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Phylogeny of Vertebrate Src Tyrosine Kinases Revealed by the Epitope Region of mAb327

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Mass fingerprinting and MS/MS analysis demonstrated that Xyk, a 57-kDa Src family tyrosine kinase that is activated within minutes of *Xenopus* egg fertilization, comprises a mixture of two Src proteins, Src1 and Src2. However, the *Xenopus* Src protein, denoted as xSrc, is hardly detectable with mAb327, a universal Src-specific antibody, whose target sequence has not yet been determined. We show that a point amino acid substitution in the Src homology 3 domain of xSrc is critical for improvement of the low efficiency of its recognition by mAb327. Namely, a point-mutated xSrc, in which Arg-121 was replaced by His that is conserved among mAb327-reactive Src in mammals and chicken, showed increased recognition by mAb327. On the other hand, a mutant chicken Src, in which the His-122 residue is replaced by Arg, showed decreased recognition by mAb327. Genomic sequencing analysis also demonstrated that reptile Src proteins are of either the R-type (snake) or H-type (caiman, turtle, and tortoise). These studies revealed, for the first time, a critical amino acid in the Src SH3 domain for mAb327 recognition, and suggest a novel scheme for the molecular evolution of Src, in which the H-type Src(s) are monophyletic and derived from the R-type Src.

Key words: fertilization, mAb327, phylogeny, Src, *Xenopus*.

Abbreviations: APMSF, *p*-(amidinophenyl)methanesulfonyl fluoride hydrochloride; KA, kinase-active; KN, kinase-negative; SFK, Src family tyrosine kinase; SH3, Src homology 3; WCL, whole cell lysate; WT, wild type; Xyk, *Xenopus* tyrosine kinase.

The Src family non-receptor tyrosine kinases (SFKs) are implicated in the regulation of a variety of cell functions, such as growth, differentiation, adhesion, vesicle trafficking and immune responses (*1*, *2*). Recent reports have demonstrated that SFKs in eggs are activated soon after fertilization (*3–6*). On fertilization, egg-sperm binding or fusion induces transient Ca^{2+} release and subsequent Ca^{2+}-dependent egg activation that includes exocytosis of cortical granules, lifting of the fertilization envelope, dephosphorylation of mitogen-activated protein kinase, and meiotic resumption (*5*, *7–9*). Fertilization is also accompanied by the tyrosine phosphorylation of a number of egg proteins (*10*). The importance of SFKs in sperm-induced egg activation has been suggested by the fact that tyrosine kinase inhibitors, such as genistein and herbimycine A, block the tyrosine phosphorylation of egg proteins and egg activation (*6*, *11–13*). In eggs of sea urchin (*14*, *15*), starfish (*16*), and zebrafish (*17*), SFK activity has been shown to increase within a few minutes post-insemination. We have demonstrated that in eggs of African clawed frog, *Xenopus laevis*, a 57-kDa SFK, named *Xenopus* tyrosine kinase (Xyk), is activated within 1 min after fertilization (*13*, *18*). Moreover, we have presented evidence that Xyk activation occurs upstream of Ca^{2+} release; *i.e.*, Xyk phosphorylates and activates phospholipase Cγ, which leads to increased production of inositol 1,4,5-trisphosphate, a second messenger for intracellular Ca^{2+} release (*19*). These results suggest that Xyk is involved in the signal transduction for egg activation. However, the molecular identity of Xyk has not yet been determined. In *Xenopus* oocytes, transcripts for several SFK genes exist. They include *src1*/*src2* (*20*, *21*), *yes* (*22*), *fyn* (*23*), *laloo* (*24*), and *lyn* (unpublished). On immunoblotting analysis, Xyk can be recognized by an anti-pepY antibody that was raised against a synthetic peptide corresponding to residues 410 to 428 of chicken c-Src, a region whose amino acid sequence is completely conserved among c-Src, Fyn and c-Yes. On the other hand, Xyk is hardly recognized by specific antibodies against c-Src, Fyn, c-Yes, c-Fgr, Lck, Lyn, Hck, or Blk proteins (*18*). Importantly, Xyk is poorly recognized by mAb327, a Src-specific monoclonal antibody that was generated in 1983 (*25*) and has been used on worldwide for Src studies. In this study, we carried out electrospray ionization mass spectrometry, peptide mass fingerprinting and tandem mass spectrometry (MS/MS) to clarify the molecular

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identity of Xyk. The purified preparation of Xyk contained Src1 and Src2 but not other SFKs. Thus, Xyk is renamed *Xenopus* Src (xSrc). By performing ectopic gene expression studies on wild-type and mutant xSrc as well as those of chicken c-Src in COS7 cells and *Xenopus* oocytes, we identified a single amino acid in the Src SH3 domain that is important for mAb327 to recognize Src. Genomic sequence analysis of *src* gene exon 4, which covers the mAb327 recognition site, suggested that the vertebrate Src proteins can be categorized into two types, mAb327-reactive and mAb327-poorly reactive types, and that the mAb327-reactive type Src was derived from the mAb327-poorly reactive type.

