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Production of Mouse ES Cells Homozygous for Cdk5-Phosphorylated Site Mutation in c-*src* Alleles

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c-Src-null mutants have not provided a full understanding of the cellular functions of c-Src, reflecting the functional redundancy among Src family members. c-Src is phosphorylated by cyclin-dependent kinase 1 (Cdk1) and Cdk5 at Ser75 in the unique amino terminal c-Src–specific domain. The specific roles of c-Src may be assessed by establishing mouse embryonic stem (ES) cells homozygous for a point mutation at Ser75. Mammalian homozygous cultured cells with a point mutation, however, have not yet been produced by gene targeting. Here we show an efficient procedure for producing ES cell clones bearing a homozygous Ser75 to Asp mutation in the c-*src* gene. This procedure was developed by combining two previously reported strategies: our procedure for introducing a point mutation into one allele with no exogenous sequence, and the high-geneticin (G418) selection procedure for introducing a mutation into both alleles. The mutant clones expressed the same levels of c-Src protein and autophosphorylation activity as wild-type cells, but the mutant c-Src was not phosphorylated on Ser75 during mitosis. This procedure is feasible for generating cells homozygous for a subtle mutation in most genes, and is expected to be applicable to other somatic cell lines.

Key words: Cdk5, c-Src, ES cells, gene targeting, homozygous mutant.

Abbreviations: ASO, allele-specific oligonucleotide; BCS, bovine calf serum; Cdk, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle medium; ES, embryonic stem; FBS, fetal bovine serum; FIAU, 1-[2′-deoxy-2′-fluoro-β-D-arabinofuranosyl]-5-iodouracil; G418, geneticin; LIF, leukemia inhibitory factor; PSL, photo-stimulated luminescence; SD, Ser75 to Asp.

c-Src protein tyrosine kinase is expressed ubiquitously in tissues and is highly expressed in platelets, neurons and osteoclasts (*1*). c-Src may be involved in diverse cellular activities through membrane and cytoskeletal modulation (*2–5*). Nevertheless, a c-Src knockout mouse shows a restricted phenotype dependent on a deficiency in osteoclast function (*6*). This may reflect functional compensation by other *src* family kinases or related molecules (*7*, *8*). To overcome this limitation, an *in vitro* cell culture system has been established from the knockout mouse, and some phenotypes have been reported for c-Src-deficient fibroblasts (*9*) and neurons (*10*). As another approach, the introduction of a point mutation into the c-*src* allele may be effective, but has not yet been reported.

c-Src is phosphorylated by Cdk1 at specific serine and threonine residues during mitosis in fibroblastic and epithelial cells (*11–13*). One of the mitotic phosphorylation sites in human c-Src, Ser75 (position 74 of the mouse sequence), is phosphorylated by Cdk5 throughout the cell cycle in Y79 human retinoblastoma cells (*14*, *15*). Although this mitosis-independent phosphorylation occurs in some neuronal and non-neuronal cultured cells (*14*, *16–19*), its physiological roles remain unknown. Ser75 exists in a unique region, the sequences of which share no homology with those of other *src* family kinases (*1*). The introduction of point mutations into the unique region may provide effective approaches for analyzing c-Src-specific functions. Therefore, we tried to produce homozygous ES cells carrying a Ser75 to Asp (SD) mutation in c-*src*.

The gene targeting technique using homologous recombination offers powerful methods for altering the structure of a gene specifically to clarify its function in the whole animal through targeted ES cells (*20*). Using this technique, methods for introducing a subtle mutation into one allele (*21–29*) and disruption into both alleles (*30–33*) have been developed for mouse ES cells and other cultured cells. The development of methods for producing mammalian cultured cells homozygous for a subtle mutation should be useful for analyses of protein structure and function. However, the production of such homozygous cells has not yet been reported.

We previously developed an efficient procedure for introducing a subtle mutation into one allele of the *transthyretin* gene in mouse F9 cultured cells by replacement and excision steps (*23*). On the other hand, a high-G418 selection procedure (*31*) has been developed for generating cells homozygous for the disrupted allele. By treating the heterozygously targeted cells bearing one *neo* copy with a high concentration of G418, cells homozygous for the targeted allele and containing two *neo* copies can be generated without a second round of targeting of the locus (*30*, *32*). Overall, we designed the procedure for producing cells homozygous for a subtle mutation by combining the above two methods.

