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Association of c-Src with p52Shc in Mitotic NIH3T3 Cells as Revealed by Src-Shc Binding Site–Specific Antibodies

Ken-ichi Sato*, Tetsushi Iwasaki and Yasuo Fukami
Research Center for Environmental Genomics, Kobe University, Nada, Kobe 657-8501

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In a previous study, we presented evidence that the adaptor protein Shc interacts with and activates the tyrosine kinase c-Src without affecting the phosphorylation state of Tyr-527 in c-Src. Here we show that Shc-mediated c-Src activation occurs in mitotic NIH 3T3 cells. Co-immunoprecipitation studies demonstrate that the c-Src–p52Shc complex involves the activation segment/inter-DFG-APE (IDA) region of c-Src and the amino-terminal region of p52Shc. The complex formation contributes to the c-Src activation, because (i) specific activity of c-Src associated with p52Shc is higher than that of the total c-Src, and (ii) a recombinant protein containing the c-Src IDA sequence disrupts the complex and decreases the c-Src activity. Anti–Src IDA antibody can activate c-Src *in vitro*, and synthetic peptides that cover the carboxyl-terminal half of the Src IDA region interfere with the kinase-activating effect of anti–Src IDA antibody. These results support the idea that dephosphorylation-independent activation of c-Src by Shc is mediated by a molecular interaction involving the c-Src IDA region.

Key words: activation segment, c-Src, inter-DFG-APE, mitosis, NIH3T3 cells, Shc.

Abbreviations: IDA, inter-DFG-APE; SFK, Src family kinase; SH, Src homology; GST, glutathione-*S*-transferase; NT, amino-terminal; DMEM, Dulbecco's modified Eagle's medium; APMSF, *p*-amidinophenyl(methansulfonyl) fluoride; BSA, bovine serum albumin; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; T-TBS, Tris-buffered saline containing Tween 20; IP, immunoprecipitation.

c-Src is a prototypical member of the Src family of protein-tyrosine kinases (SFKs) that comprises eight members in humans: c-Src, c-Yes, Fyn, Lck, Fgr, Lyn, Hck, and Blk (*1–3*). c-Src and other SFKs belong to the non-receptor kinases that have no extracellular portion and no integral plasma membrane–spanning domain. SFKs are regulated by multiple signals from the outside of cells, namely, ligand stimulation of cell surface receptors, cell–cell or cell–substratum communication, and environmental stimuli such as ultraviolet and oxidative stress. On–off switching of c-Src activity is strictly regulated, and c-Src has no ability to transform cells, but is rather involved in normal cellular functions such as cell growth, differentiation, and survival (*4*, *5*).

c-Src and other SFKs are expressed as polypeptides of about 60 kDa (~530 amino acids) polypeptides and have a common structure consisting of an amino-terminal myristoylation and/or palmitoylation signal for membrane localization, followed by a stretch of subfamily-specific sequence with unknown function, a Src homology 3 (SH3) domain that binds to a proline-rich sequence, an SH2 domain that interacts with a phosphotyrosine-containing sequence, an SH2-SH1 linker, an SH1 domain for substrate-binding and catalysis, and a carboxyl-terminal short sequence with a tyrosine residue to be phosphorylated for negative enzyme regulation. Numerous biochemical, mutational, and structural analyses have revealed that the phosphorylated C-terminal tyrosine residue of cellular SFKs (Tyr-527 in chicken c-Src) will interact intramolecularly with the SH2 domain (*6–11*). Additional intramolecular interactions involving other domains have also been demonstrated. These features of intramolecular folding contribute to the inactive conformation of SFKs, and its relief by, for example, dephosphorylation of the C-terminal tyrosine residue or protein–protein interaction, breaks the intracellular folding and activates SFKs.

Several lines of evidence indicate a general importance of c-Src and other SFKs in the normal cell cycle control. Transient activation of SFKs at growth factor-induced G_0–G_1 transition and at mitosis has been well documented (*2*, *3*). Membrane receptor–mediated cell functions such as immune responses of T- and B-cells, adhesion, and survival are also known to involve SFKs (*2–4*). Spontaneous kinase-activating mutation or unusual overexpression of SFKs, often in association with similar deregulation of receptor-type tyrosine kinases, might contribute to the malignant cell transformation (*5*, *12–16*).