MATERIALS AND METHODS

Materials—Mouse monoclonal anti-Src antibody mAb327 (*25*) and an anti-FLAG antibody were purchased from Oncogene Research Science (MA, USA) and Sigma (MO, USA), respectively. Rabbit anti–c-Src (N-16) antibody was obtained from Upstate Biotechnology (CA, USA). Rabbit anti-Src (pY416) phospho-specific antibody was purchased from Biosource (CA, USA). Anti-pepY rabbit antibody was raised against a synthetic peptide corresponding to amino acid residues 410–428. (Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gln-Gly-Ala-Lys-Phe-Pro-Ile-Lys-Trp) of chicken c-Src, as described previously (*26*). Anti-*Xenopus* Src (anti-xSrc) was raised against a synthetic peptide that corresponds to residues 22–36. (Glu-Gly-Ser-His-Gln-Pro-Phe–Thr-Ser-Leu-Ser-Ala-Ser-Gln-Thr) of xSrc1 and 2. The synthetic peptides used for immunization were purchased from Bex Corporation (Tokyo, Japan). The Cdc2 substrate peptide, which corresponds to residues 7–26. (Val-Glu-Lys-Ile-Gly-Glu-Gly-Thr-Tyr-Gly-Val-Val-Tyr-Lys-Ala-Arg-His-Lys-Leu-Ser) of the fission yeast *cdc2* gene product, was synthesized and purified as described previously (*18*). Leupeptin was purchased from the Peptide Institute (Osaka, Japan). Other reagents were obtained from Nacalai Tesque (Kyoto, Japan), Wako (Osaka, Japan), Takara Shuzo (Kyoto, Japan), or New England BioLabs (MA, USA).

Xenopus Oocytes and Purification of Xyk—Adult African clawed frogs, *Xenopus laevis*, were purchased from Hamamatsu Seibutsu Kyozai (Hamamatsu, Japan). Xyk was purified from *Xenopus laevis* oocytes as described previously (*18*). In order to concentrate the purified Xyk, we used a mouse monoclonal antibody, Clone 28, that recognizes dephosphorylated tyrosine-527 at the C terminus of c-Src, Fyn, and c-Yes (*27*). Briefly, the Xyk-containing fractions were applied to a Clone 28 IgG-Sepharose column (0.7×2.5 cm). After washing the column with phosphate-buffered saline containing 0.5 M NaCl, the bound proteins were eluted with 0.2 M Gly-HCl, pH 2.8. The eluted materials were immediately neutralized by the addition of 1 M Tris. The preparations were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*28*) on an 8% gel, and the Xyk band at the position of 57 kDa was excised and subjected to peptide mass fingerprinting (see below).

Peptide Mass Fingerprinting and MS/MS Analysis of Xyk—The details of our methodology for peptide mass fingerprinting and MS/MS analysis will be given elsewhere (*29*). Briefly, Xyk proteins in the gels were visualized with Coomassie Brilliant Blue (Wako). The bands were excised, and the proteins were reduced by incubation with 10 mM dithiothreitol and then alkylated by incubation with 40 mM iodoacetoamide. The reduced proteins were in-gel digested with either lysyl endopeptidase (Wako) or Asp-N endopeptidase (Roche Diagnostics, Tokyo, Japan) for 20–24 h. Peptide fragments were subjected to mass fingerprinting or MS/MS using a Micromass Q-Tof2 equipped with a nano-electrospray ionization source. The data obtained for peptide fragments were analyzed using MASCOT (Matrix Science, London, UK) with the NCBI database as a searching resource.

cDNA Cloning and Construction of Expression Plasmids—The *Xenopus* orthologue of Src was cloned by RT-PCR. mRNAs were purified from *Xenopus* liver using a QuickPrep Micro mRNA Purification Kit (Amersham Bioscience, NJ, USA). The first strand cDNA was generated from the purified mRNAs with a SuperScript First-strand Synthesis System for RT-PCR (GibcoBRL, MD, USA). For the cloning of xSrc, PCR was carried out with *PfuTurbo* DNA polymerase (Stratagene, CA, USA) using primers 5′-AAAAGATCTAGGGCCCATGGGTGC-CACTAAAAGCAAGCC-3′ and 5′-AAAGGTACCGGGCCC-TAAAGGTTGTCCCCAGGCTGGTA-3′. The chicken c-Src (chSrc) gene was a gift from Dr. Hidezaburo Hanafusa (Osaka Bioscience Institute). All Src mutants were constructed by oligonucleotide-mediated mutagenesis. The PCR primers used were as follows: kinase-negative (KN) xSrc, in which Lys-294 in the ATP-binding site is replaced by Met: forward, 5′-CCACTCGAGTGGCC-ATCATGACTCTGAAGC-3′ and reverse, 5′-AA-AGGTACCGGGCCCTAAAGGTTGTCCCCAGGCTGGTA-3′; constitutively kinase-active (KA) xSrc, in which Tyr-526 is replaced by Phe: forward 5′-TTTGGAATTCTCCTGACC-GAGCTCACCACC-3′, and reverse 5′-AAAGGTACCGGG-CCCTAAAGGTTGTCCCCAGGCTGGAACT-3′ xSrc SH3 mutant (the H-type xSrc), in which Arg-121 is replaced by Arg: 5′-CTGGTGGTTGGCACATTCCCTAAGCTCTG-3′ and 5′-CAGAGCTTAGGGAATGTGCCAACCACCAG-3′; and chSrc SH3 mutant (the R-type chSrc), in which His-122 is substituted by Arg: 5′-CTGGTGGCTGGCTCG-TTCCCTCACTACAG-3′ and 5′-CTGTAGTGAGGGAAC-GAGCCAGCCACCAG-3′. For the expression of carboxyl-terminal FLAG-tagged xSrc, constructs were inserted into the p3xFLAG-CMV-14 mammalian expression vector (Sigma). For expression in *Xenopus* oocytes, the cDNAs encoding xSrc wild type (WT), KN and KA were subcloned into the pBluescript (SK–) plasmid vector (Stratagene).