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Here we report, for the first time, the production of mouse ES cells bearing an SD mutation in both alleles of the c-*src* gene and the characterization of the mutant c-Src protein.

MATERIALS AND METHODS

Construction of the Targeting Vector—Mouse genomic c-*src* clone 5 was isolated from the 129SvJ mouse genomic library with a lambda FIX II vector (Stratagene) using a 1.36-kb *Nco*I–*Bam*HI fragment of human c-*src* cDNA as a probe. A 9.7-kb *Xba*I–*Not*I fragment from clone 5 was inserted into pBluescript II to generate pBMS2. An 11.9-kb *Hpa*I–*Not*I fragment of pBMS2 was self-ligated to generate pBMS4, disrupting the *Eco*52I site of pBluescript II. The SD mutation (TCC→GAC) was introduced into the second exon of c-*src* by oligo-directed mutagenesis (*34*). A 350-bp *Eco*52I-*Eco*T22I fragment carrying the mutation was ligated to the *Eco*52I-*Eco*T22I fragment of pBMS4 to generate pBMS4SD. After disrupting the *Xba*I site of pGKneo, a 1.6-kb *Hin*dIII–*Xho*I fragment was isolated, blunt-ended and inserted into the blunt-ended *Hin*dIII site of pBMS4SD to generate pBMS4SDneo. After ligating a *Hin*dIII linker to the blunt-ended *Xho*I site of the 3.2-kb *Xho*I–*Hin*dIII fragment of pBMS4, this fragment was inserted into the *Hin*dIII site of pBMS4SDneo to generate pBMS4SDneoD. A 1.9-kb blunt-ended *Xho*I–*Hin*dIII fragment from pMCtk was inserted into the blunt-ended *Eco*RI site at the 5′ end of the *neo* gene of pBMS4SDneoD to generate pBMS4SDtk/neoD. HSV-*tk*, *neo* and c-*src* are all transcribed in the same direction on the vector. The targeting vector consists of a 9.0-kb c-*src* fragment bearing a subtle mutation and a 3.4-kb cassette of pMCtk-pGKneo flanked by a 3.2-kb duplication of part of the c-*src* genomic DNA fragment.

ES Cell Culture and Transfection—CCE ES cells (*35*) were cultured on a feeder layer of SNL-STO cells, which were transfected with a *neo* and a leukemia inhibitory factor (LIF) expression construct, in ES medium containing Dulbecco's modified Eagle's medium (DMEM, GIBCO), 10% fetal bovine serum (FBS, GIBCO), 5% bovine calf serum (BCS, Hyclone), 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1% penicillin/streptomycin (GIBCO), and 1,000 units/ml LIF (GIBCO) as described previously (*36*). To introduce the targeting construct into the endogenous c-*src* gene, 20 μg of linearized DNA was electroporated into 2×10^7 cells at 200 V and 960 μF. After 72 h, the ES medium was supplemented with 200 μg/ml G418, and colonies were isolated after 6–7 d of selection.

G418 Selection for a Homozygous Mutant—The minimum concentration of G418 required for the complete inhibition of colony formation of the targeted clones was determined. Cells from each clone were plated at 200 cells per well in a 24-well plate. After 6 h, 0, 0.5,1, 2, 5, 10, 15, or 20 mg of G418 per ml was added to the media, and the cells were cultured for 7d. To produce cells homozygous for the targeted allele, cells from each clone were plated at 5×10^5 cells per 60 mm plate, and surviving clones were selected after 17 h at the minimum concentration and over.

Production of Revertants—To isolate homozygous revertants by homologous recombination within the duplications, cells homozygous for the targeted allele were expanded in G418 and then replated at 10^6 cells per 60-mm plate in non-selection medium. At 2 and 3 d after replating, 1-[2′-deoxy-2′-fluoro-β-D-arabinofuranosyl]-5-iodouracil (FIAU)-resistant clones were selected against 0.25 μM FIAU.

Southern Blot Analysis of ES Cell DNA—Genomic DNA from ES cell clones were obtained by a microextraction procedure as described previously (*37*), digested with *Eco*RI and probed with a 0.33-kb *Nco*I-*Hin*cII fragment of exon 2 (probe E2).

