

A New Monoclonal Antibody Which Selectively Recognizes the Active Form of Src Tyrosine Kinase*

(Received for publication, July 18, 1995)

Hisaaki Kawakatsu‡§, Takao Sakai¶, Yumiko Takagaki‡, Yasuhiko Shinoda‡, Masaki Saito¶, M. Koji Owada¶, and Junichi Yano‡

From the ‡Molecular Biology Department, Research Laboratories, Nippon Shinyaku Co. Ltd., 601 Kyoto, Japan, the ¶Division of Hemopoiesis, Institute of Hematology, Jichi Medical School, 329-04 Tochigi, Japan, and the §Institute of Molecular and Cellular Biology for Pharmaceutical Sciences, Kyoto Pharmaceutical University, 607 Kyoto, Japan

Phosphorylation and dephosphorylation of Tyr-530 in human c-Src (Tyr-527 in avian c-Src) is critical in regulating c-Src kinase activity. So far, it has not been possible to distinguish the active and inactive forms *in vivo*. We now report a new monoclonal antibody that selectively recognizes the active form of c-Src. This antibody, termed clone 28, recognized a region adjacent to Tyr-530 (Q⁵²⁹YQP⁵³²) in the C-terminal regulatory domain of c-Src, and its binding was hindered by phosphorylation of this tyrosine as determined by peptide competition assay. Combined immunoprecipitation/Western blotting revealed that clone 28 reacted with a 60-kDa protein that was precipitated by mAb 327, a well known monoclonal antibody against v-Src and c-Src. Cyanogen bromide cleavage and two-dimensional tryptic maps confirmed that clone 28 was specific for the active form (Tyr-530 not phosphorylated), whereas mAb 327 recognized the inactive form (Tyr-530 phosphorylated) as well as the active form. Clone 28 selectively immunoprecipitated the active form and augmented its kinase activity. Preabsorption experiments revealed that clone 28 could not completely immunoprecipitate the mAb 327 binding 60-kDa protein in either an *in vitro* or an *in vivo* phosphorylation system. These observations, taken together, strongly suggest the existence of multiple forms of c-Src as proposed by Cooper and Howell (1993) (Cooper, J. A., and Howell, B. (1993) *Cell* 73, 1051–1054).

Using clone 28, we demonstrated a distinct localization of the active form of c-Src within cultured normal fibroblast cells. In liver tissue sections, we also examined the distribution of the active form in embryonic mice. Megakaryocytes were strongly stained, in contrast to completely negative immunoreactivity in hepatocytes, reticulocytes, and granulocytes. This result provides the first direct evidence that c-Src is highly activated in platelets.

Src family tyrosine kinases are widely distributed nonreceptor tyrosine kinases that have a role in many different signal transduction pathways downstream from various types of receptor in the cell membrane; however, their exact roles in signal transduction remain unknown. Src family tyrosine kinases (for reviews, see Hunter (1987), Cantley *et al.* (1991), and Bolen *et al.* (1992)) are known both from retroviruses that have the ability to transform cells (Src, Yes, Fgr, and Lck) and from

mammalian genomes by the use of DNA probes for *src/yes/fgr* (Fyn, Lyn, Hck, Blk, and Yrk). A comparison of the corresponding cDNA sequences revealed that each member of this family has a very different sequence in the N-terminal region (50–80 amino acids from the N terminus). This region is thought to participate in specific cellular function through binding to different types of signal transducer (Resh, 1989; Shaw *et al.*, 1990; Timson-Gauen *et al.*, 1992), although the mechanism of regulation remains unknown. By contrast, the rest of the sequence is highly conserved, consisting sequentially of an Src homology (SH)¹ 3 domain, an SH2 domain, a kinase domain, and a C-terminal regulatory domain (for reviews, see Margolis (1992), Pawson and Gish (1992), Cooper and Howell (1993), and Erpel and Courtneidge (1995)).

Two tyrosine residues, Tyr-419 and Tyr-530 in human c-Src (corresponding to Tyr-416 and Tyr-527, respectively, in avian c-Src), can be phosphorylated, and their phosphorylation state influences the tyrosine kinase activity. Whereas the phosphorylation of Tyr-419 leads to a dramatic increase in kinase activity (Piwnicka-Worms *et al.*, 1987), the phosphorylation of Tyr-530 negatively regulates the kinase activity (Cooper *et al.*, 1986). The *csk* gene product is responsible for the phosphorylation of Tyr-530 (Okada and Nakagawa, 1989; Okada *et al.*, 1991). The mechanism of regulation involving these tyrosine residues is thought to be similar for other members of the Src family (Okada *et al.*, 1991). On the basis of these observations, a model that involves intramolecular binding between Tyr-530 and the SH2 region was proposed (Roussel *et al.*, 1991; Superti-Furga *et al.*, 1993; Sieh *et al.*, 1993). In this widely accepted model, subsequent signal transduction through direct binding to other proteins or by tyrosine phosphorylation is assumed to be prevented by this intramolecular binding. Consequently, the C-terminal region is important not only in the regulation of tyrosine kinase activity, but also in the association with SH2-containing proteins.

In the present study, we have generated a monoclonal antibody (mAb) specific for the C-terminal regulatory domain of c-Src. This mAb selectively recognized the active form of c-Src as demonstrated by *in vitro* and *in vivo* phosphorylation. Several different types of experiment described here illustrated the usefulness of this mAb. The antibody had a different range of reactivity in comparison with mAb 327, which has been widely used for the detection of c-Src and v-Src. In particular, clone 28 was shown to have high sensitivity to the active form of c-Src in normal cells and tissues.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 81-75-321-1111 (ext. 8932); Fax: 81-75-313-5001.

