XB130, a Novel Adaptor Protein for Signal Transduction*†‡§

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Adaptor proteins are important mediators in signal transduction. In the present study, we report the cloning and characterization of a novel adaptor protein, XB130. This gene is located on human chromosome 10q25.3 and encodes a protein of 818 amino acids. It contains several Src homology (SH) domains, a coiled-coil region, and a number of potential tyrosine or serine/threonine phosphorylation sites. Endogenous XB130 interacts with c-Src tyrosine kinase. Their co-expression in COS-7 cells resulted in activation of c-Src and elevated tyrosine phosphorylation of multiple proteins, including XB130 itself. XB130 expression in HEK293 cells enhanced serum response element- and AP-1-dependent transcriptional activity mediated by c-Src. XB130AN, an N-terminal deletion mutant lacking a putative SH3-binding motif and several putative SH2-binding sites, reduced its ability to mediate Src signal transduction. Down-regulation of endogenous XB130 with siRNA reduced c-Src activity, IL-8 production, EGF-induced phosphorylation of Akt and GSK3β, and altered cell cycles in human lung epithelial cells. These data suggest that XB130 as an adaptor may play an important role in the regulation of signal transduction and cellular functions.

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‡ The nucleotide sequence(s) reported in this paper has been submitted to GenBank™/EBI Data Bank with accession number(s) 442952.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2, Table S1, and data.

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3 The abbreviations used are: PTK, protein-tyrosine kinase; DMEM, Dulbecco’s modified Eagle’s medium; IL, interleukin; GST, glutathione S-transferase; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; UTR, untranslated region; ELISA, enzyme-linked immunosorbent assay; PH, pleckstrin homology; AFAP, actin filament-associated protein; Sin, Src-interacting/signal-integrating protein; Crk, Crk-associated substrate; mAb, monoclonal antibody; WT, wild type; EGF, epidermal growth factor; SH, Src homology; KD, kinase-deficient; SRE, serum response element.
because the apparent molecular size of the encoded protein is ~130 kDa. We further explored its potential roles as an adaptor protein by determining its interaction with c-Src via specific functional domain(s) and motif(s). We also demonstrated that the endogenously expressed XB130 plays roles in mediating intracellular signal transduction.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Other Reagents—COS-7, BEAS-2B, and A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen). Human embryonic kidney 293 cells (HEK293) were maintained in high glucose (HG)-DMEM medium (Invitrogen). Human thyroid cancer cell lines, WRO and TPC-1 cells (HEK293) were maintained in high glucose (HG)-DMEM medium (Invitrogen). Human embryonic kidney 293 (HEK293) cells were maintained in high glucose (HG)-DMEM medium (Invitrogen). HEK293 cells were maintained in high glucose (HG)-DMEM medium (Invitrogen).

To obtain the upstream sequence, 5’-RACE product in the pCR 2.1 vector and the original 3’-fragment from the EST clone were joined together into the pSPORT vector at the Xhol site. The full-length of XB130 cDNA is 3,750 bp, including 49 bp of 5’-UTR (untranslated region), 2,457 bp of coding region, 1,214 bp of 3’-UTR, and a stretch of 30 bp poly(A).

To generate C-terminal histidine (His)-tagged XB130 construct for protein expression in mammalian cells, polymerase chain reaction (PCR) was used to amplify the coding region of XB130, nucleotide 50–2506, using the full-length pSPORT-XB130 as a template. The primer pairs used were 5’-AAA GAA TTC GCC GCC ACC ATG GAG CGG TAC AAA GCC-3’ (forward primer carrying EcoRI site), and 5’-AAA AAA TCT AGA ACT TGC TCT TTT CTT CTC-3’ (reverse primer carrying XbaI site). The PCR product was digested with EcoRI and XbaI, purified, and ligated into a modified pcDNA3 vector that has a His tag at the C terminus. Mutant XB130 with N-terminal deletion (XB130ΔN) was generated by PCR, using pcDNA3-XB130/His as a template, and 5’-CCG AAT TCG CCA CCA TGG GCA TCG AGC TGA TGC GT-3’ and 5’-TCA AGC CTC TTC TCC TCT GTG TAC CG-3’ as the forward and reverse primers. The PCR product was digested and ligated into EcoRI/Xhol sites of the pcDNA3-XB130/His construct. To further determine the specific binding sites between XB130 and Src, we generated six single amino acid mutants in the N terminus of XB130, (see details under supplementary data). The nucleotide sequence of XB130 from all constructs was verified by sequencing analysis.

Transient Transfection and Luciferase Assays—For protein-protein interaction and protein-tyrosine phosphorylation studies in COS-7 cells, Lipofectamine reagent (Invitrogen) was used following the manufacturer’s protocol. Briefly, cells grown in DMEM supplemented with 10% FBS were transiently transfected at ~50–80% confluence in serum-free medium for 5 h and then maintained in DMEM containing 10% FBS for 2 days. For the 6-well plate, 1.2 μg of pCMV-c-Src and 0.8 μg of pcDNA3-XB130/His were used, and the empty vector was added to maintain the total DNA at 2 μg per well. For 100-mm Petri dishes, a total of 14 μg of DNA were used for each well.