Preparation of mRNA and Its Expression in Xenopus Oocytes—We employed a mMESSAGE mMACHINE (Ambion, TX, USA) for the synthesis of capped mRNA *in vitro*. Microinjection of oocyte with the synthetic mRNAs was performed as described by Smith *et al.* (*30*). Twenty-five nanoliters of mRNA (1 ng/nl) or water was injected into one oocyte using Nonoject (Drummond, PA, USA). The oocytes were incubated at 18°C for 6 h or 12 h in OR-2 (5 mM HEPES-NaOH, pH 7.5, 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 1 mM $CaCl_2$). The oocytes were then crushed by repeated pipetting in a 10-fold volume of 1% Triton X-100–containing Buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM Na_3VO_4, 10 μg/ml

leupeptin, and 20 μM *p*-(amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF)]. The lysate was subjected to immunoprecipitation analysis.

Cell Transfection—COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin under a humidified atmosphere containing 5% CO_2 at 37°C. Cells at 20–30% confluence (2×10^5 cell/dish) in 35 mm dishes were transfected with 2 μg of plasmid DNA per dish using Effectene (Qiagen, Germany) according to the manufacturer's standard protocol. After incubation for 24 h at 37°C, transfectants were washed twice with phosphate-buffered saline and then lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 20 μM APMSF). The cell lysates were vortex-mixed (10 s × 2), sonicated (30 s × 2), and then clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Protein concentrations were determined by means of a dye-binding assay (Bio-Rad, CA, USA). Calibration was performed with standard bovine serum albumin (Calbiochem, CA, USA).

Immunoprecipitation and Immunoblotting—Cell lysates (50 μg protein per lane) or oocyte lysates (7.5 oocytes per lane) were immunoprecipitated with 10 μg/ml anti-FLAG or 10 μg/ml mAb327 at 4°C overnight. Immunocomplexes were collected with 25 μl per sample of protein A–Sepharose by 30-min rocking at 4°C and then washed three times with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 20 μM APMSF). The immunoprecipitated proteins or original cell lysates (5 μg per lane) were separated by SDS-PAGE on 10% gels, followed by transfer to polyvinylidene difluoride membranes (Millipore, MA, USA) using a semi-dry blotter (Bio-Rad). The membranes were soaked in T-TBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) supplemented with 1% skim milk for 1 h at room temperature, and then incubated with the primary antibody specified in the text for 2 h at room temperature. Immunocomplexes on the membranes were visualized by color development that involved the catalytic reaction of alkaline phosphatase, which was conjugated to the goat anti–rabbit IgG antibody (Cappel, NC, USA) used as the secondary antibody. When a mouse antibody was used as the primary antibody, the membranes were treated with a rabbit anti–mouse IgG antibody (Cappel) for 1 h at room temperature prior to treatment with the enzyme-conjugated antibody.

In Vitro *Kinase Assay*—Immunoprecipitates prepared from Triton X-100–solubilized oocytes were incubated in a reaction mixture (25 μl) comprising 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 μM ATP, 100 kBq [γ-^{32}P]ATP, and 1 mM Cdc2 substrate peptide for 10 min at 30°C. Phosphorylated proteins and peptides were separated by SDS-PAGE and then analyzed with a BAS2500 imaging analyzer (Fuji Film, Tokyo, Japan).

Sequencing Analysis of Reptile src Exon 4—Frozen blood cells and livers from a spectacled caiman (*Caiman crocodiles*), an Indian star tortoise (*Geochelone elegans*), and a Japanese rat snake (*Elaphe climacophora*) were kindly provided by Dr. Natsuki Hama (Oji Zoo, Kobe, Japan). Frozen blood cells of a loggerhead turtle (*Caretta caretta*) were kindly provided by Mr. Tatsuya Oshika (Suma Aqualife Park, Kobe, Japan). The frozen liver of a Japanese fire-bellied newt (*Cynops pyrrhogaster*) was kindly provided by Dr. Akihiko Watanabe (Yamagata University, Yamagata, Japan). Genomic DNAs were extracted from these samples with the proteinase K/SDS dissolution mixture, and purified by phenol/chloroform extraction and ethanol precipitation as described by Sambrook and Russell (*31*). Two degenerate primers for PCR, 5′-GA(AorG)GGIGACTGGTGG(CorT)TGGC-3′ and