¹ The abbreviations used are: SH, Src homology; mAb, monoclonal antibody; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation buffer.

MATERIALS AND METHODS

Production of mAbs—A peptide corresponding to the C-terminal region of human c-Src (LEDYFTSTPEQYQPGENL, residues 519–536) was synthesized and coupled to keyhole limpet hemocyanin (Calbiochem) with the bifunctional coupling reagent sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce). This conjugate was used for mouse immunization as described by Harlow and Lane (1988). The hybridomas obtained were screened by means of enzyme-linked immunosorbent assay with a synthetic peptide as the antigen, and by Western blotting or immunoprecipitation using human cell line lysates.

Preparation of Lysates from Human Platelets—Freshly collected human blood was centrifuged at 1000 rpm for 10 min in the presence of sodium citrate as an anticoagulant. The supernatant was collected and centrifuged again at 3000 rpm for 10 min. The platelets, enriched in the pellet, were disrupted in phosphate-buffered saline (PBS(-)) containing 1% (w/v) Nonidet P-40, 20 mM NaF, 2 mM orthovanadate, 5 μ g/ml leupeptin, and 0.25 mM (*p*-aminodiphenyl)methanesulfonyl fluoride in a Teflon homogenizer. The homogenate was centrifuged at 100,000 \times *g* for 30 min and the supernatant was used for further analysis. Protein concentrations were determined by means of the bicinchoninic acid (BCA) assay (Pierce).

Western Blotting—After SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nylon membrane (Millipore, polyvinylidene difluoride, IPVH 000-10) by means of a semidry blotting system (Pharmacia Biotech Inc.) at a constant current of 0.8 mA/cm². Blocking was performed for 1 h in a Tris-buffered solution containing 2% (w/v) skim milk (Difco). After incubation with either clone 28 or mAb 327 (Lipsich *et al.*, 1983; Oncogene Science Inc., OP-07L), then with peroxidase-conjugated sheep anti-mouse immunoglobulin (Amersham Corp.), and the stain was developed with the Konica immunostain kit using 4-chloronaphthol as the substrate.

In Vitro and in Vivo Phosphorylation Assays—For *in vitro* phosphorylation, human platelets were disrupted with RIPA buffer without SDS (50 mM Tris-HCl, pH 8.2, containing 150 mM NaCl, 20 mM NaF, 10 mM EDTA, 2 mM orthovanadate, 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, 5 μ g/ml leupeptin, and 0.25 mM (*p*-aminodiphenyl)methanesulfonyl fluoride) and treated with mAbs. In each experiment, we confirmed by titration that the amount of antibody used was sufficient to precipitate all of the available protein. Immune complexes were precipitated with protein A-Sepharose CL-4B (Zymed) or anti-mouse IgG-agarose (American Corlex). After washing the precipitate three times with RIPA buffer without SDS, then twice with phosphorylation buffer (20 mM HEPES, pH 7.5, containing 150 mM NaCl and 5 mM MnCl₂), we added [γ -³²P]ATP (37 kBq (1 μ Ci); Amersham PB170) to the immune complex. For *in vivo* phosphorylation, human platelets were metabolically labeled with [³²P]PO₄³⁻ (3.7 MBq (0.1 mCi)/ml; Amersham PBS11) for 2 h. Immunoprecipitation was done by the same procedure used for *in vitro* phosphorylation, except that the RIPA buffer used for washing contained 0.1% (w/v) SDS. Peptide competition and preabsorption experiments were performed in these systems.

Phosphopeptide Mapping—Phosphorylated 60-kDa bands were excised from the dried gels and rehydrated with a solution containing 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, and 1 mM EDTA. The bands were partially digested with V8 protease in the SDS-polyacrylamide gel as described by Cleveland *et al.* (1977). Cyanogen bromide mapping was performed according to Pepinsky (1983). For two-dimensional tryptic mapping, V1 and V2 bands were excised from gels and rehydrated with several changes of 20% (v/v) isopropanol and then 100% methanol. After complete drying, gel bands were digested with trypsin treated with tosylphenylalanyl chloromethyl ketone (50 μ g/ml in 50 mM ammonium bicarbonate, pH 8.5) at 37 °C overnight. Clarified supernatants of these digests were dried down in a Speed-Vac concentrator (Savant) and washed with water by three cycles of suspension and vacuum drying. Finally, samples were resuspended in 10 μ l of electrophoresis solvent (acetic acid:formic acid:H₂O = 15:5:80) and spotted on TLC plates (silica gel 60; Merck). Tryptic peptides were first subjected to electrophoresis in the above solvent, then to chromatography in the second dimension in a different solvent (*n*-butanol:pyridine:acetic acid:H₂O = 65:50:10:40). Phosphopeptide maps were visualized by means of a Fujix BAS2000 Bio-Image Analyzer (Itoharu *et al.*, 1993).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described by Sakai *et al.* (1995), with a slight modification. Briefly, rat normal (immortalized) cell line 3Y1 was fixed in 3.5% (w/v) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 2 mM sodium orthovanadate for 20 min at 4 °C and washed with Tris-buffered saline (TBS). Cells were then permeabilized by treatment