Dot Blot Analysis of ES Cell DNA—ES genomic DNA digested with *Eco*RI was amplified by PCR using primers 1 and 2. Primer 1 was derived from the c-*src* genomic sequence upstream of the *Xba*I site preceding exon 2 (5′-TGGTACCTCTGGCTTCTCCTT-3′). Primer 2 was derived from the genomic sequence of intron 2 (5′-ACCACAGCT-CAGGGACACAC-3′). The annealing temperature was 63°C. PCR products were spotted onto duplicate membranes, denatured, neutralized and fixed. One membrane was hybridized at 48°C with the allele-specific oligonucleotide (ASO) probe *wt* to detect the normal allele, and the other was hybridized with the ASO probe *sd* to detect the mutant allele. Probes *wt* and *sd* were completely homologous to the normal sequence (5′-GTCACCTCCCCGCA-GA-3′) and to the mutant sequence (5′-TCTGCGGGTCG-GTGAC-3′), respectively, at the region around the point mutation. Probes were labeled with ^{32}P by 5′ end-labeling. The membranes were washed three times at room temperature and once at 48°C in 6× standard saline citrate.

Immunoprecipitation, Immunoblot Analysis and Autophosphorylation Assay—To remove the feeder fibroblasts, clonal ES cells were passaged four times on gelatinized plates as described previously (*36*). The feeder-free ES clonal cells were cultured on gelatinized plates in the presence of LIF, grown to late-log phase, washed with PBS and lysed as described previously (*38*). c-Src was immunoprecipitated with anti-Src antibody 327 from cell lysates (*38*). The immuoprecipitates were subjected to immunoblot analysis and autophosphorylation assay as described previously (*38*).

Radiolabeling of Nocodazole-Arrested ES Cells—Mitotic ES cells were prepared by adding nocodazole to the medium to a final concentration of 3 μg/ml as described previously (*39*). The feeder-free ES cells were plated at 6×10^6 cells on a gelatinized 60-mm dish in the presence of LIF. Two days later, the medium was renewed with or without the addition of nocodazole. Cells were cultured for 15 h at 37°C, and the medium was replaced with phosphate-free DMEM plus 10% dialyzed FCS with or without the addition of nocodazole. Mitotic and unsynchronized ES cells were labeled with 0.7 mCi/ml [^{32}P]orthophosphate for 3.5 h, washed, and lysed as described above. c-Src was immunoprecipitated and separated on SDS-10% PAGE as described previously (*38*).

Phosphopeptide Analysis—The ^{32}P-labeled c-Src was excised from unfixed gels and subjected to V8 protease mapping (*15*). The tryptic digests of the N-terminal 16-kDa V8 fragments were subjected to one-dimensional