Previously, we have demonstrated that c-Src is activated by direct interaction with the adaptor protein Shc in A431 cells (*17–19*). Shc isoforms of p52 and p66, but not p46, are capable of interacting with and activating c-Src. The inter-DFG-APE region (Src IDA region) in the c-Src kinase domain and the amino-terminal part of p52Shc, which is missing in p46Shc, have been identified as the c-Src–Shc interaction sites. Here, we attempted to determine whether the Shc-mediated c-Src activation operates in other cell systems. We show that in mitotic NIH3T3 cells, c-Src is activated through the interaction

*To whom correspondence should be addressed. Tel: +81-78-803-5953, Fax: +81-78-803-5951, E-mail: kksato@kobe-u.ac.jp

with p52Shc *via* the Src IDA region. We also evaluated the molecular mechanism by which c-Src is activated *via* molecular interaction involving the Src IDA region, by using anti–Src IDA antibody as an *in vitro* c-Src activator.

MATERIALS AND METHODS

Antibodies, Recombinant Proteins, and Synthetic Peptides—Anti-Src mouse monoclonal antibody mAb327 was obtained from Oncogene Research (San Diego, CA). Anti–Src IDA antibody, formerly named "anti-pepY antibody," was prepared by immunizing rabbit with a synthetic Src IDA peptide (see below) as described previously (*20*). A rabbit antibody against the Src homology 2 (SH2) domain of Shc (anti–Shc SH2 antibody) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–Shc NT antibody that recognizes the amino-terminal p66/p52 Shc-specific region was prepared by immunizing rabbit with a bacterially expressed and purified glutathinone-*S*-transferase (GST)-fusion Shc amino-terminal region (residues 1–45 of mouse p52Shc) (GST-NT, see below). Maintenance of antibodies, purification of IgG, and immobilization of IgG onto protein A–Sepharose (Pharmacia) were done according to the described methods (*21*). Three GST–fusion proteins, GST alone, GST-Src inter-DFG-APE (GST-IDA) (residues 404–432 of chicken c-Src), and GST-Shc amino-terminus (residues 1–45 of mouse p52Shc), were bacterially expressed and purified as described previously (*18*). Synthetic peptides used in this study were prepared with Peptide Synthesizer 430 (Applied Biosystems, Tokyo, Japan) according to the f-moc peptide synthesis chemistry. Peptides used were IDA-N (residues 400–417 of chicken c-Src), IDA (residues 410–428 of chicken c-Src), IDA-C (residues 419–437 of chicken c-Src), and Cdc2 peptide (residues 7–26 of the fission yeast Cdc2). All the synthetic peptides were purified by high-pressure liquid chromatography (Millipore). We verified sequences of all peptides by use of protein sequencer (Model 492, Applied Biosystems, Tokyo, Japan).

Cell Treatments and Subcellular Fractionation—NIH3T3 cells were grown in Dulbecco's modified Eagle's (DMEM) medium supplemented with 5% calf serum at 37°C/5% CO_2 in a humidified incubator. To obtain cells at the G_0 cell cycle stage, cells at 80% confluence were treated with DMEM medium supplemented with 0.1% calf serum for 24 h. Cells at the G_2/M cell cycle stage (mitotic cells) were prepared by incubating the G_0-stage cells in DMEM supplemented with 5% calf serum for 2 h and then in the same medium containing 0.5 μg/ml nocodazole (Sigma) for 16 h. Mitotic cells were separated by mechanical shake-off of the culture dishes. Both G_0 and mitotic cells were washed three times with phosphate-buffered saline, snap-frozen in liquid nitrogen, and stored at –80°C until required. The frozen cells were used for experiments within 2 weeks after the preparation. Subsequent manipulations were performed at 4°C or on ice. Frozen cells (5×10^8 cells) were mixed with 10 ml of buffer A containing 20 mM Tris-HCl (H 7.5), 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 20 μM *p*-amidinophenyl(methansulfonyl) fluoride (APMSF), and homogenized in a Teflon-glass homogenizer (30 strokes). The homogenates were centrifuged at 500 × *g* for 10 min, and the resulting pellet containing debris and nuclei was removed. The soluble fractions were further centrifuged at 300,000 × *g* for 10 min. The resulting supernatant fractions were collected as cytosolic fractions, while the pellets were homogenized again in buffer supplemented with 1% Triton X-100. The Triton X-100–homogenized samples were centrifuged at 300,000 × *g* for 10 min, and the supernatants were collected as detergent-solubilized membrane fractions. Protein concentrations of both cytosolic and membrane fractions were determined by the dye-binding method using a Bio-Rad Protein Assay Mixture (Bio-Rad).