For reporter activation assay, the calcium phosphate-DNA precipitation method was used. HEK293 cells were transfected in 24-well plates in triplicate, with a total of 2.1 μg of DNA (for 3 well) in 75 μl of 250 mM CaCl2, mixed with 75 μl of 2X HBS buffer (280 mM NaCl, 50 mM HEPES, and 1.5 mM sodium phosphate, pH 7.05). The constructs used included c-Src or its mutants (0.3 μg), XB130 or its mutants (0.18 μg), either alone or in combination, β-galactosidase (0.15 μg), and a reporter (0.18 μg) (SRE-Luc, AP-1-Luc, NF-κB-Luc, SBE-Luc, or IL-8-Luc). The total amount of DNA was kept constant by addition of pcDNA3 vector. After 15–20 min of incubation at room temperature, 50 μl of the precipitate were added into each well with 0.5 ml of cell culture medium and incubated overnight. The next day, cells were washed and changed to HG-DMEM containing 10% FBS for 40–48 h. In certain experiments, cells were stimulated with EGF (5 or 10 ng/ml) for 8 h. Luciferase activity in cell lysates was measured using the Berthold luminometer (Lumat LB 9507) (Oak Ridge, TN) and normalized for transfection efficiency with β-galactosidase activity.

Generation of XB130 Monoclonal Antibody—The coding sequences of XB130 was subcloned into His-tagged prokaryotic expression vector pQE30 (Qiagen) at EcoRI and XbaI sites and transformed into the Escherichia coli M15 strain. After induction with isopropyl β-D-thiogalactopyranoside (IPTG, 100 μM) for 4 h, the recombinant His-tagged XB130 was purified using Ni-NTA agarose beads (Qiagen). Balb/C mice (Charles River...
Laboratories, St. Constant, Canada) were immunized monthly
with 100 μg of recombinant XB130 over a period of three
months. Animals were sacrificed. Harvested splenocytes were
fused with myeloma cells (Inno Biotech, Toronto, Canada) to
generate hybridoma. Enzyme-linked immunosorbent assay
(ELISA) was used to select antibody-expressing hybridoma
clones. The subclass of XB130 monoclonal antibody was deter-
minded using mouse monoclonal antibody isotyping reagent
(Sigma). The quality of mAbs for Western blotting, immunoflu-
orescent staining, and immunoprecipitation was tested (see
supplemental data).

**Immunoprecipitation, Immunoblotting, and Immunostaining Microscopy**—The protocols for preparation of cell lysates, immunoprecipitation, immunoblotting have been described previously in detail (18, 21).

For immunofluorescence staining and microscopy, cells were seeded on 4-well Lab-Tek chamber slides (Nunc Inc., Naperville, IL). Two days after transfection, cells were washed with phosphate-buffered saline, fixed in 3.7% paraformaldehyde for 10 min at room temperature, and permeabilized in 0.25% Triton X-100 in phosphate-buffered saline. Cells were then incubated with an anti-His or anti-XB130 antibody for 1 h at 37 °C. After three washes in PBS, cells were incubated with FITC or Alexa 549 conjugated anti-mouse or anti-rabbit secondary antibodies for 1.5 h. Slides were then mounted with an anti-fading reagent (SlowFade, Molecular Probes), and distribution of XB130 proteins was examined by microscopy as described previously (18, 22).

**Expression, Purification of GST Fusion Protein, and GST Pull-down Assay**—Bacteria expressing GST fusion proteins containing SH2 or SH3 domain from different proteins were cultured in LB broth and induced by 100 μM isopropyl-1-thio-
β-D-galactopyranoside. The fusion proteins were purified with glutathione-Sepharose 4B beads from the soluble fraction of sonicated bacterial lysates. Cell lysates expressing His-tagged XB130 or hAFAP were incubated with GST fusion proteins immobilized on the beads. The precipitates were washed with radiolnune precipitation assay buffer, eluted in SDS sample buffer, and resolved by SDS-PAGE. The resolved proteins were transferred to nitrocellulose membranes and subjected to immunoblotting to detect XB130 or hAFAP.

**Src Kinase Activity Assay**—The protocols for Src kinase activity assay has been published (18). To determine whether XB130 can directly activate c-Src, an *in vitro* reconstitution assay was performed (18). Recombinant XB130 protein (150 ng) was incubated with Csk-inactivated c-Src (20 ng) (a gift from Dr. X. Huang, Cornell University) in Src kinase buffer containing 30 mM HEPES, pH 7.4, 5 mM MgCl2, 5 mM MnCl2, and 10 μM ATP. Together with 2 μg of Src substrate peptide (KVEKIGETGYGVVYK) and 10 μCi of [γ-32P]ATP (3,000Ci/mM), the reaction was carried out at 30 °C for 10 min, and terminated by addition of SDS sample buffer. The samples were then subjected to 20% SDS-PAGE. The phosphorylated substrate was autoradiographed on x-ray film and quantified by densitometry (GS-690, Bio-Rad).