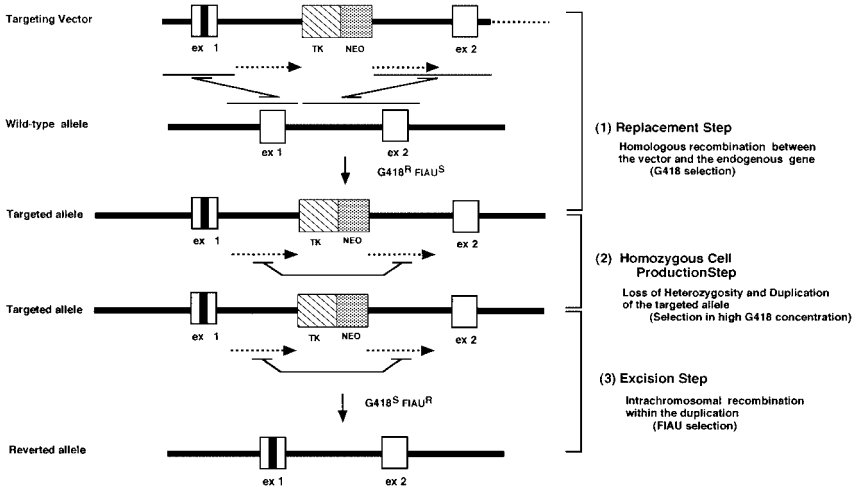

Fig. 1. **Strategy for introducing a point mutation into both alleles of an endogenous mouse gene.** The replacement step involves homologous recombinaton between the targeting vector and the endogenous gene. The vector contains a mouse genomic DNA with a *neo* and *tk* gene flanked by a duplication of the fragment of the gene. A point mutation is introduced into the first exon of the gene. The homologous recombination creates the mutation and the selection cassette flanked by the duplication in one allele. Targeted clones are selectable in G418 after transfection of CCE ES cells with the vector. In the homozygous cell production step, cells homozygous for the targeted allele are selected by growing the heterozygous cells in the presence of a high concentration of G418. In the excision step, homozygous cells bearing only the point mutation with no selection cassette are selectable in FIAU from the high-G418 surviving clones. ex, exon; TK, HSV-*tk*; NEO, *neo*; Dashed arrows localize a duplication of the fragment of the gene; thick lines, introns; dashed lines, plasmid DNA; The point mutation is represented by a thick line in the first exon; R, resistant; S, sensitive.

