examined to determine the autophosphorylated tyrosine residue in peptide II (Fig. 7). c-Yes phosphorylated only Tyr-32-containing peptides, SAGHYGVEH (1Y32) and EPVNTSAGHYGVEH (2Y32) (Fig. 7, lanes 4 and 5), but not Tyr-16-containing peptides, PAIKYTPEN (1Y16) and KENKSPAIKYTPEN (2Y16) (Fig. 7, lanes 2 and 3). Angiotensin II, a general tyrosine kinase substrate (*31, 32*), was phosphorylated by c-Yes and used as a standard for the relative mobility of phosphorylated peptides in high-voltage paper electrophoresis (Fig. 7, lane 6). The relative mobility of each radioactive spot was well correlated with the expected net charge of the respective ^{32}P-peptides. Phosphoamino acid analysis of phosphorylated peptides extracted from each spot showed that only phosphotyrosine was detected (data not shown). These results suggest that the N-terminal autophosphorylation site in rat liver c-Yes is Tyr-32.

DISCUSSION

In this study, c-Yes was purified to a single protein band on SDS-PAGE (Fig. 4) by introducing column chromatography on casein-Toyopearl and HPLC on hydroxylapatite, which effectively removed contaminating proteins (Figs. 1 and 2). The purified enzyme not only quantitatively reacted with MAb 3H9, but also contained an amino acid sequence, YTPENPTEPV, specific for c-Yes among other *src*-family tyrosine kinases, confirming that the purified 60-kDa protein-tyrosine kinase was indeed c-Yes.

The purified c-Yes did not react with a monoclonal antibody against phosphotyrosine under conditions where autophosphorylated c-Yes was immunoprecipitated, indicating that the purified c-Yes contains negligible phosphotyrosine residues (Table II), possibly because of dephosphorylation during purification in the absence of protein-tyrosine phosphatase inhibitors.

It is known that the protein-tyrosine kinase activity of c-Src is suppressed by phosphorylation of the C-terminal tyrosine residue, Tyr-527 (*1-3*). The phosphorylation is catalyzed by either the C-terminal Src kinase, Csk (*11, 12*) or autophosphorylation (*13*). The C-terminal tyrosine residue, and its surrounding sequence is known to be conserved in c-Yes (*4-9*). Therefore, c-Yes may also be negatively regulated by phosphorylation at the C-terminal tyrosine residue corresponding to Tyr-527 of c-Src. Using the dephosphorylated form of c-Yes, we could not find any autophosphorylation of the C-terminal tyrosine residue under the conditions employed. The phosphorylation of the C-terminal tyrosine residue may be catalyzed by a protein-tyrosine kinase, such as Csk. Csk has been reported to be active toward not only c-Src, but also Lyn, Fyn (*33*), and Lck (*34*).

In this paper, the *in vitro*-autophosphorylation sites of c-Yes were determined to be Tyr-424 and possibly Tyr-32. Tyr-424 corresponds with the *in vitro*-autophosphorylation site of c-Src, Tyr-416 (*30*), whose surrounding sequence is well-conserved in all *src*-family tyrosine kinases, including c-Yes (*35*). The importance of Tyr-416 phosphorylation for c-Src function has been strongly suggested, even though stable phosphorylation is not observed *in vivo* (*3, 13, 14*). On the other hand, Tyr-32 and its surrounding sequence are conserved in c-Yes from all the species so far examined (*4-9*) and are unique to c-Yes among other *src*-family tyrosine-protein kinases. The rate of autophosphorylation of the N-terminal tyrosine residue, possibly Tyr-32, was comparable to that of Tyr-424 (Fig. 5).

A synthetic peptide derived from p34^{cdc2}, KVEKIGEGTYGVVKK-amide was found to be a specific and efficient substrate for *src*-family tyrosine kinases (*32*). The sequence, GTYGV, of the phosphorylation site is similar to the sequence surrounding Tyr-32, GHYGV, in rat c-Yes. Songyang *et al.* derived some specificity requirements governing substrates for *src*-family tyrosine kinases using peptide libraries (*36*). They reported that the optimal substrate for *src*-family tyrosine kinases would contain the sequence EE (or G) IYGE (or V) FF, although the scoring of additional residues in various positions suggests considerable flexibility. In fact, a peptide with the sequence of only 5 residues, AcIYGEF-amide, showed significant substrate activity for c-Src (*37*). Such sequence specificity of *src*-family tyrosine kinases is illustrated in Fig. 7, where c-Yes phosphorylated only Tyr-32-containing peptides whose sequences have specific substrate determinants at Y−2(G), Y+1(G), and Y+2(V), but not Tyr-16-containing peptides which have no such determinants. These results suggest that the N-terminal autophosphorylation site in c-Yes could be Tyr-32.