Residues		Observed (MH+)
19-41	xSrc1 DIVEGSHQPFTSLSASQTPNKSL	2443.15
	xSrc2 DIAEGSHQPFTSLSASQTPSKSL	2388.15
42-55	xSrc1 DSHRPPAQPPFGGNC(Cam)	1539.70
	xSrc2 DSHRPSGQPPFGGNC(Cam)	1515.66
56-66	xSrc1 DLTPFGGINFS	1167.56
	xSrc2 DLTPFGGVNFS	1153.54
98-115	xSrc1 DLSFKKGERLQIVNNTEG	2048.02
	xSrc2 DLSFRKGERLQIVNNTEG	2076.02

Fig. 1. **Xyk comprises a mixture of *Xenopus* Src1 and Src2.** Shown are the amino acid sequences of eight peptide fragments that were obtained from Xyk digested with Asp-N endopeptidase. Assignment using the MASCOT algorithm revealed these peptides are parts of either *Xenopus* Src1 (xSrc1) or Src2 (xSrc2). The numbering of amino acid residues for each fragment and the observed masses of singly protonated peptides (MH+) are indicated. The shadowed amino acids are conserved in xSrc1 and xSrc2. Note that in peptides of residues 42–55, the carboxyl-terminal cysteine is carboxyl-amino methylated (Cam).

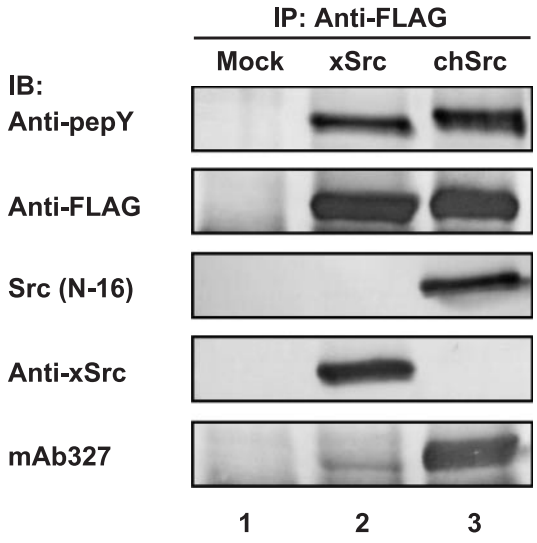

Fig. 2. **Distinct recognition of xSrc and chicken Src by anti-Src specific antibodies.** Triton X-100–solubilized extracts were prepared from mock-transfected COS7 cells (Mock, lane 1), and from cells expressing either FLAG-tagged *Xenopus* Src (xSrc, lane 2) or FLAG-tagged chicken c-Src (chSrc, lane 3) as described under "MATERIALS AND METHODS." The extracts (50 μg/lane) were immunoprecipitated (IP) with an anti-FLAG antibody, and the immunoprecipitates were analyzed by immunoblotting (IB) with anti-pepY antibody (1:100 dilution of serum), anti-FLAG antibody (0.8 μg/ml IgG), Src(N-16) antibody (1 μg/ml IgG), anti-xSrc antibody (1:100 dilution of serum), or mAb327 (1 μg/ml IgG) as described under "MATERIALS AND METHODS."

5′-TC(CorT)TCAGCCTG(AorGorT)ATGGAGTC-3′, were used to amplify nucleotide sequences that corresponded to exon 4 of *src* gene. The amplified PCR fragments were inserted into the pCR2.1-TOPO vector (Invitrogen). DNA sequences were analyzed with an ABI3100 or ABI310 genetic analyzer (Applied Biosystems). Phylogenetic analysis was performed by the neighbor-joining method using *MEGA* version 3.0 (*32*).

RESULTS AND DISCUSSION

Molecular Identification of Xyk by Mass Spectrometry— The purified preparation of Xyk was separated by SDS-PAGE and then subjected to in-gel digestion with Asp-N endopeptidase. The proteolytic fragments were analyzed by peptide mass fingerprinting using the MASCOT program or further subjected to MS/MS. The observed mass values for peptide fragments demonstrated the presence of protein products of the *Xenopus src1* and *src2* genes (xSrc1 and xSrc2) (Fig. 1), whose mRNAs have been shown to be present in *Xenopus* oocytes (*20*, *21*). Four pairs of Asp-N peptide fragments, which were similar to but distinct from each other, were assigned as either xSrc1 or xSrc2. Analysis of a peptide fragment that corresponds to residues 517 to 528 of xSrc1/2 demonstrated that C-terminal tyrosine residue 526 is unphosphorylated