Immunoprecipitation, SDS-PAGE, and Immunoblotting—For immunoprecipitation, protein samples (500–2000 μg protein) were incubated for 3–5 h at 4°C with the specified amount of antibody immobilized onto protein A–Sepharose that had been washed three times with buffer A containing 3 mg/ml bovine serum albumin (BSA). After the incubation, non-specifically bound materials were removed by washing the Sepharose beads three times with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 20 μM APMSF. The washed beads containing specifically bound proteins were mixed with a concentrated SDS sample buffer (*22*) and boiled for 3 min. When the subcellular fractions or recombinant proteins were directly analyzed by immunoblotting, these protein samples (1–20 μg protein) were directly mixed with a concentrated SDS sample buffer. The SDS-treated proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels and transferred to polyvinylidene difluoride membranes using a semi-dry blotting apparatus (Bio-Rad). Membranes were blocked with T-TBS buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, and 3 mg/ml BSA for 1 h and then incubated for 2–4 h with a primary antibody diluted in T-TBS as specified in the text. After the primary antibody treatment, the membranes were washed with BSA-free T-TBS and then incubated for 1 h with T-TBS containing ^{125}I-labeled protein A (50 kBq/ml) (ICN Biochemicals, Irvine, CA). After washing with BSA-free T-TBS (four times), immune complexes were visualized by analyzing the membranes using a BAS2000 Bioimaging Analyzer (Fujifilm, Tokyo, Japan).

In Vitro Kinase Assay—Kinase activity of c-Src was assessed by incubating enzyme preparations in the kinase reaction mixture containing 2 μM [γ-^{32}P]ATP (5 μCi/assay, Moravek Biochemicals, Brea, CA) and 5 mM $MgCl_2$, in the absence or the presence of 1 mM Cdc2 peptide, an exogenous substrate. Enzyme preparations used were immunoprecipitates prepared from NIH3T3 cells (500–2,000 μg protein/assay, see above) or the bovine brain c-Src (10 ng protein/assay). After proceeding at 30°C for 20 min, the kinase reaction was terminated by incubating the reaction mixture with SDS sample buffer at 98°C for 5 min. Phosphorylated c-Src and Cdc2 peptide were separated by SDS-PAGE on 8% gels and 16% gels, respectively, and visualized and quantified with a BAS2000 Phosphoimage Analyzer. When the effects of IgG and/or synthetic peptide were examined, enzyme preparations were preincubated with these effectors at 30°C for 10 min and subjected to *in vitro* kinase assay.

RESULTS AND DISCUSSION

To analyze the site-specific function of c-Src and Shc, we employed specific antibodies, synthetic peptides, and a recombinant protein (Fig. 1). Anti–Src IDA antibody was raised against a synthetic Src IDA peptide that covers the entire Src IDA region (residues 410–428 of the chicken c-Src), while anti–Shc NT antibody was raised against a bacterially expressed protein containing the amino-terminal portion of p52Shc (residues 1–45 of the mouse p52Shc). Anti–Shc NT antibody recognizes both p52Shc and p66Shc, but not p46Shc (data not shown). We also employed anti-Src antibody mAb327 and anti–Shc SH2 antibody as control antibodies. A recombinant GST-tagged Src IDA peptide (GST-IDA) and synthetic Src IDA-N and IDA-C peptides that cover the amino-terminal half and the carboxyl-terminal half of the Src IDA region, respectively, were also used.