Using the purified c-Yes preparation which was essen-

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tially free from phosphotyrosine residues, we found that c-Yes was autophosphorylated with Mg^{2+}·ATP exclusively at tyrosine residues with a concomitant increase in the kinase activity (Table II). However, it is not clear whether the stimulation of the enzyme activity requires autophosphorylation of one or both of the two sites, Tyr-32 and Tyr-424. Furthermore, the rates of phosphorylation of tyrosine-glutamate copolymers, catalyzed by the purified c-Yes were proportional to the square of enzyme concentration, suggesting that c-Yes undergoes autophosphorylation through intermolecular catalysis, resulting in stimulation of the enzyme activity. The physiological significance of such activation through intermolecular catalysis may be for the amplification of c-Yes activity after rescue from suppression by tyrosine dephosphorylation, presumably at the C-terminal Tyr-535. PDGF treatment of quiescent fibroblasts results in transient complex formation between c-Yes and PDGF receptors, followed by the activation of c-Yes (*38*). c-Yes also participates in platelet signal transduction by association with transmembrane glycoprotein IV (CD36) on the inner surface of plasma membranes (*39*). Ligand-binding to the transmembrane proteins at the outer surface of the cell may result in dimerization or oligomerization of the transmembrane proteins as well as the associated c-Yes, whose activity might then be stimulated by intermolecular cross-phosphorylation.

The autophosphorylation of the purified platelet c-Src mainly occurred at Tyr-416 in the presence of Mn^{2+}/Mg^{2+}·ATP, being accompanied by an approximately twofold increase in the apparent V_{max} value using casein as the substrate (*16*). Dilution experiments with v-Src (*40*) and platelet c-Src (*41*) purified by immunoaffinity chromatography have indicated that autophosphorylation at Tyr-416 is intramolecular. On the other hand, *in vivo* and *in vitro* experiments with c-Src mutants expressed in yeast indicate that autophosphorylation at Tyr-416 in c-Src can be intermolecular (*42*). Using c-Src purified from recombinant bacteria, much of the Tyr-416 autophosphorylation was intermolecular (*13*). Recombinant mouse Lyn, purified from Sf9 cells infected with a baculovirus carrying the *lyn* cDNA, was autophosphorylated through an intermolecular mechanism at Tyr-397 which corresponds to Tyr-416 in c-Src, and was consequently activated up to 17-fold (*43*). As in the *src*-family tyrosine kinases, autophosphorylation at Tyr-1162 of the insulin receptor, which occurs by an intermolecular mechanism, is a prerequisite for activation (*44*). The sequence homology surrounding the autophosphorylation sites suggest that these kinases follow similar molecular mechanisms of autophosphorylation and autoactivation (*43, 44*).

It is known that c-*yes* expression is high in mammalian neural cells and epithelial cells, particularly in basal keratinocytes (*45-47*). During Ca^{2+}-induced keratinocyte differentiation, the protein-tyrosine kinase activity of c-Yes is inhibited, while c-Src is activated (*17*). Although c-Src is activated through dephosphorylation of its Tyr-527 by the elevated protein-tyrosine phosphatase activity, c-Yes is inactivated through a Ca^{2+}-dependent association with cellular proteins, which seems to override the activation resulting from C-terminal tyrosine dephosphorylation. These Ca^{2+}-dependent inhibitor proteins did not bind to c-Src and were suggested to interact with the unique N-terminal region in c-Yes. For understanding the mechanism of Ca^{2+}-induced c-Yes inactivation, it is important to know the effect of Tyr-32 autophosphorylation on the binding of c-Yes to Ca^{2+}-dependent inhibitor protein. Autophosphorylation at Tyr-32 in the unique N-terminal region of c-Yes may also be involved in another differential regulation of c-Yes and other *src*-family protein-tyrosine kinases in the same cell.