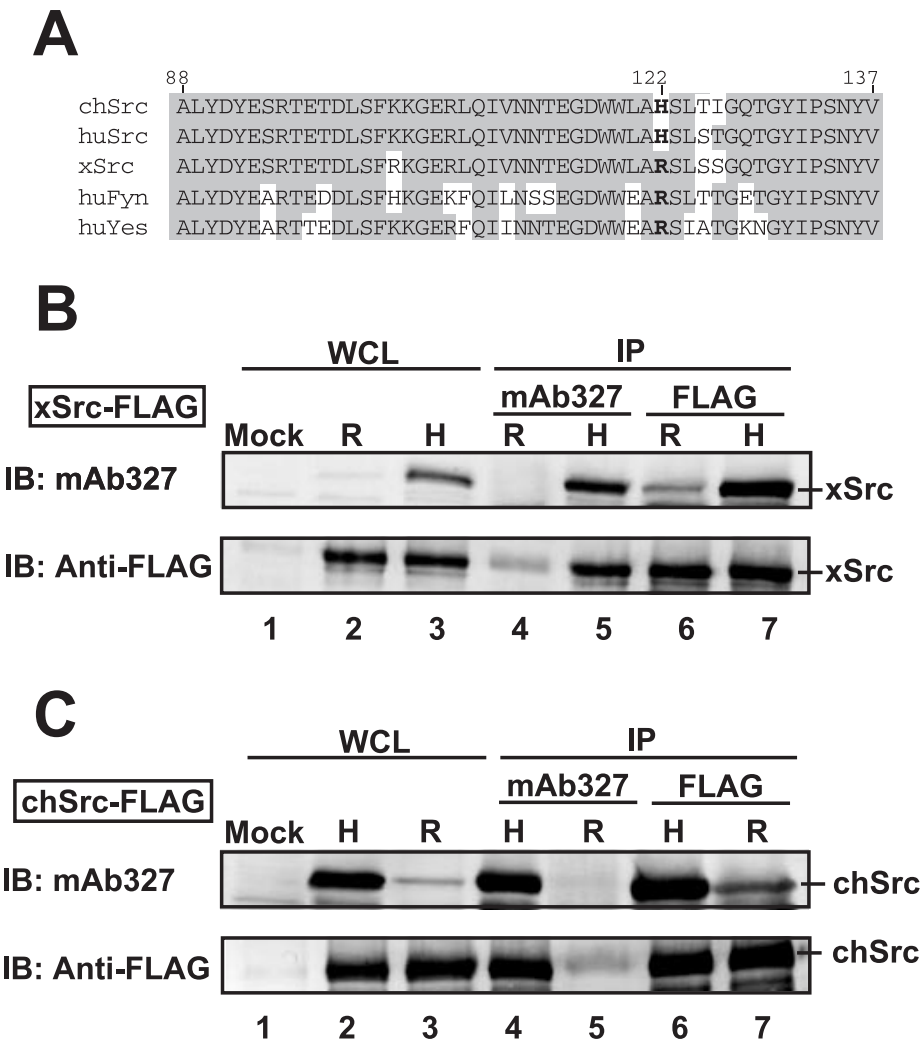

Fig. 3. **A point mutation in the Src SH3 domain affects its recognition by mAb327.** (A) Shown is amino acid-alignment of the SH3 domains of Src family members: chicken Src (chSrc), DDBJ/EMBL/GenBank accession number V00402; human Src (huSrc), BC011566; *Xenopus* Src2 (xSrc), M23422; human Fyn (huFyn), M14333; and human Yes (huYes), M15990. Conserved amino acid residues are shadowed. Bold letters indicate the position of a histidine residue that is conserved in chicken and human Src. This residue is substituted by an arginine residue in xSrc, human Fyn, and human Yes. Numbering above the alignment indicates the amino acid residue numbers of chSrc. (B) Triton X-100–solubilized extracts were prepared from mock-transfected COS7 cells, and from COS7 cells expressing wild type xSrc, which contains arginine at position 121, the R-type xSrc (R), or the H-type xSrc, in which arginine-121 was substituted by histidine (H). Immunoprecipitates of the extracts (IP, 50 μg/lane) obtained with either mAb327 (mAb327) or anti-FLAG (FLAG) antibody as well as a whole cell lysate (WCL, 5 μg/lane) were analyzed by immunoblotting (IB) with mAb327 or anti-FLAG antibody. The positions of xSrc are indicated. (C) Mock-transfected COS7 cells and COS7 cells expressing wild type chSrc, which contains histidine-122 (H), or R-type chSrc (R), in which histidine 122 was replaced by arginine, were prepared. Immunoprecipitation and immunoblotting analyses were carried out as in panel B. The positions of chSrc are indicated.

(data not shown). This is consistent with that the Xyk preparation was prepared using an antibody toward the unphosphorylated C-terminus of SFKs (*27*) (see "MATERIALS AND METHODS"). MS/MS of the same Xyk preparation digested with Lys-C endopeptidase demonstrated that a peptide fragment containing the amino-terminal glycine residue of xSrc1/2 is myristoylated (data not shown). Importantly, peptide fragments for no other SFKs or protein kinases were detected. Thus, we conclude that the purified Xyk is a mixture of xSrc1 and xSrc2, and thus is renamed xSrc.