**Cytokine Assays**—BEAS-2B cells (2 × 105) were seeded on 6-well plates. The next day, DMEM plus 10% FBS (1 ml) with or without Src inhibitors, PP2 (10 μM) or SU6656 (2 μM) (VWR, Mississauga, Canada), was added into each well. Culture medium was collected after 8 h for cytokine measurement. For siRNA transfection, 75 nM of XB130 siRNA or scrambled dou-
ble-stranded RNA (5′-NNG AUA CCG CUG AUA AGU GAC-
G-3′, Dharmacon Inc, Dallas, TX) was used as we described (18, 21). Two siRNAs were designed: 1) sequence is 5′-NNG AUU CUU GAC CAG GAG AAC-3′, and 2) is 5′-NNC UAC GAG UCC UAC GAU GAA-3′. The medium containing siRNA was replaced with fresh medium after 72 h. The cell lysate was prepared for Western analysis. IL-8 released into the culture media was determined using ELISA kits from BIOSOURCE, following the manufacturer's instruction (23). LiquiChip cytokine assay was performed by Host Defense Research Center (Toronto, Canada) to analyze multiple cytokines in the culture media, using a LiquiChip Work station (Qiagen).

**Cell Cycle Analysis**—A549 cells were transfected with 50 nM pooled XB130 siRNAs for 48 h. Cells were subjected to serum starvation overnight and then stimulated with EGF (50 ng/ml) for 2 h. Cells were collected and fixed with 70% EtOH, permeabilized with 0.1% Triton X-100, and stained with 50 mg/ml propidium iodine for 30 min. Cells from different cell cycle stages were counted by flow cytometer (Coulter FC500, Beck-
man Coulter).

**Statistical Analyses**—Data are expressed as mean ± S.D. from at least three experiments and analyzed by Student’s *t* test with significance defined as *p* < 0.05.

**RESULTS**

**Molecular Cloning and Sequence Analysis of XB130**—While searching for the human *AFAP* gene in GenBank®, several human EST clones that share similarities with chicken AFAP were found. One of the clones (GenBank® accession number: 1154093) contains a partial C-terminal open reading frame and 1.2 kb of 3′-UTR. Following 5′-RACE using mRNA isolated from human lung alveolar epithelial cells as a template, an mRNA transcript with novel nucleotide sequence was obtained (Fig. 1). The canonical start codon, ATG, was identified based on Kozak consensus context (24). Sequence structure analysis revealed 23 putative tyrosine phosphorylation sites. The N-terminal portion of XB130 protein contains one proline-rich motif (PDLPPPKKMIP), which is followed by two tyrosine-containing sites (YEEA and YDEE). In addition, two pleckstrin homology (PH) domains were identified, which were designated as PH1 and PH2. Following the PH2 domain, there is another tyrosine motif (YDYV). The C-terminal portion of XB130 contains a coiled-coil region that might be important for membrane traf-
ficking or multimerization (25, 26). Moreover, there are 27 putative phosphorylation targets for serine/threonine kinases, including protein kinase C, protein kinase A, and casein kinase 2. In the 3′-UTR, there are two consensus AU-rich elements (AREs), which have been suggested to play a critical role in the regulation of translation and mRNA stability (27, 28). The first ARE is a pentamer (AUAUAUAUUA) located 656-bp downstream of the stop codon; the second, an enneamer (UUAAUUUAUUA), is 124-bp downstream of the first ARE. *XB130* encodes a protein of 818 amino acids, and the molecular mass measured by immunoblotting is ~130 kDa. Therefore we designated the newly identified protein as XB130. As XB130 does not contain
any recognizable enzymatic domain, but is composed of numerous protein modules for protein-protein interactions, we defined it as an adaptor type protein. The sequence was deposited into GenBank™ data base (accession number 442952).

Chromosomal Localization, mRNA Expression, and Subcellular Distribution of XB130—To determine the chromosomal localization of XB130, fluorescence in situ hybridization analysis was performed using a fluorescence dye-labeled cDNA probe of XB130 sequence. XB130 is localized on human chromosome 10q25.3. A GenBank™ data base search confirmed this location and revealed that XB130 gene contains 19 exons, which are separated by 18 introns over 40 kb in length. Northern blot analysis showed that XB130 mRNA transcript is ~4 kb, and is highly expressed in human spleen, thyroid, and relatively lower expression in the kidney, brain, lung, and pancreas (data not shown).

To determine the subcellular distribution of XB130 protein, we expressed His-tagged XB130 in 3Y1 cells. Immunofluorescence analysis confirmed this localization and revealed that XB130 gene contains 19 exons, which are separated by 18 introns over 40 kb in length. Northern blot analysis showed that XB130 mRNA transcript is ~4 kb, and is highly expressed in human spleen, thyroid, and relatively lower expression in the kidney, brain, lung, and pancreas (data not shown).
cent staining with an anti-His antibody demonstrated that XB130 was distributed primarily in the cytoplasm (Fig. 2A). We raised monoclonal antibodies against XB130 (see supplemental Fig. S1). Similar subcellular distribution of endogenous XB130 was confirmed with confocal microscopy in BEAS-2B cells (Fig. 2B), and similar distribution pattern was observed in multiple cell lines, such as TPC-1 and WRO cells (Fig. 2C).

Although XB130 was identified using chicken AFAP as a search query, the overall amino acid sequence similarity shared between these two proteins was 45%. Yet, their structural compositions are quite similar; both contain a proline-rich motif, several potential SH2-binding sites, and two PH domains. However, in the C terminus, AFAP contains a leucine zipper as well as an actin-binding motif, whereas XB130 has a coiled-coil region. Consistent with this particular structural difference, when BEAS-2B cells were double-stained for XB130 and F-actin, XB130 was not associated with actin filaments (Fig. 2D). To compare the intracellular localization between XB130 and AFAP, we double-stained BEAS-2B cells with XB130 mAb and a polyclonal antibody for AFAP. The distribution of AFAP appeared to be along the stress fiber (Fig. 2E, right), whereas XB130 did not (Fig. 2E, left).