Interaction of c-Src and p52Shc in Mitotic NIH3T3 Cells Is Mediated by the Src IDA (Inter-DFG-APE) Region—Several reports have demonstrated that c-Src activation is induced at the mitotic phase of the cell cycle (*23–27*). In this system, dephosphorylation of Tyr-527, a negative regulatory phosphorylation site of c-Src, has been suggested to be the predominant, but not the sole, mechanism of c-Src activation. Therefore, we wanted to determine whether c-Src activation in G_2-M phase of the cell cycle involves c-Src–Shc interaction. To this end, we prepared cytosolic fractions and Triton X-100–solubilized membrane fractions from serum-starved or mitotic NIH 3T3 cells that had been treated with nocodazole. Proteins of normalized amount in each fraction were immunoprecipitated with mAb327, anti–Src IDA, anti–Shc SH2, or anti–Shc NT, and the immunoprecipitates were analyzed by immunoblotting with either mAb327 or anti–Shc SH2 antibody. c-Src is predominantly localized to the membrane fractions in both serum-starved and mitotic cells (Fig. 2A, upper panels, lanes 1, 6, 11, and 16). On the other hand, three isoforms of Shc (p46, p52, and p66) are present in both fractions irrespective of the cell conditions (Fig. 2A, lower panels, lanes 1, 6, 11, and 16).

Co-immunoprecipitation (co-IP) experiments demonstrated that membrane-associated p52Shc, but not other Shc isoforms, are associated with c-Src in mitotic cells (upper panel, lane 19 and lower panel, lane 17), while no interaction is observed in serum-starved cells (lanes 2–5 and 7–10). Co-IP of c-Src and Shc was only seen when mAb327 or anti–Shc SH2 antibody was used for IP, but not when anti–Src IDA antibody or anti–Shc NT antibody was used (upper panel, lane 20, and lower panel, lane 18). These results suggest that c-Src and p52Shc in mitotic NIH 3T3 cells interact with each other through the Src IDA region and the Shc NT region. This is supported by the fact that the efficiency of IP of c-Src with anti–Src IDA antibody and that of p52Shc with anti–Shc NT antibody is lower in mitotic cells than serum-starved cells (Fig. 2A, upper panel, lanes 8 and 18, and lower panel, lanes 10 and 20). On the other hand, no change is observed when mAb327 or anti–Shc SH2 antibody is used for IP (upper panel, lanes 7 and 17, and lower panel, lanes 9 and 19).

We also performed a similar set of experiments to look for interaction between c-Src and Shc isoforms in cells

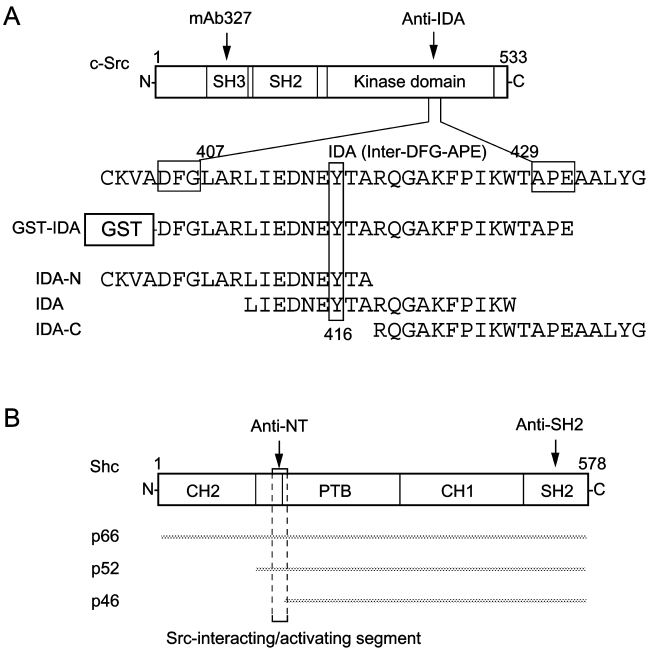

Fig. 1. **Antibodies, recombinant protein, and synthetic peptides used in this study.** A: The tyrosine kinase c-Src contains amino-terminal Src homology (SH) 3 and SH2 domains as well as a carboxyl-terminal kinase domain. MAb327, a Src-specific monoclonal antibody, recognizes the c-Src SH3 domain, while the anti-IDA antibody recognizes the inter-DFG-APE (IDA) region (residues 407–429) that encompasses the autophosphorylation site (Y416) in the kinase domain. GST-fusion Src-IDA protein (GST-IDA) was prepared with a bacterial expression system as described in "MATERIALS AND METHODS." Synthetic Src-IDA peptides (IDA-N, IDA, and IDA-C) were also prepared. B: The adaptor protein Shc comprises three isoforms, p66, p52, and p46, which contain each three functional domains: a phosphotyrosine-binding (PTB) domain, a central collagen homology domain (CH1), and an SH2 domain. With an extension in the amino-terminus, the p52 and p66 isoforms are both capable of interacting with and activating the tyrosine kinase c-Src. In addition to the Src-interacting/activating segment, the p66 isoform has one more CH domain (CH2) in the amino-terminus. We employed two anti-Shc antibodies: anti-NT antibody, which recognizes only p52 and p66 isoforms, and anti-SH2 antibody, which recognizes all three isoforms.