Immunochemical Characterization of xSrc Expressed in COS7 Cells—To characterize the structure and function of xSrc, we employed the COS7 cell expression system. We constructed an expression plasmid encoding xSrc2, hereafter denoted as xSrc for simplicity, which was tagged with the FLAG sequence at its C-terminus. As can be seen in Fig. 2, we performed immunoblotting analysis with several antibodies of Triton X-100–solubilized extracts of COS7 cells expressing nothing (Mock, lane 1), xSrc-FLAG (xSrc, lane 2), or chicken c-Src-FLAG (chSrc, lane 3). The data obtained with an anti-pepY antibody, *i.e.*, a pan-SFK antibody that recognizes the autophosphorylation site of SFKs, and an anti-FLAG antibody showed that almost equal amounts of 62 kDa xSrc-FLAG and 63 kDa chSrc-FLAG were detected. As we expected, an anti–mammalian Src-specific antibody, Src(N-16), and an anti-xSrc antibody showed an exclusive pattern of recognition. On the other hand, a monoclonal anti-Src antibody, mAb327, showed only limited efficiency as to recognition of xSrc-FLAG.

Identification of His-122 as a Crucial Residue for mAb327 Recognition—The results in Fig. 2 are consistent with our previous finding that Xyk is hardly detectable with mAb327 (*18*), and indicate that the amino acid sequence for the epitope region of mAb327 in chSrc may differ from that of xSrc. It has been believed that the epitope for mAb327 resides in the SH3 domain of chSrc, which comprises 50 amino acids and is highly conserved among SFKs. As shown in Fig. 3A, however, we found that a histidine residue at position 122 (His-122) in chSrc, which corresponds to His-125 in human c-Src, is substituted by an arginine residue in xSrc (Arg-121). A similar substitution was also found in human Fyn and c-Yes (Fig. 3A), both of which are also hardly detectable with mAb327 (*18*). To determine whether or not the histidine residue in the Src SH3 domain is important for the immunoreactivity of Src toward mAb327, we prepared COS7 cells expressing a point-mutated version of xSrc, the H-type xSrc, in which Arg-121 in the SH3 domain was replaced by histidine. Triton X-100–solublized cell lysates were analyzed by immunoblotting with either mAb327 or anti-FLAG antibody before (whole cell lysate, WCL) or after immunoprecipitation (IP) with either the mAb327 or anti-FLAG antibody (Fig. 3B). The H-type xSrc exhibited stronger immunoreactivity toward mAb327 compared to the wild type (the R-type) xSrc (IB: mAb327, lanes 2 and 3). The efficiency of immunoprecipitation of the H-type xSrc with mAb327 was also greatly improved; comparable amounts of xSrc were detected in the mAb327 and anti-FLAG immunoprecipitates (lanes 5 and 7), while the R-type xSrc was barely detected in the mAb327 immunoprecipitates (lane 4). On the other hand, we observed that a point-mutated chSrc (R-type chSrc) that contains an arginine residue at position-122 instead of histidine residue exhibited a dramatic reduction in immunoreactivity toward mAb327 (Fig. 3C). From these