XB130 Interacts with c-Src Both in Vitro and in Vivo—XB130 contains several potential Src SH2/SH3-binding motifs (Fig. 1). To determine whether XB130 interacts with SH2 or SH3 domain-containing proteins, cellular extracts from XB130-transfected COS-7 cells were incubated with GST fusion proteins containing either an SH2 or SH3 domain from Src, GAP, and Nck. XB130 was able to interact with either SH2 or SH3 domain of Src or GAP, but not of Nck (Fig. 3A, upper panel). In contrast to XB130, hAFAP was able to bind the SH2 or SH3 domain of Src, GAP or Nck fused with GST (data not shown). These results suggested that SH2/SH3-binding motifs of XB130 interact specifically with some, but not all, SH2/SH3 domain-containing proteins.

When FLAG-tagged c-Src and His-XB130 were co-expressed in COS-7 cells, immunoprecipitation of Src, using an anti-FLAG or anti-Src antibody, allowed detection of XB130 by immunoblotting with an anti-His antibody (Fig. 3B). To confirm the Src-XB130 interaction, we also transfected cells with non-tagged c-Src and XB130, used the anti-His antibody to pull-down XB130, and then blotted for Src. Binding of these two proteins was seen in cells co-expressing Src and XB130, but not in cells expressing XB130 alone (Fig. 3C, upper panel). In the anti-Src blot of whole cell lysates, a weak signal was detected in cells transfected with XB130 alone, which probably reflected the level of endogenous Src. Similar levels of XB130 proteins in XB130 transfection alone or XB130/Src co-transfections were demonstrated in both whole cell lysates and immu-

**FIGURE 2. Intracellular distribution of the XB130 protein**. A, overexpression of XB130 in 3Y1 cells. 3Y1 cells transfected with pcDNA3-XB130-His and immunostained with an anti-His mAb (left) and counterstained with F-actin (right). B, localization of endogenous XB130 in BEAS-2B cells was examined by confocal microscopy. C, WRO or TPC-1 cells were stained with an anti-XB130 mAb or with IgG2a as a negative control (not shown). D, XB130 is not associated with actin filaments. BEAS-2B cells stained with an anti-XB130 mAb (left) and counterstained to reveal F-actin structures (right). E, XB130 and AFAP do not co-localize. BEAS-2B cells were double-stained with mAb against XB130 and polyclonal antibody against AFAP.

**FIGURE 3. In vitro and in vivo interactions of XB130 with c-Src**. A, in vitro interactions of XB130 with SH2 and SH3 domains of Src and GAP. Bacterial expressed GST fusion proteins on glutathione-Sepharose 4B beads, containing SH2 or SH3 domain of c-Src, GAP, or Nck, were incubated with lysates from COS-7 cells expressing His-tagged XB130. Interacting XB130 was separated on SDS/PAGE and immunoblotted using anti-His antibody (upper panel). The levels of the GST fusion proteins were shown in the lower panel. B, in vivo interactions of XB130 with c-Src detected by immunoprecipitation of Src. COS-7 cells were transfected with FLAG-tagged c-Src and/or His-tagged XB130. Following immunoprecipitation using an anti-FLAG or anti-Src (GD11) antibody, the presence of XB130 in the precipitates was detected by immunoblotting with an anti-His antibody (upper panel). The membranes were stripped and blotted with the Src antibody used for immunoprecipitation (lower panel). C, in vivo interactions of XB130 with c-Src detected by immunoprecipitation of XB130. COS-7 cells were transfected with either His-tagged XB130 alone or in combination with c-Src (non-tagged). Cell lysates were subjected to immunoprecipitation with an anti-His antibody, followed by immunoblotting with an anti-Src. To document XB130 and c-Src protein levels in whole cell lysates (WCL) or immunoprecipitation samples, the blot was blotted with anti-His antibody (lower panel). D, interaction of endogenous XB130 and c-Src in BEAS-2B cells. Cell lysates were immunoprecipitated with an anti-Src antibody (GD11), and immunoblotted with mAbs for XB130 and Src.
results suggested that the increased protein tyrosine phosphorylation of c-Src alone. Cell lysates were immunoprecipitated with Src mAb. Half of immunoprecipitates was used for an ELISA-based tyrosine kinase assay (3). c-Src alone, or cells that co-expressed XB130 and SrcKD, a kinase-deficient mutant of c-Src, were analyzed for protein tyrosine phosphorylation and pTyr, anti-phosphotyrosine (4G10); anti-AFAP or anti-p85 (Fig. 4). The level of Src protein in the SrcKD sample was higher than that in others (Fig. 4A, panel 4). It has been shown that activated Src is targeted to ubiquitin proteasome-mediated degradation (31, 32). Therefore, a lack of kinase activity of SrcKD may lead to a higher steady state level of the protein compared with the wild-type c-Src. Because dephosphorylation of Src Tyr527 has also been shown to lead to activation of Src tyrosine kinase (3), we examined the level of tyrosine phosphorylation on Tyr527 in all samples. Phosphorylation of Tyr527 was not reduced by the presence of XB130 (Fig. 4A, 3rd panel), suggesting that XB130-induced Src activation is not a consequence of dephosphorylation of Tyr527 by activation of tyrosine phosphatase. Similar results have been found in HIV-1 Nef-in-