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REFERENCES

1. Cooper, J.A. and King, C.S. (1986) Dephosphorylation or antibody binding to the carboxy terminus stimulates $pp60^{c\text{-}src}$. *Mol. Cell. Biol.* **6**, 4467-4477
2. Courtneidge, S.A. (1985) Activation of the $pp60^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. *EMBO J.* **4**, 1471-1477
3. Kmiecik, T.E. and Shalloway, D. (1987) Activation and suppression of $pp60^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell* **49**, 65-73
4. Sukegawa, J., Semba, K., Yamanashi, Y., Nishizawa, M., Miyajima, N., Yamamoto, T., and Toyoshima, K. (1987) Characterization of cDNA clones for the human c-*yes* gene. *Mol. Cell. Biol.* **7**, 41-47
5. Sudol, M., Kieswetter, C., Zhao, Y.-H., Dorai, T., Wang, L.-H., and Hanafusa, H. (1988) Nucleotide sequence of a cDNA for the chick *yes* proto-oncogene: Comparison with the viral *yes* gene. *Nucleic Acids Res.* **16**, 9876
6. Zheng, X., Podell, S., Sefton, B.M., and Kaplan, P.L. (1989) The sequence of chicken c-*yes* and $p61^{c\text{-}yes}$. *Oncogene* **4**, 99-104
7. Steele, R.E., Irwin, M.Y., Knudsen, C.L., Collett, J.W., and Fero, J.B. (1989) The *yes* proto-oncogene is present in amphibians and contributes to the maternal RNA pool in the oocyte. *Oncogene Res.* **4**, 223-233
8. Hannig, G., Ottilie, S., and Schartl, M. (1991) Conservation of structure and expression of the c-*yes* and *fyn* genes in lower vertebrates. *Oncogene* **6**, 361-369
9. Klages, S., Adam, D., Eiseman, E., Fargnoli, J., Dymecki, S.M., Desiderio, S.V., and Bolen, J.B. (1993) Molecular cloning and analysis of cDNA encoding the murine c-*yes* tyrosine protein kinase. *Oncogene* **8**, 713-719
10. Fischer, E.H., Charbonneau, H., and Tonks, N.K. (1991) Protein tyrosine phosphatases: A diverse family of intracellular and transmembrane enzymes. *Science* **253**, 401-406
11. Okada, M. and Nakagawa, H. (1988) Identification of a novel protein tyrosine kinase that phosphorylates $pp60^{c\text{-}src}$ and regulates its activity in neonatal rat brain. *Biochem. Biophys. Res. Commun.* **154**, 796-802
12. Nada, S., Okada, M., MacAuley, A., Cooper, J.A., and Nakagawa, H. (1991) Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of $p60^{c\text{-}src}$. *Nature* **351**, 69-72
13. Osusky, M., Taylor, S.J., and Shalloway, D. (1995) Autophosphorylation of purified c-Src at its primary negative regulation site. *J. Biol. Chem.* **270**, 25729-25732
14. Piwnica-Worms, H., Saunders, K.B., Roberts, T.M., Smith, A.E., and Cheng, S.H. (1987) Tyrosine phosphorylation regulates the biochemical and biological properties of $pp60^{c\text{-}src}$. *Cell* **49**, 75-82
15. Kmiecik, T.E., Johnson, P.J., and Shalloway, D. (1988) Regulation by autophosphorylation site in overexpressed $pp60^{c\text{-}src}$. *Mol. Cell. Biol.* **8**, 4541-4546
16. Reuter, C., Findik, D., and Presek, P. (1990) Characterization of purified $pp60^{c\text{-}src}$ protein tyrosine kinase from human platelets. *Eur. J. Biochem.* **190**, 343-350
17. Zhao, Y., Uyttendaele, H., Krueger, J.G., Sudol, M., and