treated with either serum or platelet-derived growth factor, both of which are known c-Src activators (*2*, *3*). However, no interaction was observed (data not shown).

The c-Src–p52Shc Interaction Contributes to c-Src Activation in Mitotic NIH3T3 Cells—We next performed *in vitro* kinase assay of the immunoprecipitates prepared with either mAb327 or anti-SH2 antibody from the membrane fractions of serum-starved or mitotic cells. We determined specific activity of c-Src in the immunoprecipitates by quantifying autophosphorylation of c-Src that was normalized by the quantity of the immunoprecipitated c-Src. Figure 2B shows that total activity of c-Src, as judged by IP-kinase assay with mAb327, increased 2.8-fold in mitotic cells. We also detected c-Src activity in the anti–Shc SH2 immunoprecipitates in mitotic cells. Importantly, specific activity of c-Src in the Shc immunoprecipitates was higher (4.5-fold increase) than that obtained with mAb327, indicating that the anti–Shc SH2 immunoprecipitates contain a pool of activated c-Src.

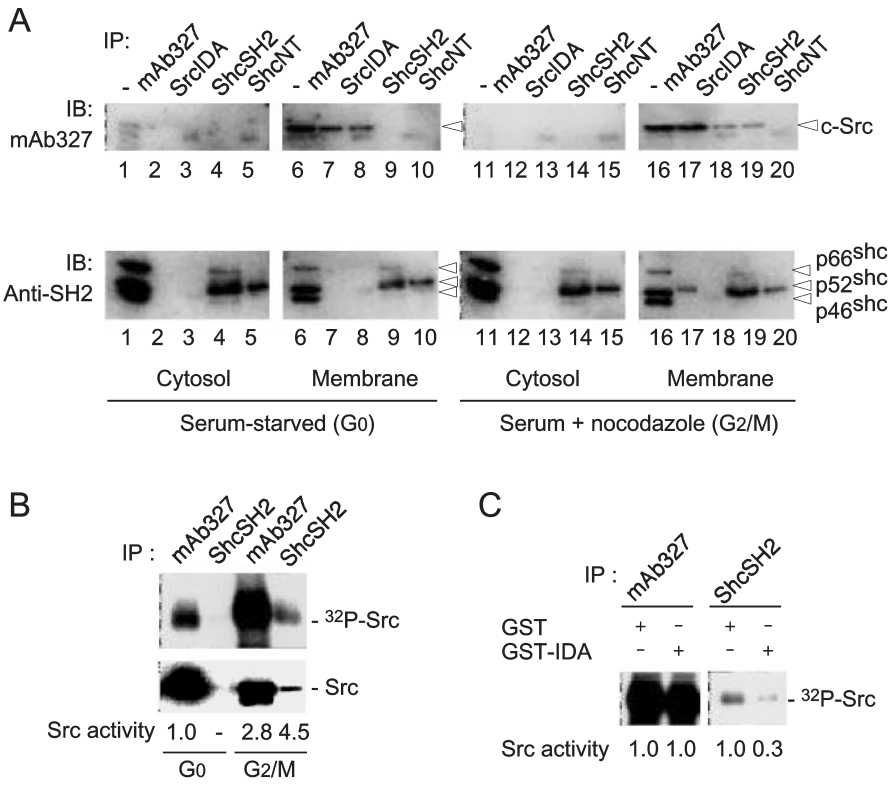

Fig. 2. **Site-specific association of c-Src and Shc and activation of c-Src in mitotic NIH3T3 cells.** A: Immunoprecipitation of c-Src and Shc was performed with cytosolic and Triton X-100–solubilized membrane fractions (each 500 μg/lane) prepared from serum-starved (G_0) or serum/nocodazole-treated mitotic (G_2/M) NIH3T3 cells as described in "MATERIALS AND METHODS." Antibodies used for immunoprecipitation were mAb327 (lanes 2, 7, 12, and 17) and anti-IDA antibody (lanes 3, 8, 13, and 18) (for detection of c-Src), and anti-SH2 (lanes 4, 9, 14, and 19) and anti-NT (lanes 5, 10, 15, and 20) antibodies (for detection of Shc). The presence of c-Src or Shc in the immunoprecipitates, as indicated by arrowheads, was analyzed by immunoblotting with mAb327 (upper panel) or anti-SH2 antibody (lower panel). The cell fractions were also directly analyzed by immunoblotting to show the presence of c-Src and Shc in each sample (20 μg/lane: lanes 1, 6, 11, and 16). B: Kinase activity of c-Src was assessed by *in vitro* kinase assay using the mAb327 or anti-SH2 immunoprecipitates of the membrane fractions prepared as in panel A. The upper panel shows a representative image of autophosphorylated c-Src (^{32}P-Src) visualized by a BAS2000 Bioimaging Analyzer (Fujifilm, Japan). The lower panel shows the amount of c-Src immunoprecipitated. Specific activity of c-Src (autophosphorylated c-Src/protein amount of c-Src) in each condition is shown. Kinase activity of the mAb327 immunoprecipitates prepared from serum-starved cells was taken as 1.0. C: Immunoprecipitated samples by using mAb327 or anti-SH2 antibody were prepared from the membrane fractions of mitotic cells and subjected to *in vitro* kinase assay in the presence of either 100 μM GST or 100 μM GST-IDA. The position of the autophosphorylated c-Src (^{32}P-Src) and the specific activity of c-Src (autophosphorylated c-Src/protein amount of c-Src) are shown.