**FIGURE 4.** Src activation and protein tyrosine phosphorylation induced by XB130. A, Src activation induced by XB130. COS-7 cells transfected with XB130, WT c-Src, Src KD (a kinase-deficient mutant of c-Src), or in combination. Whole cell lysates were subjected to SDS/PAGE and immunoblotted with various antibodies as indicated: α-pTyr, anti-phosphotyrosine (4G10); α-pY416, anti-Src phospho-Tyr416; α-Tyr527, anti-Src phospho-Tyr527. Co-expression of XB130 with c-Src, but not with SrcKD, increased protein tyrosine phosphorylation, and Src Tyr527 phosphorylation. Co-expression of XB130 and c-Src increased tyrosine phosphorylation of endogenous AFAP and p85 subunit of PI3 kinase, as determined by immunoprecipitation and immunoblotting. B, co-expression of XB130 with c-Src increased Src activity. COS-7 cells were transfected with XB130/c-Src or c-Src alone. Cell lysates were immunoprecipitated with Src mAb. Half of immunoprecipitates was used for an ELISA-based tyrosine kinase assay (upper panel). The other half was immunoblotted with mAb for Src (lower panel). C, XB130 directly activates c-Src in vitro. Recombinant XB130 was incubated with Csk-inactivated c-Src. Phosphorylation of a Src substrate peptide was visualized by autoradiography following SDS/PAGE. *, p < 0.05 versus c-Src only, n = 3 separate experiments.

noproprecipitated samples (Fig. 3C, lower panel). The interaction between endogenously expressed XB130 and c-Src was shown in BEAS-2B cells, as detected by co-immunoprecipitation with anti-XB130 mAb followed by immunoblotting with anti-Src antibody (GD11) (Fig. 3D).

Co-expression of XB130 with c-Src Activates c-Src—It has been demonstrated that disruption of intramolecular interactions of c-Src by heterologous binding partners, through SH2 or SH3 domain-mediated interactions, is able to activate Src tyrosine kinase (7, 29, 30). To investigate whether XB130/c-Src interaction plays a role in the regulation of Src activity, we expressed c-Src, with or without XB130, in COS-7 cells. Cell lysates were analyzed for protein tyrosine phosphorylation and Src activation. When wild-type c-Src was co-expressed with XB130, tyrosine phosphorylation was increased in multiple proteins (Fig. 4A, top panel, lane 4), compared with cells that expressed XB130 or Src alone, or cells that co-expressed XB130 and SrcKD, a kinase-deficient mutant of c-Src (lane 5). These results suggested that the increased protein tyrosine phosphor-

ylation might be the consequence of Src activation. Autophosphorylation of Src Tyr416 in the activation loop is a critical step for full activation of Src kinase, which stabilizes the extended conformation of Src and facilitates the access of substrates to the Src kinase domain (3). Src Tyr416 phosphorylation was markedly increased in the presence of c-Src/XB130 co-expression (Fig. 4A, second panel, lane 4) compared with Src expression alone (lane 3). The phosphorylation of Src Tyr416 was not increased when SrcKD was co-expressed with XB130 (lane 5). The level of Src protein in the SrcKD sample was higher than that in others (Fig. 4A, panel 4). It has been shown that activated Src is targeted to ubiquitin proteasome-mediated degradation (31, 32). Therefore, a lack of kinase activity of SrcKD may lead to a higher steady state level of the protein compared with the wild-type c-Src. Because dephosphorylation of Src Tyr527 has also been shown to lead to activation of Src tyrosine kinase (3), we examined the level of tyrosine phosphorylation on Tyr527 in all samples. Phosphorylation of Tyr527 was not reduced by the presence of XB130 (Fig. 4A, 3rd panel), suggesting that XB130-induced Src activation is not a consequence of dephosphorylation of Tyr527 by activation of tyrosine phosphatase. Similar results have been found in HIV-1 Nef-in-

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To verify the effect of XB130 on Src PTK activation, COS-7 cells were transfected with c-Src alone or c-Src together with XB130. Cell lysates were immunoprecipitated with an anti-Src antibody, Src activity in the precipitated protein was determined with an ELISA-based assay. XB130/c-Src co-expression significantly increased tyrosine phosphorylation of endogenous AFAP and p85 subunit of PI3 kinase, as demonstrated by immunoprecipitation with anti-phosphotyrosine (4G10) and immunoblotted with anti-AFAP or anti-p85 (Fig. 4A, bottom panels).
other proteins, XB130 alone was able to increase the tyrosine kinase activity of Src (Fig. 4C).