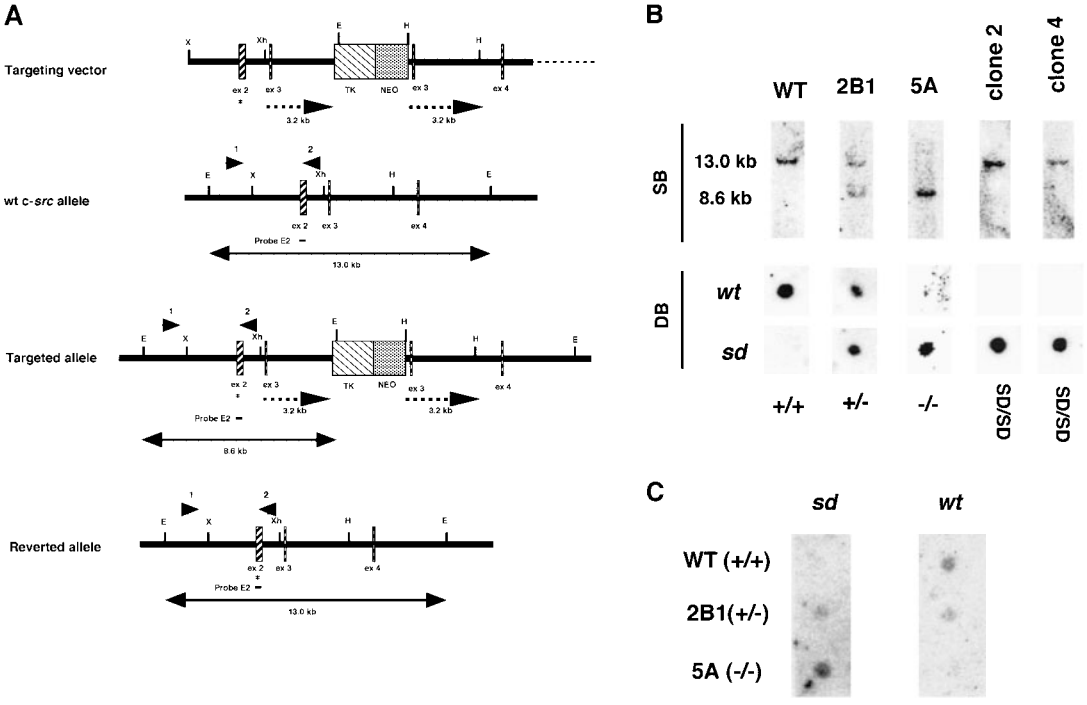

Fig. 2. **Generation of ES cells homozygous for the SD mutation in c-*src*.** (A) Structures of wild-type, targeted and reverted alleles, and the targeting vector. The targeting vector contains 9.0-kb mouse c-*src* sequences, a 3.4-kb HSV-*tk*-*neo* cassette flanked by a 3.2-kb duplication containing a part of intron 2, exon 3 and a part of intron 3 of c-*src*. Exon 2 includes a 2-bp SD mutation (TCC→GAC). Probe E2 for Southern analysis is the 0.33-kb *Nco*I–*Hin*cII fragment of exon 2 of c-*src*. The sizes of the fragments recognized by probe E2 are predicted to be 13.0-kb for the wild-type allele and 8.6-kb for the targeted allele. Dashed lines, pBluescript II DNA; Arrowheads with 1 and 2 correspond to primers 1 and 2, respectively; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; X, *Xba*I; Xh, *Xho*I; asterisk, 2-bp mutation in exon 2; wt, wild-type. Other symbols are as described in the legend to Fig. 1. (B) Southern blot analysis of the c-*src* region (upper panel) and dot blot analysis of the PCR-amplified products by the hybridization of ASO probes (lower panel). Genomic DNA was isolated from ES cell clones by the microextraction procedure, and 40% of the extracts was subjected to *Eco*RI digestion and then Southern blot analysis using probe E2. The *Eco*RI-digested genomic DNA was amplified by PCR using primers 1 and 2. PCR products were spotted onto duplicate membranes, denatured, neutralized and fixed. One membrane was hybridized at 48°C with the ^{32}P-labeled ASO probe *wt* to detect the normal allele, and the other was hybridized with the ^{32}P-labeled ASO probe *sd* to detect the mutant allele. (C) ASO probe hybridization using the same amount of PCR products from clones WT, 2B1 and 5A. The same amounts of DNA were subjected to dot blot analysis as described above. The radioactivity of the respective spots was quantitated by determining the photo-stimulated luminescence (PSL) values on a BAS 2000 image analyzer (Fuji). The PSL values were 1635 (2B1) and 3436 (5A) for *sd*; 694 (2B1) and 1550 (WT) for *wt*. The genotypes of cells are indicated as +/+ (wild-type), +/– (heterozygous), –/– (homozygously targeted) and SD/SD (homozygously SD mutated). WT, wild-type ES cells; *wt*, ASO probe *wt*; *sd*, ASO probe *sd*; SB, Southern blot; DB, dot blot.

analysis on Tricine-SDS-PAGE (16.5%/T/3%C) as described previously (*15*).

RESULTS

Strategy to Introduce a Point Mutation into Both Alleles of a Mouse Endogenous Gene—The procedure for introducing the point mutation into both alleles of the gene comprises three steps: (i) replacement, (ii) homozygous cell production, and (iii) excision (Fig. 1). The targeting vector consists of mouse genomic sequences of the gene modified to include the point mutation, an HSV-tk-neo cassette flanked by a duplication containing a part of the gene. In the first step, the homologous recombination introduces the mutation and the cassette flanked by the duplication into one allele. The targeted clones are selectable in G418 after transfection of CCE ES cells with the vector. In the second step, cells homozygous for the targeted allele are selectable from the heterozygous cells grown in a high concentration of G418. In the third step, intrachromosomal recombination within the duplication in both alleles generates the homozygous mutant clones with no selectable marker. The excision events are selectable in FIAU for the loss of HSV-*tk*.

Introduction of SD Mutation into Both Alleles of c-src in ES cells: Replacement Step—ES cells were cultured, transfected with the linearized vector, and G418 resistant clones were isolated. These clones were screened for homologous recombination by Southern blot analysis of the genomic DNA with probe E2 (Fig. 2A). This probe hybridizes to a 13.0-kb fragment of the wild-type allele and an 8.6-kb fragment of a correctly targeted allele (Fig. 2A). Moreover, the digests of clones revealing two expected fragments from the wild-type and targeted alleles were subjected to PCR amplification. PCR primers were derived from a c-*src* genomic sequence upstream from the *Xba*I site at the 5′ end of the targeting vector and from a sequence in intron 2 downstream from the mutation site. ASO probes were dot-blot hybridized to the PCR products to confirm the heterozygosity.

A 13.0-kb band was detected in wild-type cells, and an additional 8.6-kb band was detected in a G418-resistant clone, 2B1 (Fig. 2B). The PCR product from the wild type reacted only with probe *wt*, and that of 2B1 reacted with both probes *wt* and *sd* (Fig. 2B). Six clones corresponding to the expected homologous recombination event were obtained from 477 G418-resistant clones. The frequency is slightly higher than that reported for the targeted disruption of the similar c-*src* locus (*6*), suggesting that neither the length of the insertion of non-homologous sequences nor the presence of the duplication affects the targeting efficiency as described previously (*23*).