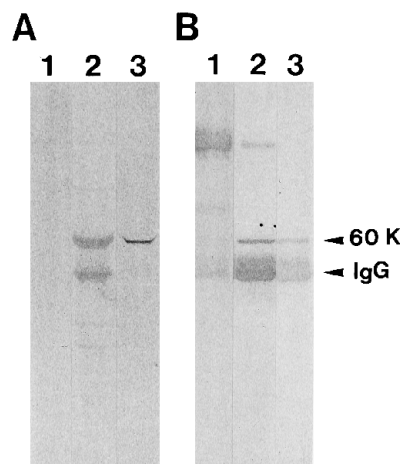


FIG. 1. Reactivity of clone 28 and mAb 327 with human platelet lysates. A, Western blotting with control mouse IgG (lane 1), clone 28 (lane 2), and mAb 327 (lane 3) in human platelets. B, combined immunoprecipitation/Western blotting in human platelets. Platelet lysates were immunoprecipitated with control mouse IgG (lane 1), clone 28 (lanes 2 and 3), and immune complexes were subjected to electrophoresis for Western blotting by mAb 327 (lanes 1 and 2) or clone 28 (lane 3).

with 0.2% (v/v) Triton X-100 in TBS containing 2 mM sodium orthovanadate for 15 min at 4 °C. They were washed with TBS containing 0.5 mM sodium orthovanadate and incubated in TBS containing 1% (w/v) bovine serum albumin for 30 min to block nonspecific protein binding. Slides were then incubated with primary monoclonal antibodies for 60 min at room temperature (normal mouse IgG was used as a negative control antibody). The slides were washed with TBS containing 0.5 mM sodium orthovanadate and were treated with fluorescein-labeled, affinity-purified goat anti-mouse IgG (MBL, Nagoya, Japan) for 40 min at room temperature. Slides were briefly washed with TBS, mounted with glass coverslips, and viewed with a BX60 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Immunocytochemistry—Immunocytochemical observations were performed as described by Sakai *et al.* (1993) with a slight modification. Deparaffinized sections were immersed in methanol containing 0.3% (v/v) hydrogen peroxide to inactivate endogenous peroxidases. After washing with PBS(-), the sections were incubated in PBS containing 5% (v/v) normal goat serum to block nonspecific protein binding, then pretreated with a biotin/avidin blocking kit (Vector Laboratories Inc.) to block avidin binding to endogenous biotin. They were then treated with antibodies and left overnight at 4 °C (normal mouse IgG was used as a negative control primary antibody). The sections were treated with a secondary antibody, biotinylated goat anti-mouse IgG (Organon Teknica), and then with peroxidase-conjugated streptavidin (Histofine, Nichirei Corp.). The color was developed with a freshly prepared chromogen solution containing 0.1% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories) and 0.02% (v/v) hydrogen peroxide in 50 mM Tris-HCl, pH 7.6. Sections were counterstained with hematoxylin, mounted with glass coverslips, and observed under a New Vanox-s microscope (Olympus Optical Co., Ltd.).

RESULTS

Clone 28 Detected a 60-kDa Protein Recognized by mAb 327—Of five hybridomas screened by means of an enzyme-linked immunosorbent assay system with the peptide that was used for immunization (LEDYFTSTPEQYQPGENL, amino acids 519–536 in human c-Src), one clone, termed clone 28, was sensitive enough to detect a 60-kDa band in a human platelet lysate by Western blotting (Fig. 1A, lane 2). By combined immunoprecipitation/Western blotting, this 60-kDa protein was recognized by mAb 327 (Fig. 1B, lane 2), whose epitope is thought to be in the SH3 domain of v-Src and c-Src. The 60-kDa protein in the immunoprecipitate of clone 28 was still recognized by clone 28 in the above system, demonstrating that clone 28 retains its specificity even when the c-Src protein is denatured (Fig. 1B, lane 3).

Clone 28 Sensitive Detected the Active Form of c-Src—Clone 28 immunoprecipitated phosphorylated 60-kDa protein from

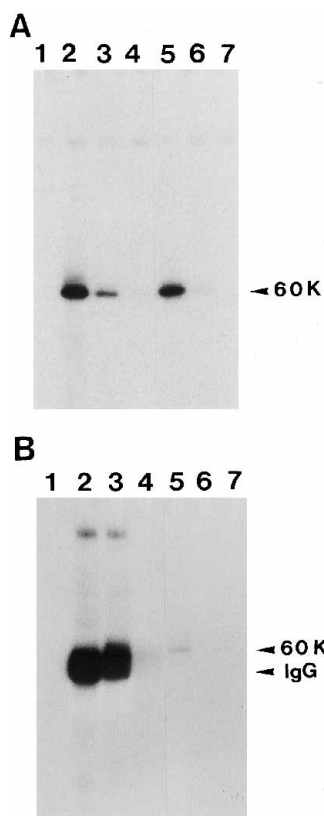


FIG. 2. Detection of phosphorylated proteins from human platelets. A, *in vivo* phosphorylation assay. Platelets were metabolically labeled with [32 P] PO_4^{3-} , then immunoprecipitated with control mouse IgG (lane 1, 10 μ g), clone 28 (lane 2, 10 μ g; lane 3, 1 μ g; lane 4, 0.1 μ g), or mAb 327 (lane 5, 10 μ g; lane 6, 1 μ g; lane 7, 0.1 μ g). B, *in vitro* phosphorylation assay. In *in vitro* kinase activity was assessed in human platelet lysates immunoprecipitated by control mouse IgG (lane 1, 10 μ g), clone 28 (lane 2, 10 μ g; lane 3, 1 μ g; lane 4, 0.1 μ g), or mAb 327 (lane 5, 10 μ g; lane 6, 1 μ g; lane 7, 0.1 μ g).