Co-expression of XB130 with c-Src Leads to Tyrosine Phosphorylation of XB130 and SRE- or AP-1-dependent Transcriptional Activation—c-Src is able to phosphorylate tyrosines on diverse adaptor proteins, such as AFAP, CAS, and Sin (7, 20, 30). Through tyrosine phosphorylation of these substrates, Src exerts its regulation in a variety of cellular events including cytoskeleton reorganization, transcriptional activation, and DNA synthesis (7, 29, 34). As XB130 has several tyrosine-containing motifs that might serve as a target of tyrosine kinases, we examined whether XB130 is also a Src substrate. We co-expressed His-tagged XB130 with or without FLAG-tagged c-Src in COS-7 cells. Cell lysates were immunoprecipitated with anti-His antibody to pull-down XB130, followed by anti-phosphotyrosine immunoblotting. When expressed alone, tyrosine phosphorylation of XB130 was not observed; co-expression with c-Src, however, made tyrosine-phosphorylated XB130 readily detectable (Fig. 5A, top right). Consistent with this finding, phosphorylation of XB130 was not observed; co-expression with c-Src and/or XB130. Luciferase activity is expressed as the mean ± S.D. of triplicates. Values were normalized by activity of β-galactosidase. Similar results were obtained from three independent experiments.

Enhanced Transcriptional Activation of AP-1 and SRE Reporters Requires Src Kinase Activity and Is XB130 Dose-dependent—To determine the role of c-Src in XB130/Src co-expression induced transcriptional activation, we co-expressed XB130 with either wild type, constitutively activated, or kinase-deficient c-Src, and assessed AP-1 or SRE reporter activity. A significant increase in luciferase activity was observed in cells co-transfected with WB130 and wild-type c-Src for both AP-1 and SRE reporters (Fig. 6A). In contrast, co-transfection of WB130 with SrcKD displayed a
level of transcriptional activity that was comparable to XB130 expression alone (Fig. 6A), indicating that the tyrosine kinase activity of Src was required for the transcriptional activation. On the other hand, cells expressing the SrcCA dramatically stimulated the reporter activities at a much higher level, regardless of the XB130 expression (Fig. 6A). This suggested that SrcCA is fully activated; the presence of XB130 may not further affect Src-induced SRE/AP-1 activation.

To examine whether the enhancement of Src-mediated transcriptional activation in the presence of XB130 was specifically contributed by XB130, we assessed AP-1 or SRE activation using increasing amounts of XB130 constructs, which expressed elevating levels of XB130 protein and produced increasing levels of Src Tyr416 phosphorylation (data not shown). We demonstrated that in the absence of c-Src, increasing amount of XB130 expression did not increase reporter activation. However, in the presence of c-Src, increasing XB130 correlated with increasing levels of transcriptional activation of both SRE and AP-1 reporters (Fig. 6B). These results indicated that XB130 specifically contributed to the cooperative effect in c-Src-mediated transcriptional activation.

The Src SH2- and SH3-binding Motifs of XB130 Are Required for Src Activation and Src-mediated Transcriptional Activation—

To gain more insights into the function of XB130 in Src activation and transcriptional activation we constructed XB130ΔN, a mutant that lacks 168 residues in the N-terminal region, thereby removing one proline-rich motif and two tyrosine phosphorylation sites (Fig. 7A). The binding of XB130ΔN to c-Src was significantly reduced compared with that of the wild-type XB130, as determined by co-immunoprecipitation and immunoblotting (Fig. 7B, top panel). Anti-His blotting of whole cell lysates showed that the XB130ΔN was expressed at a lower level relative to the wild-type XB130 (bottom panel). When the ratio of these bands were quantified by densitometry, the binding of XB130ΔN to c-Src was only about 7% of that of wild-type XB130.

To determine whether the loss of interaction of XB130ΔN with Src affects activation of Src tyrosine kinase, we then examined tyrosine phosphorylation of total protein and Src Tyr416. The total protein tyrosine phosphorylation was reduced when XB130ΔN was co-expressed with Src (Fig. 7C, top panel). These results suggested that XB130ΔN is less capable of activating Src tyrosine kinase. In support of this notion, Src phosphorylation on Tyr416 was also significantly decreased when XB130ΔN was co-expressed with c-Src (Fig. 7C, 2nd panel).

To investigate whether the N terminus of XB130 is important for Src-mediated transcriptional activation, we examined SRE-dependent transcription activation by c-Src in the presence of wild-type XB130 or XB130ΔN. The activation was dramatically decreased when Src was co-expressed with XB130ΔN (Fig. 7D). In comparison with Src alone, co-expression of XB130ΔN and Src led to higher SRE activity, suggesting that other functional domains and motifs in the rest of XB130 also mediate SRE transactivation. To determine whether XB130 and/or Src could mediate signals initiated by external stimuli, cells were transfected with either vector, XB130, XB130ΔN, c-Src alone, or together with c-Src. Cells were then treated with EGF (either 5 or 10 ng/ml). Compared with vector control, XB130, XB130ΔN, or c-Src alone moderately increased EGF-induced SRE activation. Co-expression of XB130 and c-Src dramatically enhanced EGF-induced SRE activation. Importantly, this effect was reduced when XB130ΔN was co-expressed with c-Src (Fig. 7E). This suggests that the interaction between XB130 and c-Src may play an important role in mediating signal transduction induced by EGF.