Homozygous Cell Production Step—The minimum G418 concentration for the complete inhibition of colony formation in the targeted clone 2B1 was 5 mg/ml, and so cells were selected at 5, 6 and 7 mg/ml G418. The frequency of the appearance of the high-G418 resistant colonies was 6×10^{-5} at 5 mg/ml. Few surviving clones were obtained at 6 and 7 mg/ml. The surviving clones at 5 mg/ml were analyzed for loss of heterozygosity by Southern and ASO probe hybridizations. The 8.6-kb band was detected, whereas the 13.0-kb band was not, in clone 5A (Fig. 2B). The ASO probe *sd* dot-hybridized to the amplified DNA from clone 5A, whereas probe *wt* did not (Fig. 2B). These results show that clone 5A has no wild-type allele. The amounts of DNA loaded using the microextraction procedure varied in the Southern blot analyses. To estimate the copy number of the targeted allele in clone 5A, ASO probe hybridization using the same amounts of PCR products from the clones was conducted. The quantity of the product was measured by the minigel method (*40*). The relative intensities of the wild-type and the targeted alleles from clone 2B1 were 0.45 (1.0 for wild-type CCE) and 0.48 (1.0 for clone 5A), respectively (Fig. 2C). The levels of the wild-type and targeted alleles in 2B1 were almost the same in the Southern blot analysis (Fig. 2B), suggesting that clone 5A contains two copies of the targeted allele. Two desired homozygous clones were obtained from 16 surviving clones selected at 5 mg/ml, suggesting that this step occurs efficiently retaining the mutation.

Excision Step—This step excises the selection cassette by homologous recombination between the duplicaton at both alleles. Clone 5A was expanded in the absence of G418 for 48 or 72 h. FIAU-resistant clones were then selected and screened for the excision by Southern blot and ASO probe hybridization analyses (Fig. 2A). The data for clones 2 and 4 are shown in Fig. 2B. The 13.0-kb bands from the reverted allele were detected, but not the 8.6-kb bands from the targeted allele. The probe *sd* dot-hybridized to the amplified DNA, whereas the probe *wt* did not. These results indicate that these clones carry only the mutant allele with no selectable marker. Three desired clones were obtained from12 resistant clones isolated after the 72-h pre-culture without G418. No desired clones were obtained from 9 resistant clones from the 48-h pre-culture.

Expression of c-Src in the Mutants—To verify that the mutation in the c-*src* in mutant clones 2 and 4 was a homozygous mutation, we examined the levels of the c-Src protein and its autophosphorylation activity in undifferentiated cell clones. The quantitative data are summarized in the legends to Fig. 3. The c-Src protein levels in clones 2 and 4 were nearly equal to those of the wild-type ES cells. The autophosphorylation activity levels in mutant clones 2 and 4 also were nearly equal to those of the wild type (Fig. 3, A and C). The two levels in the heterozygous clone 2B1 in the replacement step were nearly half of those of the wild type; The high-G418 surviving clone 5A showed no detectable level of the protein or its kinase activity (Fig. 3, B and D). These results indicate that the mutation introduced into the c-*src* gene in clones 2 and 4 is a homozygous mutation, as could be expected. The wild-type and the SD/SD mutant cells display the same level of active c-Src, suggesting that the point mutation does not affect the kinase activity.

Lack of Ser75 Phosphorylation in the SD/SD Mutant c-Src—c-Src is phosphorylated at Ser75 during mitosis by Cdk1 kinase in anchorage-dependent cultured cells. On the other hand, this site is phosphorylated constitutively in c-Src from retinoblastoma Y79 (*14*). The SD/SD mutation is expected to lead to the blocking the mitosis-specific phosphorylation. We examined the alteration of the phosphorylation patterns of c-Src from SD/SD mutant cells during mitosis by phopsphopeptide mapping. The V8 peptide maps of *in vivo* phosphorylated c-Src from the