lysates of human platelets metabolically labeled with [32 P] PO_4^{3-} (Fig. 2A). The detection sensitivity of clone 28 was comparable to that of mAb 327 as determined by titration of each antibody. In contrast to this *in vivo* phosphorylation, only the clone 28 precipitate displayed strong *in vitro* phosphorylation. A human platelet lysate was subjected to immunoprecipitation by either clone 28 or mAb 327. After a thorough wash with RIPA buffer without SDS, and then with phosphorylation buffer, [γ - 32 P]ATP was added for *in vitro* phosphorylation. The fluorograph obtained (Fig. 2B) showed that the clone 28 precipitate was heavily phosphorylated compared with the mAb 327 precipitate. As determined by titration of the antibody concentration, the clone 28 precipitate underwent 50 times as much autophosphorylation as did the mAb 327 precipitate. Taken together, these results show clone 28 to be a sensitive and selective detector of the activated form of c-Src (Tyr-530 not phosphorylated). Augmentation of the kinase activity was confirmed by mixing clone 28 and purified c-Src in the *in vitro* phosphorylation system (data not shown).

The Epitope of Clone 28 Was Assigned to a Region Adjacent to the C-terminal Tyrosine Residue of c-Src—In order to define the site within the C-terminal region of c-Src to which clone 28 binds, we synthesized a series of peptides corresponding to segments of the peptide used for immunization (Table I). The *in vitro* phosphorylation assay was used to detect competition by each peptide for clone 28 (Fig. 3A). Antigen recognition was completely inhibited by the peptides QYQPGENL (amino acids 529–536), QFQPGENL (amino acids 529–536, Tyr 530 re-

TABLE I
Peptides used in competition studies

Residues in bold type differ from the corresponding residues in human c-Src. In the epitope region, c-Fyn, c-Src, and c-Yes share the sequence QYQPGENL, whereas c-Fgr has a different sequence, QYQPGDQT.

c-Src	Peptide	Numbering in human
L E D Y F T S T E P Q Y Q P G E N L	*	519–536
L E D Y F T S T	E P Q Y Q P G	519–526
	Q Y Q P G E N L	527–533
	Q F Q P G E N L	529–536
	Q Y Q P G D Q T	529–536

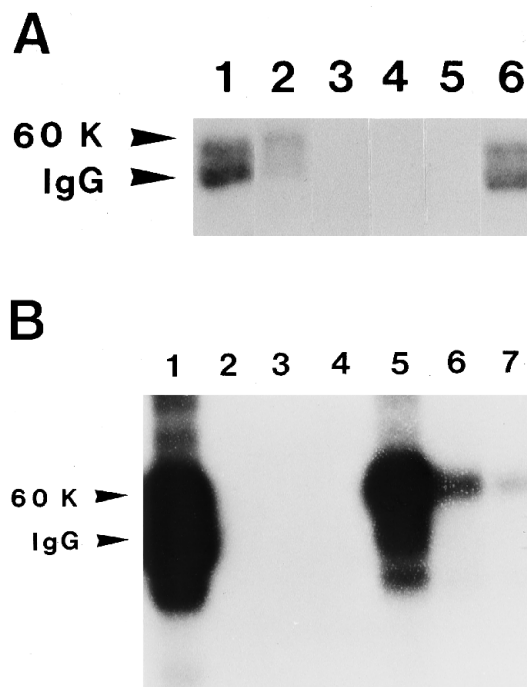


FIG. 3. Competition for antibody binding in *in vitro* phosphorylation systems. We synthesized a series of short peptides as illustrated in Table I and determined their ability to inhibit immunoprecipitation by clone 28 in the *in vitro* kinase assay (A). Immunoprecipitation was performed in the presence of 0.1 mg/ml LE-DYFTST (519–526, lane 1), EPQYQPG (527–533, lane 2), QYQPGENL (529–536, lane 3), QF*QPGENL (529–536, lane 4), or QYQPGD*Q*T* (529–536, lane 5) or in the absence of synthetic peptides (lane 6). We also compared the inhibition of immunoprecipitation caused by the non-phosphorylated peptide QYQPGENL (B, lanes 2–4) and the corresponding phosphotyrosine-containing peptide (B, lanes 5–7). The concentration of each peptide was 1 μ g/ml (lanes 2 and 5), 10 μ g/ml (lanes 3 and 6), or 100 μ g/ml (lanes 4 and 7). The control level was determined in the absence of any peptide (lane 1).

placed by Phe) and QYQPGDQT (amino acids 529–536, ENL replaced by DQT, identical to the corresponding region in c-Fgr), and partially inhibited by EPQYQPG (amino acids 527–533), all at a concentration of 100 μ g/ml. The N-terminal eight amino acids of the peptide used for immunization, LEDYFTST (amino acids 519–526), did not influence antigen recognition. We therefore concluded that clone 28 bound to the C-terminal region of c-Src in the vicinity of Tyr-530. For further confirmation of the specificity of this clone, we used the peptide QYQPGENL in either the nonphosphorylated or the phosphorylated form for *in vitro* phosphorylation. Whereas the nonphosphorylated peptide completely inhibited antigen recognition even at the lowest concentration used (1 μ g/ml; Fig. 3B, lanes 2–4), the phosphorylated peptide only partially inhibited the phosphorylation (27% at 1 μ g/ml and 88% at 10 μ g/ml; Fig. 3B, lanes