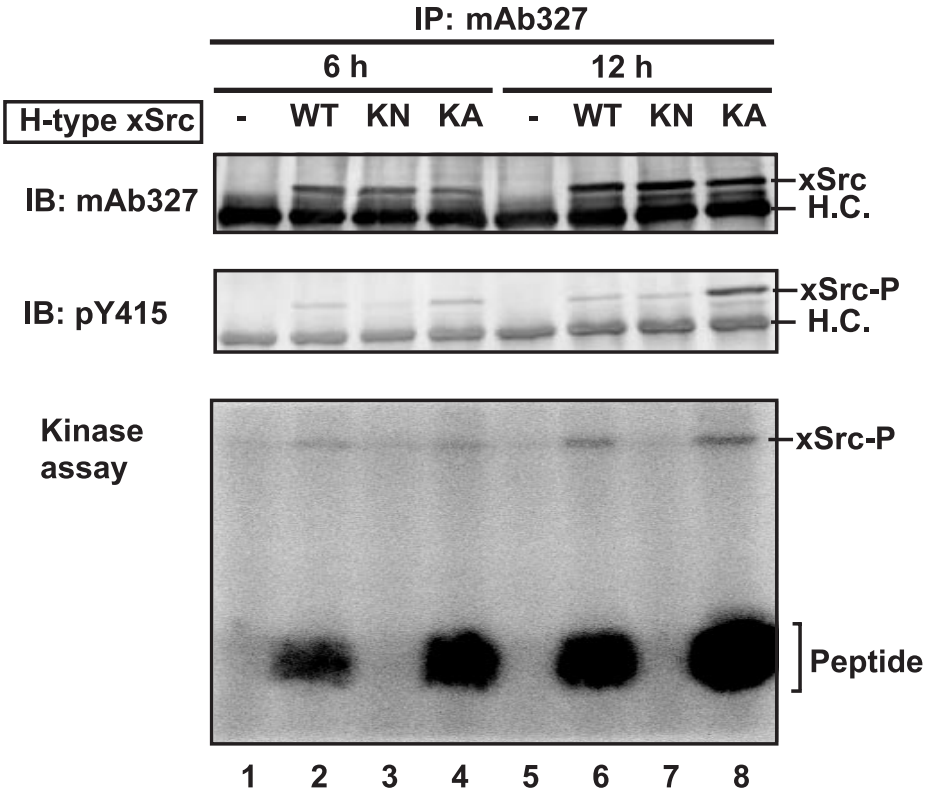

Fig. 4. **Expression of mAb327-reactive xSrc in *Xenopus* oocytes.** *Xenopus* oocytes were injected with water alone (lanes 1 and 5), or mRNA (lanes 2–4 and 6–8, 25 ng per oocyte) encoding the H-type xSrc of kinase wild type (WT, lanes 2 and 6), kinase-negative (KN, lanes 3 and 7), and constitutively kinase-active (KA, lanes 4 and 8). At 6 or 12 h after mRNA injection, Triton X-100–solubilized oocyte extracts were prepared and immunoprecipitated (IP, 7.5 oocyte per lane) with mAb327. The immunoprecipitates were analyzed by immunoblotting (IB) with mAb327 (top panel) or anti-Src phosphotyrosine 415 specific antibody (pY415, middle panel), or the *in vitro* kinase assay (bottom panel) as described under "MATERIALS AND METHODS." The positions of xSrc, heavy chains of IgG (H.C.), and the phosphorylated xSrc (xSrc-P) and substrate peptide (peptide) are indicated.

data, we conclude that a histidine residue at position 122 in the chSrc SH3 domain is critical for the immunoreactivity of Src toward mAb327.

Expression of mAb327-Immunoreactive xSrc in Xenopus Oocytes—We next wanted to determine whether or not the H-type, i.*e.*, mAb327-immunoreactive type xSrc, could be expressed functionally in *Xenopus* oocytes. To this end, we performed microinjection of *Xenopus* stage VI oocytes with poly(A)$^+$-mRNA encoding different H-type xSrc constructs: wild type (WT), kinase-negative (KN), and kinase-active (KA). At 6 h or 12 h after injection, oocytes were collected and extracted in the presence of 1% Triton X-100. The oocyte extracts were then immunoprecipitated with mAb327 and the immunoprecipitates were analyzed by immunoblotting or with the *in vitro* kinase assay. As shown in Fig. 4, all H-type xSrc proteins could be recovered from oocytes, as judged from the mRNA- and injection time-dependent appearance of an mAb327-immunoreactive band (Fig. 4, IB: mAb327). To assess the catalytic activity of xSrc expressed in oocytes, the immunoprecipitates were analyzed by immunoblotting with an antibody specific to the phosphorylated Y415 of Src, a hallmark of the active state of Src. Consistently, an anti–pY415-immunoreactive band could be detected for the mAb327 immunoprecipitates of WT and KA. It should also be noted that KN (Fig. 4, IB: pY415) exhibited immunoreactivity with the anti-pY415 antibody (Fig. 4), suggesting that endogenous oocyte xSrc or other tyrosine kinases can phosphorylate Tyr-415 of the H-type KN xSrc intermolecularly. The *in vitro* kinase assay demonstrated that the immunoprecipitates containing xSrc WT and KA, but not KN, are capable of autophosphorylation and phosphorylation of the Cdc2 peptide (Fig. 4, Kinase assay). Thus, we conclude that mAb327-reactive xSrc can be successfully expressed in *Xenopus* oocytes with an expected functional property.

Sequencing and Phylogenetic Analysis of Exon 4 of src *Genes in Vertebrates*—As shown in this study, *Xenopus* Src