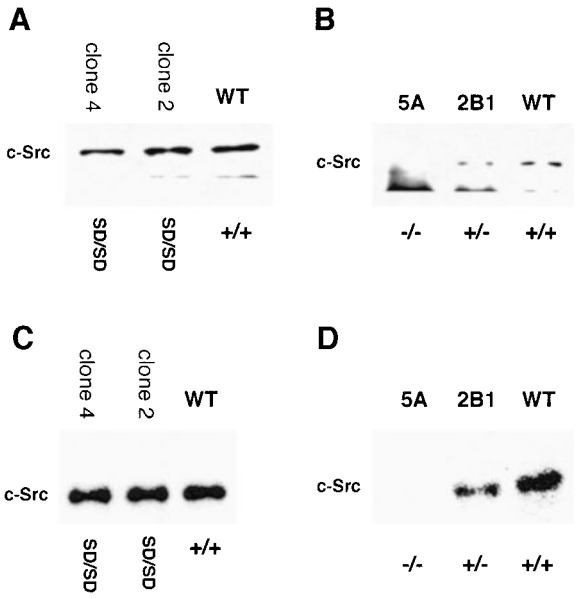

Fig. 3. **c-Src expression and autophosphorylation kinase assay in wild-type and mutant cells.** The feeder-free ES cell clones were cultured, washed and lysed. c-Src was immunoprecipitated from the cell lysate with anti-Src antibody 327. The immunoprecipitates were subjected to anti-Src immunoblot analysis (A, B) and autophosphorylation assay (C, D). The c-Src levels were quantitated using an FMBIO-100 Image Analyzer (TAKARA). The densitometry values were 10837 (WT), 9991 (clone 2) and 8987 (clone 4) for (A); 8470 (WT), 36 (5A) and 4626 (2B1) for (B). The autophosphorylation activity levels were quantitated using a BAS 2000 image analyzer (Fuji). The PSL values were 4804 (WT), 4681 (clone 2) and 4306 (clone 4) for (C); 182458 (WT), 95939 (2B1) and 14 (5A) for (D). Amounts of lysate proteins were 400 (A,C), 300 (B) and 200 μg (D).

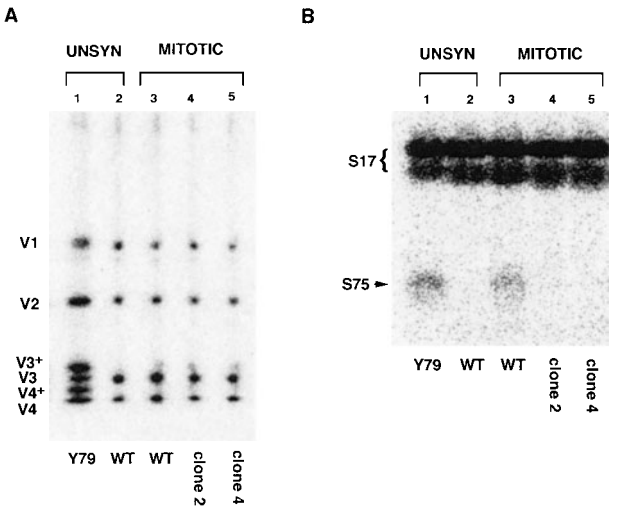

Fig. 4. **Phosphopeptide maps of c-Src from wild-type and SD/SD mutant cells.** Unsynchronized (UNSYN) and mitotic (MITOTIC) cells were labeled with [^{32}P]orthophosphate, washed and lysed. Phosphorylated c-Src was immunoprecipitated, separated on SDS-PAGE and subjected to V8 protease mapping (A). The tryptic digests of the V4 fragments were subjected to one-dimensional analysis on Tricine-SDS-PAGE (B). V1, N-terminal 34-kDa fragments; V2, C-terminal 26-kDa fragments; V3 and V4, N-terminal 18- and 16-kDa fragments, respectively, derived from further cleavage of V1; V3$^+$and V4$^+$, slightly retarded forms of V3 and V4, respectively, derived from a neuronal form of Y79 c-Src (*12*), which has six additional amino acids inserted at position 117 of the mouse c-Src. S17, phosphoserine 17; S75, phosphoserine 75.

mitotic mutant cells were identical to those of c-Src from unsynchronized and mitotic wild-type ES cells (Fig. 4A), consistent with the reported pattern of fibroblastic c-Src and not that of altered forms, such as the neuronal form, of Y79 c-Src (*14*, *16*). To reveal the Ser 75 phosphorylation in ES c-Src, we performed the tryptic phosphopeptide mapping of the N-terminal 16-kDa V8 fragments of mitotic c-Src. As shown in Fig.4B, phosphoserine 75–containing peptide 7 (*15*) was not detected in the mitotic mutant c-Src, whereas the phosphorylation was detected in mitotic wild-type ES and unsynchronized Y79 c-Src. These results indicate that mitotically active Cdk1 or a related kinase phosphorylates the wild-type c-Src at Ser75 and not the mutant c-Src during mitosis.