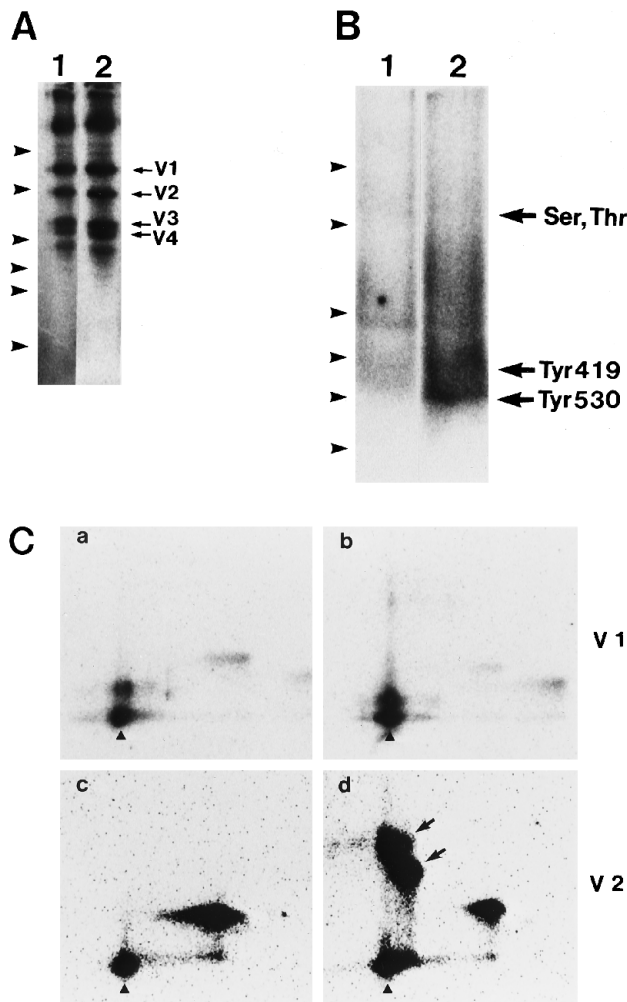


FIG. 4. **Phosphopeptide mapping.** Excised 60-kDa bands from lanes 2 (clone 28 precipitate) and 5 (mAb 327 precipitate) in Fig. 2A were subjected to V8 protease partial digestion (A) or cyanogen bromide cleavage (B). Lanes 1 correspond to clone 28 and lanes 2 to mAb 327. Excised V1 and V2 bands from each lane in A were further analyzed by two-dimensional tryptic peptide mapping (C).

5–7). Thus we could localize the epitope to the center of critical C-terminal regulatory domain.

Comparison of Phosphorylation State of the Immunoprecipitates by Peptide Mapping and Preabsorption—For the further analysis of the phosphorylation state of c-Src recognized by clone 28 or mAb 327, each 60-kDa band was excised from the gel shown in Fig. 2A and subjected to partial digestion by V8 protease (Fig. 4A). The phosphorylation of both the V1 fragment (which contains phosphorylated Ser-12, Ser-17, Thr-34, and Thr-46 in c-Src; Courtneidge and Smith (1984)) and the V2 fragment (which contains phosphorylated Tyr-419 and Tyr-530 in c-Src) was observed in both precipitates by this mapping procedure. Cyanogen bromide cleavage confirmed this result and also showed that Tyr-530 was phosphorylated only in the mAb 327 precipitate (Fig. 4B). Tryptic maps of V2 fragments derived from clone 28 and mAb 327 precipitates also confirmed this result; additional spots not seen in the clone 28 precipitate were detected in the mAb 327 precipitate (Fig. 4C, c and d), whereas tryptic maps of V1 fragments from both precipitates displayed almost identical patterns (Fig. 4C, a and b).

We also performed preabsorption experiments in *in vitro* and *in vivo* systems. Lysates of human platelets metabolically labeled with [32 P] PO_4^{3-} were pretreated with normal mouse IgG or clone 28, then subjected to clone 28 and mAb 327 precipita-

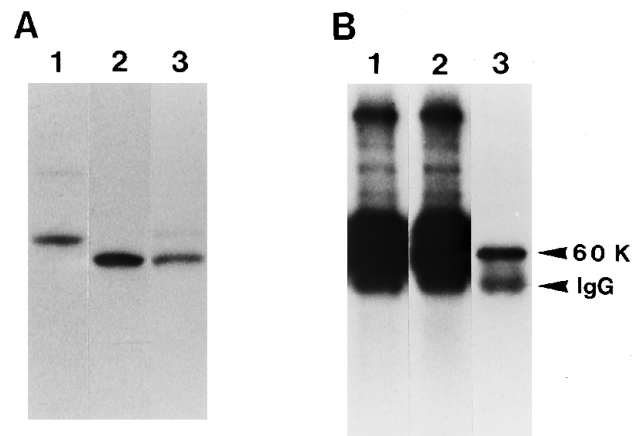


FIG. 5. **Preabsorption of human platelet lysates.** Human platelet lysates were pretreated with control mouse IgG (lanes 1 and lanes 2) or clone 28 (lanes 3). After preabsorption, lysates were further incubated with clone 28 (lanes 1) or mAb 327 (lanes 2 and lanes 3). *In vivo* (A) or *in vitro* (B) phosphorylation assays were performed as in Fig. 2.