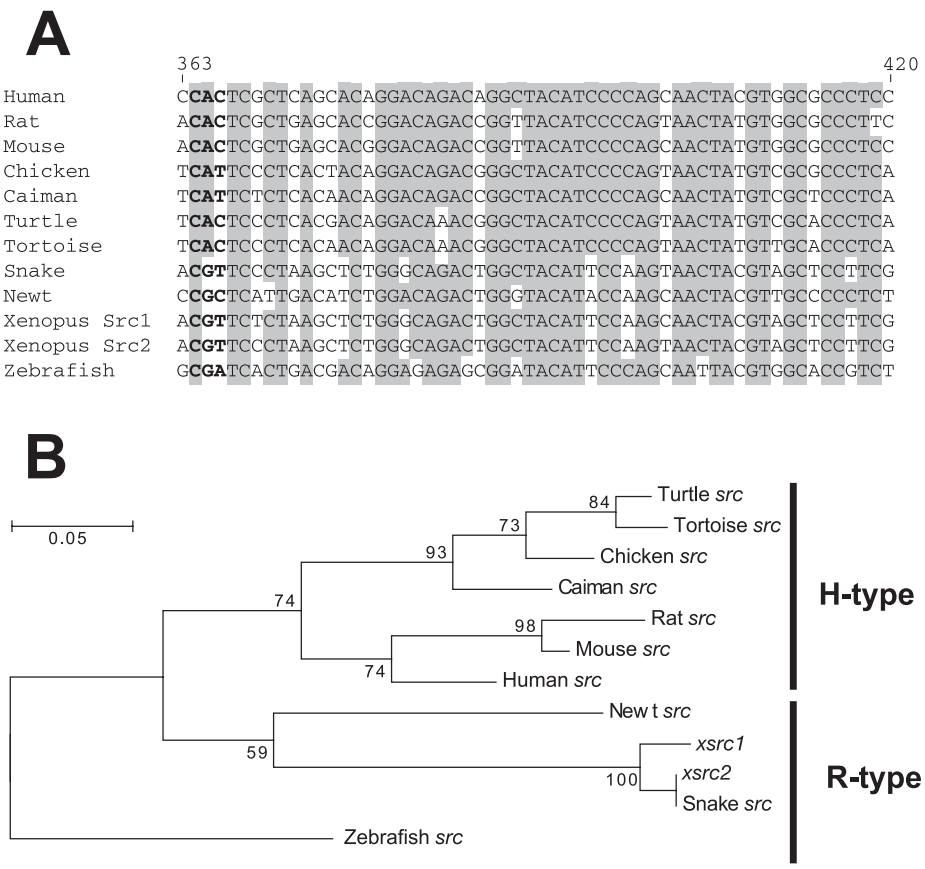

Fig. 5. **Comparison of the exon 4 of *src* genes in amphibians and reptiles.** (A) Shown are the nucleotide sequences of *src* exon 4: human, *Homo sapiens c-src* (Human); rat, *Rattus norvegicus c-src* (Rat); mouse, *Mus musculus c-src* (Mouse); chicken, *Gallus gallus c-src* (Chicken); spectacled caiman, *Caiman crocodilus c-src* (Caiman); loggerhead turtle, *Caretta caretta c-src* (Turtle); Indian star tortoise, *Geochelone elegans c-src* (Tortoise); Japanese rat snake, *Elaphe climacophora c-src* (Snake); Japanese fire-bellied newt, *Cynops pyrrhogaster* (Newt); African clawed frog, *Xenopus laevis src1* (xSrc1); *Xenopus laevis src2* (xSrc2); and zebrafish *Danio rerio* (Zebrafish). Shadowed are those that are conserved among more than 7 genes. Shown in bold letters are those encoding His or Arg residues. Numbering above the alignment indicates the nucleotide numbers of chSrc. (B) Phylogenetic tree inferred from 12 nucleotide sequences of *src* exon 4 based on Kimura's 2-parameter distance. Numbers on the branch indicate 1,000 bootstrap values (%) determined with the maximum neighbor-joining method. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the following accession numbers: *Homo sapiens c-src*, BC011566; *Rattus norvegicus c-src*, AF130457; *Mus musculus c-src*, BC039953; *Gallus gallus c-src*, V00402; *Caiman crocodilus c-src*, AB193187; *Geochelone elegans c-src*, AB193185; *Caretta caretta c-src*, AB193182; *Elaphe climacophora c-src*, AB193189; *Cynops pyrrhogaster c-src*, AB193184; *Xenopus laevis src1*, M24704; *Xenopus laevi src2*, M23422; and *Danio rerio c-src*, AJ620750.

proteins, xSrc1 and xSrc2, are of the R-type. Likewise, zebrafish Src has also been determined to be of the R-type (unpublished). On the other hand, Src in both mammalian and avian species such as human, mouse, rat and chicken are of the H-type. In order to determine the distribution pattern of the H-type and R-type in amphibians and reptiles, we performed genomic PCR-sequencing analysis of *src* gene exon 4, which encodes the mAb327-recognition site. The results obtained are summarized in Fig. 5A. A urodele amphibian, the Japanese fire-bellied newt (*Cynops pyrrhogaster*), possesses an R-type *src* like *Xenopus*. On the other hand, the *src* genes in reptiles are heterogeneous: *src* in the Japanese rat snake (*Elaphe climacophora*), a lepidosaurian reptile, is of the R-type, while *src* in the spectacled caiman (*Caiman crocodilus*), loggerhead turtle (*Caretta caretta*), and Indian star tortoise (*Geochelone elegans*) are all of the H-type. The phylogenetic tree inferred from the exon 4 sequences of vertebrate *src* genes demonstrated that the H-type and R-type Src can be separated into two clades with relatively high bootstrap value (Fig. 5B). Turtle, tortoise, caiman and chicken form a monophyletic group with a 93% bootstrap value. This relationship supports the estimation by Hedges and Poling (*33*). These results suggest that the H-type Src was derived from R-type Src through replacement of Arg (CGC, CGT or CGA) by His (CAC or CAT).

Since mAb327 was established by Lipsich *et al.* 23 years ago (*25*), it has been the most popular anti-Src monoclonal antibody worldwide. We showed here for the first time that for Src-recognition by mAb327, the histidine residue within the SH3 domain of Src (His-122 in chicken Src) is very important, and that a phylogenetic feature of the vertebrate Src could be inferred by analyzing the amino acid sequence surrounding this histidine (or arginine in xSrc) residue. The histidine or arginine residue in the Src SH3 domain is located just nearby the proline-rich sequence-binding region and is exposed on the molecular surface (*34*). Some SH3-interacting molecules can augment Src activity (*35*). Therefore, arginine/histidine variation in this region may influence the Src SH3 interaction with other signaling molecules and/or regulation of the catalytic activity of Src. Our results suggest that the H-type Src evolved from the R-type Src, and was selected in mammalian, avian and some reptile species (Fig. 5). It is supposed that the H-type and R-type Src were positively selected in each species through adaptive evolution together with some physiological and biochemical changes such as improvement of the immune system, homoiothermilization, acquisition of an efficient metabolic system, and so on. The functional differences between R-type and H-type Src(s), however, remain unclear, and thus are under investigation.

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