DISCUSSION

We have succeeded in establishing cell clones bearing the homozygous Ser75 to Asp mutation in the c-*src* gene. The mutant cells expressed the same levels of c-Src and its kinase activity as wild-type cells and lost the mitotic Ser75 phosphorylation.

Mice in which all cells carry a homozygous subtle mutation can be generated by the breeding heterozygous mice generated using ES cell technology (*20*). Homozygous mutant cells can be established from the tissues of the mutant mice, unless the mutation is lethal. However, we can not always establish a desired cell line with ease from mouse tissues. Moreover, the generation of transgenic organisms using ES cell technology is not successful in animals other than mouse. Therefore, the development of a procedure to produce these cells without using homozygous animals is very important.

Our procedure for establishing mutant cell clones bearing the desired homozygous mutation consists of two events: the introduction of a point mutation into one allele and the production of homozygous mutant cells. As the first event, we adopted our previously reported procedure (*23*) to generate homozygous mutant cells with no selectable marker, because it is possible that selection markers affect the expression of a targeted locus and closely linked genes (*41*). In some procedures (*21*, *22*, *24*–*29*), selectable sequences and vector components are also removed. However, a double replacement procedure/tag-and-exchange procedure (*21*, *24*, *26*, *28*) may give a low frequency of gene replacement with the desired mutation, because of the double requirement of homologous recombination between the target allele and the targeting vector. In Cre/*loxP*- and Flp/FRT-mediated procedures (*22*, *25*), the selectable sequence is excised using site-specific recombinases Cre and Flp, respectively, at high efficiency, but the recombination sequences remain in the targeted genome. Recent studies have shown that pseudo-*loxP* sites are present in the mammalian genome (*42*, *43*) and that Cre mediates DNA damage in mammalian cultured cells (*44*). The hit and run/in-out procedure (*27*, *29*) is a method similar to ours, in which the marker sequence is excised by homologous recombinaton within a duplication inserted into the target locus. However, loss

of the designed mutation frequently occurs during the excision step, due to the location of the mutation within the duplication (*45*).

In the excison step, the frequency (5×10^{-5}) of the appearance of FIAU-resistant colonies in the homozygous clone 5A bearing two copies of the selection cassette was 4 to 5 times lower than that of the heterozygous clone 2B1 bearing one copy of the selection cassette (Kato, G. and Maeda, S., unpublished data). Moreover, even a 48-h pre-culture of clone 2B1 produced the revertant clones at a frequency of 4×10^{-1} from the resistant colonies (Kato, G. and Maeda, S., unpublished data); the 48-h pre-culture of clone 5A produced no clones. Nevertheless, the 72-h pre-culture of clone 5A efficiently generated revertant clones at a frequency of ~10^{-1}. This indicates that double excision of the selection cassettes on both c-*src* gene alleles is practicable. Furthermore, Src antibody 327, which is specific for the src homology 3 domain derived from exons 3 and 4, recognized the wild-type and mutant c-Src in an identical manner. There is no difference in the the patterns of the V3 and V4 fragments derived from exons 2 to 5 between the immunoprecipitated wild-type and mutant c-Src in V8 peptide mapping. Tryptic phosphopeptide mapping analysis of the mutant c-Src supports the substitution of Ser75 by Asp. Overall, the events of loss of heterozygosity and homologous recombination between the duplication in this procedure cause no alteration of gene structure and no loss of the designed mutation in these mutants. On the other hand, gene targeting in human somatic cells can be done as easily and efficiently as in mouse ES cells (46). The rate of the production of loss of heterozygosity in high-G418 selection in these cells is similar to that shown in other cultured somatic cells (*31*). The excision step in our procedure is applicable to a wide variety of mouse genes (*23*). Taken together, our procedure provides a feasible way to generate a homozygous mutant in both alleles of any gene and is expected to be applicable to other somatic cell lines.

Because ES cells are derived from the inner cell mass of the blastocyst and have totipotency to differentiate into various types of cells *in vitro* (*36*), they are useful for analyzing the function of a protein in early embryonic cells and the cell types of the later embryo. In this report, we indicate that ES cells produce a normal c-Src protein with its kinase activity, suggesting that c-Src plays some roles in the less specified cells of the early embryo. The homozygous SD mutant cells will be useful for elucidating the role of Ser75 phosphorylation.

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