tion (Fig. 5A). Lanes 1 and 2 show the basal level of clone 28 and mAb 327 binding activity toward the phosphorylated 60-kDa form when normal mouse IgG was used in preabsorption step. Even when excess clone 28 was used in the preabsorption step, substantial phosphorylated 60-kDa bands remained that were recognized by mAb 327 (lane 3). This result suggested that substantial amounts of c-Src were phosphorylated at Tyr-530 in human platelets. (If a significant amount of the observed phosphorylation were due to Ser or Thr, we would have expected a reduction in the intensity of the 60-kDa band (see Fig. 4A).) In addition, we pretreated cold platelet lysates with normal mouse IgG or clone 28 and then subjected them to clone 28 and mAb 327 precipitation (Fig. 5B). Under conditions when both clone 28 and mAb 327 showed high autophosphorylation activity (lanes 1 and 2), substantial kinase activity was observed in the mAb 327 precipitate even when excess clone 28 was used in the preabsorption step (lane 3).

Immunocytochemical Localization of the Active Form of c-Src Tyrosine Kinase—We examined the cellular distribution of the active form of c-Src tyrosine kinase in normal rat 3Y1 cells. In addition to the expected submembranous localization, we also observed intense staining on the outer nuclear membrane and the cytoskeletal components (Fig. 6A). In contrast to clone 28, the staining with mAb 327 was too weak to show any distinct localization within the cell (Fig. 6B).

In order to test the utility of the clone 28 directly in an *in vivo* system, we examined formalin-fixed, paraffin-embedded C3H-mouse fetal liver (16 days) by an immunocytochemical method. Fig. 7 clearly shows the presence of the active form of c-Src in megakaryocytes, but completely negative immunoreactivity in hepatocytes, reticulocytes, and granulocytes in the fetal liver. We also detected high level of the active form in neuronal tissues.²

DISCUSSION

In order to clarify the activation mechanism of Src family tyrosine kinases, we set out to produce mAbs that could be used to distinguish the active and inactive forms of these tyrosine kinases in various systems. Activation is associated with dephosphorylation of the tyrosine residue nearest the C terminus (Courtneidge, 1985; Cooper and King, 1986), and subsequent signal transfer is presumed to involve a dissociation between this tyrosine-containing region and an SH2 region. We have

² T. Sakai and H. Kawakatsu, unpublished observation.

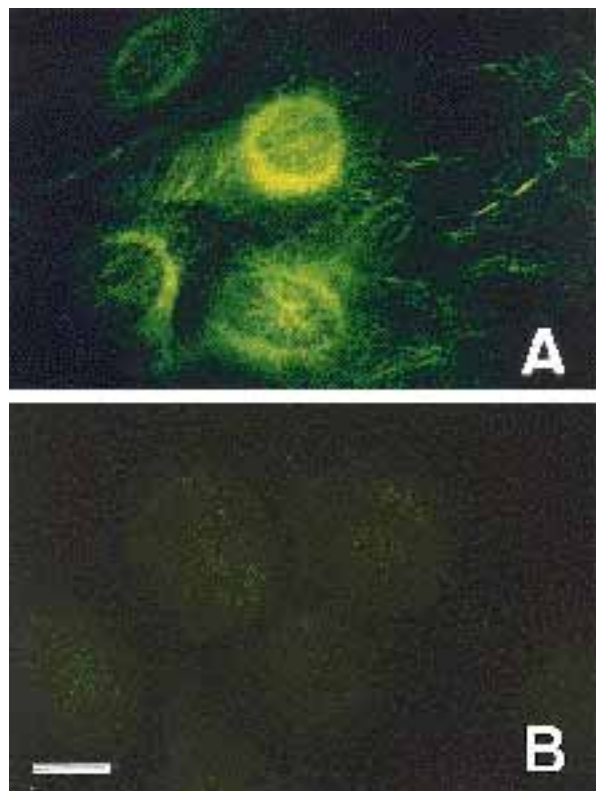


FIG. 6. The distribution of c-Src within normal rat fibroblast 3Y1 cells. Comparison between clone 28 and mAb 327 in an immunocytochemical analysis. The 3Y1 cells were stained with clone 28 (A) or mAb 327 (B). Bar, 15 μ m.

generated a single mAb, termed clone 28, that could selectively recognize the active form of c-Src. We have characterized clone 28 and demonstrated its usefulness as follows: 1) clone 28 sensitively detected the active form of c-Src as judged by titration in an *in vitro* phosphorylation experiment (Fig. 2); 2) a competition study using phosphorylated and nonphosphorylated synthetic peptides confirmed the specificity of clone 28 for the active (Tyr-530 nonphosphorylated) form (Fig. 3); 3) the phosphorylation site of the clone 28 precipitate was clearly distinguishable from that of the mAb 327 precipitate (Fig. 4); 4) preabsorption experiments with clone 28 and mAb 327 in *in vitro* and *in vivo* phosphorylation systems suggested that there are at least two different conformations that retain kinase activity (Fig. 5); and 5) the distribution of the active form in rodent tissues and cultured cells could be directly observed by an immunocytochemical method (Figs. 6 and 7).