At this point, it was not clear whether molecular interaction between c-Src and p52Shc contributes to c-Src activation in mitotic cells. Thus, we performed in vitro kinase assay of the immunoprecipitates prepared as above in the absence (GST alone) or presence of GST-IDA, which would disrupt the c-Src–p52Shc interaction. As we expected, c-Src activity associated with Shc was inhibited more than 3-fold in the presence of GST-IDA (Fig. 2C, IP: anti–Shc SH2). On the other hand, c-Src activity in the mAb327 immunoprecipitates was not affected under the same conditions (Fig. 2C, IP: mAb327). These results are consistent with our previous finding that GST-IDA can bind to p52Shc in NIH3T3 cells (*18*). So, we conclude that c-Src activation in mitotic cells involves a novel, Src IDA region–mediated interaction with p52Shc.

In Vitro Activation and Inhibition of c-Src by an Anti–Src IDA Antibody—In our previous studies, we have shown that not only full-length p52Shc but also an amino-terminal segment of p52Shc interacts with and activates c-Src through its binding to the c-Src IDA region (*18*). To analyze further the relationship between the Src IDA–mediated molecular interaction and c-Src activation, we performed *in vitro* kinase assays using inactive c-Src enzymes. As an *in vitro* activator of c-Src, we employed the anti–Src IDA antibody instead of p52Shc, because anti–Src IDA antibody interacts with the Src IDA region and activates c-Src *in vitro* as p52Shc does.

As shown in Fig. 3A, c-Src activity toward a synthetic peptide substrate can be stimulated up to 4.5-fold in the presence of the anti–Src IDA antibody (1 μg IgG per assay). Interestingly, however, a larger amount of the anti–Src IDA antibody (5–10 μg IgG per assay) causes the c-Src activity to decrease almost to basal level. A similar pattern of activation and inhibition of c-Src was also observed when autophosphorylation of c-Src was examined (data not shown). This effect is not due to inhibition by excess IgG, because the same amount of preimmune IgG does not show such an effect (Fig. 3A). Rather, anti-Src IDA antibody may contain a fraction of IgG that is inhibitory to c-Src activity. In fact, the following experiments support this idea.