To further dissect the XB130/c-Src interaction, we constructed six mutants with single amino acid mutated within the N terminus. The mutant M4, which carries a mutation from tyrosine (Y) to phenylalanine (F) in a tyrosine-containing site (YEEA), showed lower binding with c-Src, as determined by immunoprecipitation and immunoblotting, and reduced SRE transcriptional activation when co-expressed with c-Src (see
XB130, a Novel Adaptor Protein—The overall structural similarity between XB130 and AFAP suggests the possibility that they may belong to a new family of adaptor proteins. A common feature of XB130 and AFAP is the presence of a proline-rich domain that shares partial similarity with the leucine zipper motif. Another striking feature is that they both have two PH domains. PH domains may target host proteins to cellular membranes, perhaps through interactions with phosphatidylinositol 4,5-bisphosphate and membrane-associated proteins (47, 48). The third common feature of this group of adaptor proteins is found in their C termini. XB130 has a coiled-coil domain that shares partial similarity with the leucine zipper domain in AFAP. In general, both coiled-coil and leucine zipper motifs are known to be important for host defense by producing a variety of cytokines and chemokines (44). We used human lung small airway BEAS-2B cells as a model system to determine whether XB130 is involved in the regulation of cytokine production. We first treated cells with two Src PTK inhibitors, PP2 (10 μM) and SU6656 (2 μM), IL-8 in the culture medium was measured by ELISA. C, reducing XB130 protein level led to reduced Src activity. Cells were pretreated with siRNA against XB130 or nonspecific dsRNA control (75 nM, 72 h). siRNA reduced XB130 protein level, SrcTyr416 phosphorylation, but not total Src protein level. D, reducing XB130 inhibited IL-8 release in the culture medium as measured by ELISA. *, p < 0.05 compared with the control group.

Lung epithelial cells are known to be important for host defense by producing a variety of cytokines and chemokines (44). We used human lung small airway BEAS-2B cells as a model system to determine whether XB130 is involved in the regulation of cytokine production. We first treated cells with two Src PTK inhibitors, PP2 (10 μM) and SU6656 (2 μM) for 8 h. Both inhibitors significantly reduced IL-8 release (Fig. 8B). We then pretreated cells with two siRNAs against XB130; both significantly reduced XB130 protein levels, in comparison with cells treated with nonspecific control double strand RNA (Fig. 8C). Furthermore, siRNA treatment reduced SrcTyr416 phosphorylation, but has no effect on total Src protein levels (Fig. 8D). This treatment also significantly reduced the IL-8 released into the culture medium measured by ELISA (Fig. 8D). To determine whether this effect is specific to IL-8, we measured cytokines in the culture media with a LiquiChip multiple cytokine assay. In addition to IL-8, the protein level of IL-6 was also reduced by siRNA treatment. In contrast, the levels of IL-1β, IL-2, IL-4, IL-10, TNFα, IFNγ, and GM-CSF were not affected (data not shown).

It has been shown that c-Src is involved in EGF-induced VEGF expression (45) and EGF receptor-mediated cell migration (46) through PI3 kinase (PI3K) pathway. To determine whether XB130 is involved in mediating exogenous signals, we incubated A549 cells with either Src PTK inhibitor PP2 or its inactivated analogue PP3 (10 nM for 30 min) followed by EGF stimulation (50 ng/ml, 10 min). PP2 reduced Src Tyr416 phosphorylation, and phosphorylation of Akt on Ser473 and phosphorylation of GSK3β on Ser9. Both are molecules in the PI3K signaling pathway. Whereas PP3 had no such effects (Fig. 9A). We transfected A549 cells with pooled XB130 siRNA (50 nM for 48 h). EGF was then added into the medium (50 ng/ml for 10 min) after 5 h of serum starvation. Knocking-down XB130 by siRNA reduced phosphorylation of SrcTyr416, Akt Ser473, and GSK3β Ser9 at baseline and also partially reduced EGF-induced phosphorylation of Akt Ser473 and GSK3β Ser9 (Fig. 9B). Furthermore, down-regulation of XB130 with siRNA prior to EGF stimulation in A549 cells resulted in accumulation of cells in G1 phase, reduction of cells in G2/M and S phases (Fig. 9C). These data suggest that XB130 is involved in EGF-induced PI3K signaling and cell cycle progression in A549 cells.

DISCUSSION

XB130, a Novel Adaptor Protein—The overall structural similarity between XB130 and AFAP suggests the possibility that they may belong to a new family of adaptor proteins. A common feature of XB130 and AFAP is the presence of a proline-rich region and several putative Src SH2-binding sites in their N termini. Another striking feature is that they both have two PH domains. PH domains may target host proteins to cellular membranes, perhaps through interactions with phosphatidylinositol 4,5-bisphosphate and membrane-associated proteins (47, 48). The third common feature of this group of adaptor proteins is found in their C termini. XB130 has a coiled-coil domain that shares partial similarity with the leucine zipper domain in AFAP. In general, both coiled-coil and leucine zipper
motifs are thought to be involved in protein oligomerization and DNA binding (25, 26, 27, 28). Despite these similarities, XB130 does not behave like an actin filament-associated protein. The actin-binding site that is present in the C terminus of AFAP (49) is only partially present in XB130. Through its interaction with actin filaments, AFAP transmits physical force and mediates mechanical stretch-induced c-Src activation (18, 50). The diffuse distribution of XB130 in the cytoplasm suggests that XB130 plays a different role in signal transduction and cellular functions.