Cooper and Howell (1993) proposed a new activation mechanism for Src family tyrosine kinases involving allosteric activators or inhibitors. They presumed that phosphorylation of the tyrosine residues might be a consequence, and not a cause, of changes in activity. Site-directed mutagenesis studies (Hirai and Varmus, 1990; O'Brien *et al.*, 1990; Seidel-Dugan *et al.*, 1992) support this notion, because Src could be activated by introducing mutations in other domains such as SH2 or SH3. The detection of kinase activity both before and after preabsorption by clone 28 (Fig. 5) also showed good agreement with this previous work, since excess clone 28 could not completely immunoprecipitate the active form, even though this clone was specific for the active form. Erpel and Courtneidge (1995) also discussed yet another activation mechanism and proposed three possible pathways to the active form.

In platelets, V8 phosphopeptide mapping facilitated further analysis of the phosphorylation state of the clone 28 and the

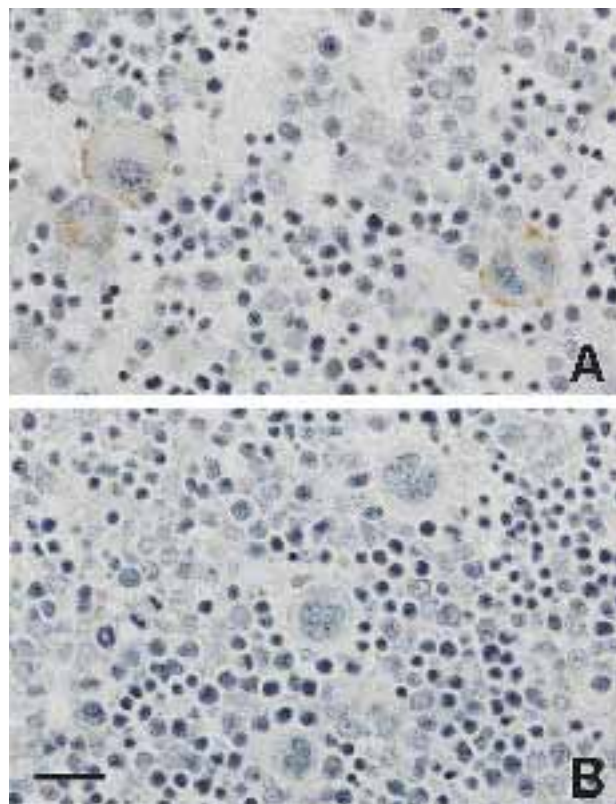


FIG. 7. Immunoreactive localization of the active form of c-Src in the liver of developing mouse embryo (16 days). Sections were stained with clone 28 (A) or normal mouse IgG (B, as a negative control). The active form of c-Src was found preferentially in inside the plasma membrane of megakaryocytes. Bar, 20 μ m.

mAb 327 precipitates: the patterns for both precipitates were virtually identical (Fig. 4A). Cichowski *et al.* (1992) detected Src, Yes, and Fyn, but not Fgr, in thrombin-stimulated human platelets. They co-precipitated Yes and Fyn with an antibody against GTP-activating protein, but the member of the Src family responsible for platelet activation has not been identified. We also investigated several antibodies against each member of the c-Src family in order to identify which were active in the platelet system, but none of the antibodies demonstrated satisfactory sensitivity or specificity. However, clone 28 could give us valuable information on alterations in the kinase activity and cellular localization of Src family tyrosine kinases; such information could help us understand the redundancy and compensation *in vivo* among the members of the Src family that possess the QYQPG motif. Primary cells derived from knockout mice (*e.g.* *src*, *fyn*, and *yes*) might provide information about the role of each member of the Src family when tested with our new antibody.

Polyclonal antibodies against the C-terminal region of c-Src have previously been reported (Courtneidge and Smith, 1984; Cooper and King, 1986). One of these antibodies (Courtneidge and Smith, 1984) could be used both for the precipitation of the phosphorylated form of the kinase and for the autophosphorylation assay. However, there was no mention of cross-reactivity among members of the Src family. Another antibody (Cooper and King, 1986) stimulated the kinase activity upon antibody binding and could also be used for the precipitation of the phosphorylated form. For these polyclonal antibodies, it is possible that several epitopes coexist, some containing Tyr-530 and some not. The results obtained with these antibodies are consistent with our results. Since clone 28 recognized only a single epitope (adjacent to Tyr-530) and was specific for the

nonphosphorylated, active form, the phosphorylation state of c-Src was more readily characterized with this antibody.

In addition to its usefulness in biochemical systems, we have also demonstrated the application of clone 28 in *in vivo* systems. In particular, Fig. 7 clearly shows a restricted distribution of the active form in megakaryocytes, the progenitors of platelets. The tyrosine kinase activity of c-Src might be essential for platelet formation in the fetal liver, because c-Src was activated at all the developmental stages we observed (data not shown). Our data show good agreement with previous work, which reported high kinase activity in platelets by an *in vitro* kinase assay (Oda *et al.*, 1992; Horvath *et al.*, 1992; Clark and Brugge, 1993). In contrast to the intense staining in established cell lines such as 3Y1 (Fig. 6), immunohistochemical observations with mouse tissue sections revealed a restricted distribution even in the fetal stage, where cellular growth and differentiation actively occur (Fig. 7). These data, taken together, suggest that c-Src kinase activity would be suppressed in the steady state and activated transiently by extracellular stimuli in major tissues.