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Hanafusa, H. (1993) Inactivation of c-*yes* tyrosine kinase by elevation of intracellular calcium levels. *Mol. Cell. Biol.* **13**, 7507-7514
18. Schultz, A.M., Henderson, L.E., Oroszlan, S., Garber, E.A., and Hanafusa, H. (1985) Amino terminal myristylation of the protein kinase p60^{src}, a retroviral transforming protein. *Science* **227**, 427-429
19. Sefton, B.M. and Buss, J.E. (1987) The covalent modification of eukaryotic proteins with lipid. *J. Cell Biol.* **104**, 1449-1453
20. Azuma, K., Ariki, M., Miyauchi, T., Usui, H., Takeda, M., Semba, K., Matsuzawa, Y., Yamamoto, T., and Toyoshima, K. (1991) Purification and characterization of a rat liver membrane tyrosine protein kinase, the possible protooncogene c-*yes* product, p60$^{c\text{-}yes}$. *J. Biol. Chem.* **266**, 4831-4839
21. Miyauchi, T., Ariki, M., Usui, H., Semba, K., Matsuzawa, Y., Yamamoto, T., Toyoshima, K., and Takeda, M. (1992) Purification and characterization of a possible protooncogene *fyn* product, p59^{fyn}, from a rat brain particulate fraction. *J. Biochem.* **112**, 729-732
22. Sukegawa, J., Akatsuka, T., Sugawara, I., Mori, S., Yamamoto, T., and Toyoshima, K. (1990) Monoclonal antibodies to the amino-terminal sequence of the c-*yes* gene product as specific probes of its expression. *Oncogene* **5**, 611-614
23. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299
24. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
25. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
26. Takeya, T. and Hanafusa, H. (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
27. Kawakami, T., Pennington, C.Y., and Robbins, K.C. (1986) Isolation and oncogenic potential of a novel human *src*-like gene. *Mol. Cell. Biol.* **6**, 4195-4201
28. Semba, K., Nishizawa, M., Miyajima, N., Yoshida, M.C., Sukegawa, J., Yamanashi, Y., Sasaki, M., Yamamoto, T., and Toyoshima, K. (1986) *Yes*-related protooncogene, *syn* belongs to the protein-tyrosine kinase family. *Proc. Natl. Acad. Sci. USA* **83**, 5459-5463
29. Marth, J.D., Peet, R., Krebs, E.G., and Perlmutter, R.M. (1985) A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA. *Cell* **43**, 393-404
30. Smart, J.E., Oppermann, H., Czernilofsky, A.P., Purchio, A.F., Erikson, R.L., and Bishop, J.M. (1981) Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60$^{v\text{-}src}$) and its normal cellular homologue (pp60$^{c\text{-}src}$). *Proc. Natl. Acad. Sci. USA* **78**, 6013-6017
31. Wong, T.W. and Goldberg, A.R. (1983) *In vitro* phosphorylation of angiotensin analogs by tyrosyl protein kinases. *J. Biol. Chem.* **258**, 1022-1025
32. Cheng, H.-C., Nishio, H., Hatase, O., Ralph, S., and Wang, J.H. (1992) A synthetic peptide derived from p34^{cdc2} is a specific and efficient substrate of *src*-family tyrosine kinases. *J. Biol. Chem.* **267**, 9248-9256
33. Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., and Nakagawa, H. (1991) CSK: A protein-tyrosine kinase involved in regulation of *src* family kinases. *J. Biol. Chem.* **266**, 24249-24252
34. Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N.A., Amrein, K.E., Autero, M., Burn, P., and Alitalo, K. (1992) The human p50^{csk} tyrosine kinase phosphorylates p56^{lck} at Tyr-505 and down regulates its catalytic activity. *EMBO J.* **11**, 2919-2924
35. Hanks, S.K., Quinn, A.M., and Hunter, T. (1988) The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52
36. Songyang, Z., Carraway, K.L., III, Eck, M.J., Harrison, S.C., Feldman, R.A., Mohammadi, M., Schlessinger, J., Hubbard, S.R., Smith, D.P., Eng, C., Lorenzo, M.J., Ponder, B.A.J., Mayer, B.J., and Cantley, L.C. (1995) Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature* **373**, 536-539
37. Edison, A.M., Barker, S.C., Kassel, D.B., Luther, M.A., and Knight, W.B. (1995) Exploration of the sequence specificity of pp60$^{c\text{-}src}$ tyrosine kinase. *J. Biol. Chem.* **270**, 27112-27115
38. Kypta, R.M., Goldberg, Y., Ulug, E.T., and Courtneidge, S.A. (1990) Association between the PDGF receptor and members of the *src* family of tyrosine kinases. *Cell* **62**, 481-492
39. Huang, M.-M., Bolen, J.B., Barnwell, J.W., Shattil, S.J., and Brugge, J.S. (1991) Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. *Proc. Natl. Acad. Sci. USA* **88**, 7844-7848
40. Sugimoto, Y., Erikson, E., Graziani, Y., and Erikson, R.L. (1985) Inter- and intramolecular interactions of highly purified Rous sarcoma virus-transforming protein, pp60$^{v\text{-}src}$. *J. Biol. Chem.* **260**, 13838-13843
41. Feder, D. and Bishop, J.M. (1990) Purification and enzymatic characterization of pp60$^{c\text{-}src}$ from human platelets. *J. Biol. Chem.* **265**, 8205-8211
42. Cooper, J.A. and MacAuley, A. (1988) Potential positive and negative autoregulation of p60$^{c\text{-}src}$ by intermolecular autophosphorylation. *Proc. Natl. Acad. Sci. USA* **85**, 4232-4236
43. Sotirellis, N., Johnson, T.M., Hibbs, M.L., Stanley, I.J., Stanley, E., Dunn, A.R., and Cheng, H.-C. (1995) Autophosphorylation induces autoactivation and a decrease in the Src homology 2 domain accessibility of the Lyn protein kinase. *J. Biol. Chem.* **270**, 29773-29780
44. Hubbard, S.R., Wei, L., Ellis, L., and Hendrickson, W.A. (1994) Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* **372**, 746-754
45. Zhao, Y.-H., Krueger, J.G., and Sudol, M. (1990) Expression of cellular-*yes* protein in mammalian tissues. *Oncogene* **5**, 1629-1635
46. Krueger, J., Zhao, Y.-H., Murphy, D., and Sudol, M. (1991) Differential expression of p62$^{c\text{-}yes}$ in normal, hyperplastic and neoplastic human epidermis. *Oncogene* **6**, 933-940
47. Zhao, Y.-H., Baker, H., Walaas, S.I., and Sudol, M. (1991) Localization of p62$^{c\text{-}yes}$ protein in mammalian neural tissues. *Oncogene* **6**, 1725-1733

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