In Fig. 3B, we examined the effect of 1 μg per assay of either anti–Src IDA or preimmune antibody in the presence of different concentrations of synthetic Src IDA peptides: IDA-N, IDA, and IDA-C (see Fig. 1A). As we expected, IDA peptide, which was used for preparation of anti–Src IDA antibody, canceled the antibody-induced c-Src activity in a dose-dependent manner. IDA-C had a similar effect. The results indicate that anti–Src IDA antibody stimulates c-Src activity through binding to the carboxyl-terminal half of the Src IDA region. Conversely, IDA-N peptide further stimulated the antibody-activated c-Src. This result suggests that a fraction of the anti–Src IDA antibody is reactive toward the amino-terminal half of the Src IDA region and that the anti–Src IDA "amino-

terminal" antibody is inhibitory toward Src activity. The presence of both anti–Src IDA "amino-terminal" as well as "carboxyl-terminal" IgG has been confirmed by the fact that both IDA-N and IDA-C peptides react with anti–Src IDA antibody, as judged by immunoblotting of recombinant GST-IDA with anti–Src IDA antibody in the presence of IDA peptides (Fig. 3C). It should also be noted that IDA and IDA-C peptides, but not IDA-N peptide, are capable of stimulating c-Src activity (up to 150% of control) when added in the presence of preimmune antibody (Fig. 3B). We suggest that IDA or IDA-C peptide interferes with the interaction between the Src IDA region and an IDA-interacting segment(s) in Src molecules.

One important point that remained unclear was which part of the Src IDA region is targeted by p52Shc. We have previously shown that interaction between c-Src and epidermal growth factor receptor, in which Shc may act as a bridge between them, can be disrupted by a full-length IDA peptide, but not by IDA-N and IDA-C peptides (*23*). In fact, neither IDA-N nor IDA-C peptides is capable of interacting with p52Shc (data not shown). Also, it should be noted that a mutant p52Shc M46P, in which methionine-46 is replaced by proline, is not able to activate c-Src, although it can bind to c-Src (*18*). These results suggest that p52Shc binds to the center of the Src IDA region, which cannot be reconstituted by the N-terminal or C-terminal fragment.

Summary and Perspectives—Here we provide evidence that c-Src activation in mitotic NIH3T3 cells is at least partly due to protein–protein interaction involving p52Shc, and that the molecular interaction mediated by the Src IDA region and its binding partners (possibly the amino-terminal portion of p52Shc) is responsible for c-Src activation. Mitotic c-Src activation has been well documented and it may involve dephosphorylation of the carboxyl-terminal Tyr-527 (*24–26*) and/or cdc2 phosphorylation of the amino-terminal region of c-Src molecules (*27*, *28*). In both cases, however, it is not clear whether the phosphorylation/dephosphorylation-dependent regulation is the dominant event in mitotic c-Src activation. Our present study demonstrates that mitotic c-Src activation as observed in the c-Src–p52Shc complex can be canceled by disruption of the complex. Therefore, we conclude that the c-Src–p52Shc interaction can be added to the multiple events involved in mitotic c-Src activation in NIH3T3 cells. Further study will be directed to determine how the c-Src–p52Shc interaction is induced and canceled in mitotic cells. In this connection, our preliminary experiments have shown that the mitotic c-Src–p52Shc complex is enriched in the nuclear fraction of NIH3T3 cells (our unpublished results), suggesting that mitotic interaction of c-Src with p52Shc is a subcellular localization-specific event.

By using the anti–Src IDA antibody as an in vitro Src IDA-binder and activator, we have shown that the carboxyl-terminal half of the Src IDA region is the target of its kinase-activating partner (*i.e.*, p52Shc, anti–Src IDA IgG). Most protein–protein interactions with c-Src occur through the SH2 and SH3 domains, and many of them