With clone 28, our immunofluorescence studies demonstrated distinctive staining of c-Src in normal rat cells, whereas with mAb 327, only a weak, diffuse staining pattern was observed. With mAb 327, it is very difficult to detect c-Src unless the protein is overexpressed by gene transfection (David-Pfeuty and Nouvian-Dooghe, 1990; David-Pfeuty *et al.*, 1993; Kaplan *et al.*, 1994). Our new mAb could provide valuable information about early events in carcinogenesis and other diseases in which Src family tyrosine kinases or associated SH2-containing proteins play a crucial role.

Acknowledgments—We thank G. E. Smyth for critical reading of this manuscript and M. Todoroki for outstanding photographic assistance.

REFERENCES

- Bolen, J. B., Rowley, R. B., Spana, C., and Tsygankov, A. Y. (1992) *FASEB J.* **6**, 3403–3409.
- Brugge, J. A., Lustig, A., and Messer, A. (1987) *J. Neurosci. Res.* **18**, 532–538.
- Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64**, 281–302.
- Cichowski, K., McCormick, F., and Brugge, J. S. (1992) *J. Biol. Chem.* **267**, 5025–5028.
- Clark, E. A., and Brugge, J. S. (1993) *Mol. Cell. Biol.* **13**, 1863–1871.
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106.
- Cooper, J. A., and Howell, B. (1993) *Cell* **73**, 1051–1054.
- Cooper, J. A., and King, C. S. (1986) *Mol. Cell. Biol.* **6**, 4467–4477.
- Courtneidge, S. A. (1985) *EMBO J.* **4**, 1471–1477.
- Courtneidge, S. A., and Smith, A. E. (1984) *EMBO J.* **3**, 585–591.
- David-Pfeuty, T., and Nouvian-Dooghe, Y. (1990) *J. Cell Biol.* **111**, 3097–3116.
- David-Pfeuty, T., Baglodia, S., and Shalloway, D. (1993) *J. Cell Sci.* **105**, 613–628.
- Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) *Cell* **71**, 1081–1091.
- Erpel, T., and Courtneidge, S. A. (1995) *Curr. Biol.* **7**, 176–182.
- Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Hirai, H., and Varmus, H. E. (1990) *Mol. Cell. Biol.* **10**, 1307–1318.
- Horvath, A. R., Muszbek, L., and Kellie, S. (1992) *EMBO J.* **11**, 855–861.
- Hunter, T. (1987) *Cell* **49**, 1–4.
- Itohara, S., Mombaerts, P., Lafaille, J., Iacomini, J., Nelson, A., Clarke, A. R., Hooper, M. L., Farr, A., and Tonegawa, S. (1993) *Cell* **72**, 337–348.
- Kaplan, K. B., Bibbins, K. B., Swedlow, J. R., Arnaud, M., Morgan, D. O., and Varmus, H. E. (1994) *EMBO J.* **13**, 4745–4756.
- Lipsich, L. A., Lewis, A. J., and Brugge, J. S. (1983) *J. Virol.* **48**, 352–360.
- Margolis, B. (1992) *Cell Growth Diff.* **3**, 73–80.
- O'Brien, M. C., Fukui, Y., and Hanafusa, H. (1990) *Mol. Cell. Biol.* **10**, 2855–2862.
- Oda, A., Druker, B. J., Smith, M., and Salzman, E. W. (1992) *J. Biol. Chem.* **267**, 20075–20081.
- Okada, M., and Nakagawa, H. (1989) *J. Biol. Chem.* **264**, 20886–20893.
- Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., and Nakagawa, H. (1991) *J. Biol. Chem.* **266**, 24249–24252.
- Pawson, T., and Gish, G. D. (1992) *Cell* **71**, 359–362.
- Pepinsky, R. B. (1983) *J. Biol. Chem.* **258**, 11229–11235.
- Piwnicka-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E., and Cheng, S. H. (1987) *Cell* **49**, 75–82.
- Resh, M. D. (1989) *Cell* **58**, 281–286.
- Roussel, R. R., Brodeur, S. R., Shalloway, D., and Laudano, A. P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10696–10700.
- Sakai, T., Kawakatsu, H., Hirota, N., Yokoyama, T., Taakaoka, T., Sakakura, T., and Saito, M. (1993) *Exp. Cell Res.* **206**, 244–254.
- Sakai, T., Ohta, M., Furukawa, Y., Saga, Y., Aizawa, S., Kawakatsu, H., and Saito, M. (1995) *J. Cell. Physiol.* **165**, 18–29.
- Seidel-Dugan, C., Meyer, B. E., Thomas, S. M., and Brugge, J. S. (1992) *Mol. Cell. Biol.* **12**, 1835–1845.
- Shaw, A. S., Chalupny, J., Whitney, J. A., Hammond, C., Amrein, K. E., Kavathas, P., Sefton, B. M., and Rose, J. K. (1990) *Mol. Cell. Biol.* **10**, 1853–1862.
- Sieh, M., Bolen, J. B., and Weiss, A. (1993) *EMBO J.* **12**, 315–321.
- Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) *Cell* **64**, 693–702.
- Sudol, M., Kuo, C. F., Shigematsu, L., and Alvarez-Buylla, A. (1989) *Mol. Cell. Biol.* **9**, 4545–4549.
- Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S. A., and Draetta, G. (1993) *EMBO J.* **12**, 2625–2634.
- Timson-Gauen, L. K., Tony-Kong, A.-N., Samelson, L. E., and Shaw, A. S. (1992) *Mol. Cell. Biol.* **12**, 5438–5446.