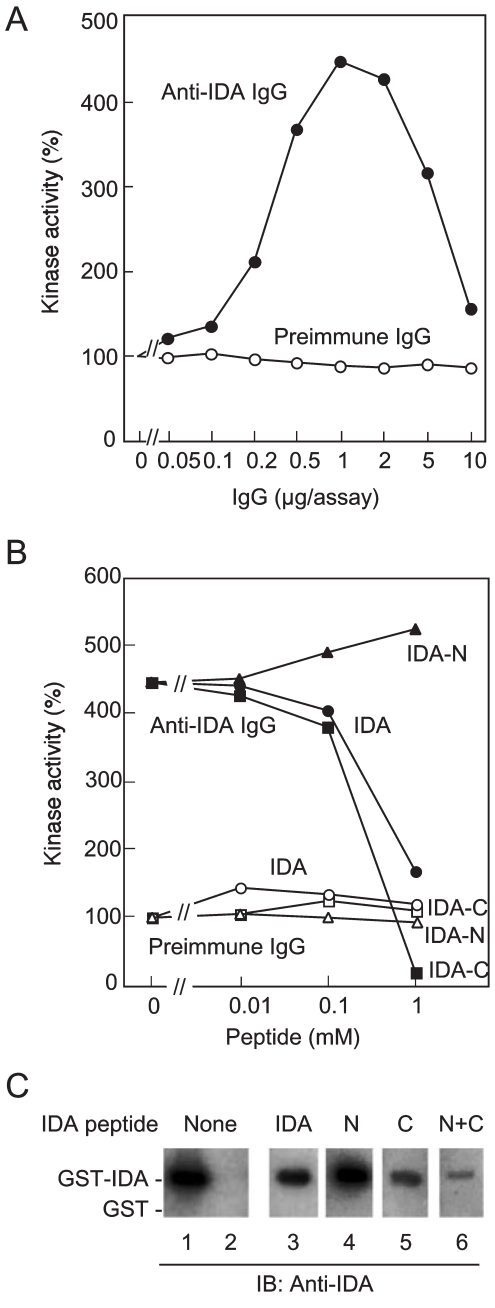

Fig. 3. **Characterization of anti-Src IDA antibody that binds to and stimulates the c-Src activity.** A: Shown is a representative trace of the dose-dependent effect of anti–Src IDA IgG on the kinase activity of the purified c-Src (closed circles), as determined by phosphorylation of a substrate peptide as described in "MATERIALS AND METHODS." Total volume of the reaction mixture of *in vitro* kinase assay was 25 μl. Therefore, the final concentration of IgG is, for example, 40 μg/ml when the amount of IgG indicated in the graph is 1 μg/assay. The effect of IgG prepared from the preimmune serum was also examined as a control experiment (open circles). B: The effect of anti–Src IDA IgG (closed symbols) or preimmune IgG (open symbols), each at 1 μg/assay, was examined as in A in the absence or the presence of the Src IDA peptides; IDA-N (triangles), IDA (circles), or IDA-C (squares), each at the indicated final concentration. C, Immunoblotting of GST-IDA (lanes 1, 3, 4, 5, and 6) and GST alone (lane 2) (all 1 μg/lane), which were both bacterially expressed and purified, with anti–Src IDA antibody (10 μg/ml IgG) was done in the absence (lanes 1 and 2) or the presence of 100 μM IDA (lane 3), 100 μM IDA-N (lane 4), 100 μM IDA-C (lane 5), or 100 μM IDA-N plus 100 μM IDA-C (lane 6). The positions of GST-IDA and GST are indicated.

disrupt the intramolecular interactions that maintain the inactive conformation of the enzyme (*1*, *2*). On the other hand, several cellular ligands for the c-Src kinase domain have been described: they include the transforming polyoma virus middle T-antigen (*29*), c-Cbl (*30*), β-arrestin 1 (*31*), the trimeric Gα proteins (*32*), phospholipase D (*33*), and Shc (*18*, this study). A more recent report describes a novel ligand for the Src kinase domain, E4orf4, adenovirus type 2 early region 4 ORF4 (*34*). In all cases, except for c-Cbl, c-Src activity is activated or stabilized by these binding partners. However, among these cellular ligands, only Shc (p52Shc and p66Shc) has shown which part of the Src kinase domain is responsible for molecular interaction and kinase activation. In this connection, further study should determine whether other cellular ligands for the Src kinase domain also target the Src IDA region. We also show that the remaining part of the Src IDA region, which is covered by the IDA-N peptide, has an opposite function, which may be related to the maintenance of catalytic activity. In fact, our present study has shown that the antibody binding to this region results in the inhibition of Src kinase activity. This result is consistent with the previous finding by Gentry *et al.* (*35*) and others (*36*) showing that antibodies with similar sequence-specificity shows an inhibitory effect toward v-Src or receptor-type tyrosine kinase activity. The results also suggest that cellular ligands for this particular segment would be inhibitors of Src kinase (c-Cbl could be a candidate for this idea).

We believe that identification and characterization of cellular ligands for the Src IDA region and other regions in the Src kinase domain would be a unique and important research area for not only Src but for every protein kinase that possesses specific IDA sequences in the kinase domain (*37*).

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