**Activation of c-Src by XB130**—XB130 activated c-Src when they were co-expressed in COS-7 cells, and recombinant XB130 directly activated c-Src in vitro. Thus, XB130 could function as a direct activator of Src or Src family PTKs. Using GST fusion proteins we demonstrated that XB130 binds both Src SH3 and SH2 domains. This is supported by the finding that XB130ΔN mutant, containing a deletion of the N-terminal SH3- and SH2-binding sites, significantly reduced c-Src binding, activation, and c-Src-mediated SRE transcriptional activation. To a lesser extent, a single amino acid mutation in the YEEA site reduced binding between XB130 and c-Src, and SRE activation.

An important structural feature of XB130 is that the proline-rich region and the YEEA motif in its N terminus are in a close proximity. Previous studies have demonstrated that peptides containing both SH2- and SH3-binding sites of FAK exhibited higher affinity to Src (51); SH2- and SH3-binding sites on Sin cooperatively activated c-Src signaling potential (7). A mode of bipartite binding of Src to adaptor protein CAS or Sin has been suggested; the initial interaction between Src SH3 domain and a polyproline motif in adaptor proteins was followed by tyrosine phosphorylation of the adaptor proteins, creating additional binding sites for the Src SH2 domain (7, 30). The dual binding serves to stabilize c-Src in an activated conformation and allow adaptor proteins to function as both activator and substrate of c-Src (29). The dual functions of adaptor proteins have also been observed in other studies of adaptors, such as CAS (30), Sin (7), and AFAP (18, 20).

**XB130/c-Src Induced AP-1/SRE Activation**—Transcriptional activation mediated through SRE and AP-1 transcription regulatory elements has been shown to be responsive to various signaling pathways, including growth factor receptor tyrosine kinases, G-protein-coupled receptors, and intracellular signaling mediators such as non-receptor Src family PTKs (36, 37). XB130/c-Src interaction led to transactivation of AP-1 and SRE. This effect is dependent upon the level of XB130 expression. Furthermore, XB130/c-Src enhanced EGF-induced SRE transcriptional activation. Therefore, the interaction between XB130 and c-Src may be physiologically regulated.

### FIGURE 9. Function of endogenous XB130 in A549 cells

**A**, Src inhibitor PP2 blocks phosphorylation of Akt and GSK3β. After 5 h of serum starvation, A549 cells were incubated with Src PTK inhibitor PP2 or its inactivate analogue PP3 (both at 10 nM) for 30 min, followed by EGF (50 ng/ml) stimulation for 10 min. Whole cell lysate was collected and immunoblotted with antibodies against phospho-SrcTyr416; total Src; phospho-Akt Ser473; total Akt; phospho-GSK3βSer9.

**B**, down-regulation of XB130 reduced phosphorylation of Akt and GSK3β. A549 cells were transfected with pooled XB130 siRNA (50 nM) for 48 h. EGF (50 ng/ml) was added into the medium after 5 h of serum starvation and incubated for 10 min. Knocking-down XB130 by siRNA reduced the EGF-induced phosphorylation of Akt and GSK3β. C, down-regulation of XB130 altered progression of cell cycle. A549 cells were transfected with pooled XB130 siRNA (50 nM) for 48 h, subjected to serum starvation overnight, and then stimulated with EGF (50 ng/ml) for 2 h. siRNA treatment increased accumulation of cells in G1 phase and reduced cells in G2/M and S phases. dsRNA, nonspecific double-stranded RNA; siRNA, small interference RNA against XB130. *, p < 0.05; **, p < 0.01, compared with siRNA-treated groups.
Other adaptor proteins, such as Sin (7) and CAS (29) are also capable of activating c-Src when overexpressed in the cells. However, only Sin-induced c-Src activation led to AP-1/SRE activation (12). Full-length CAS cannot mediate c-Src induced AP-1/SRE, but it effectively mediated v-Src-induced SRE activation (38). Therefore, the roles of different adaptor proteins in activating c-Src and mediating Ssrc-induced transcriptional activities could be unique and have different consequences in signal transduction. Activating Ssrc and mediating Ssrc-related transcriptional activity can be separate functions for each adaptor protein. The signaling mechanisms of XB130/c-Src-induced transcriptional activation of AP-1 and SRE remain to be investigated.

Role of Endogenous XB130 in Regulation of Cellular Functions—in addition to the artificial reporter constructs for AP-1 and SRE, we also demonstrated that XB130/c-Src co-expression enhanced transcriptional activity of IL-8-luc reporter, in which the luciferase gene is under the control of a naturally existing IL-8 promoter. It has been shown that AP-1- and NF-kB-binding sites are the primary cis-acting elements in the IL-8 promoter and they synergistically regulate gene transcription of IL-8 (43). The IL-8 promoter activation appears to be specific, because the XB130AN mutant did not have the same effect. Furthermore, we demonstrate that by reducing the protein level of XB130, the endogenous Ssrc activity and IL-8 production were also reduced. IL-8 is an important chemokine for neutrophil recruitment and activation. Our results implicate that XB130/Ssrc interaction may play a role in determining IL-8 production from airway epithelial cells.

XB130 may also be involved in the receptor PTK-related signal transduction. Ssrc PTK is involved in EGF receptor-Pi3K-Akt-GSK3β signaling in several cell types (46, 52). We showed that either alone, or in cooperation with c-Src, XB130 expression enhanced EGF-induced SRE transactivation. In A549 cells, basal and/or EGF-induced phosphorylation of Akt and GSK3β was partially reduced by either Ssrc inhibitor or XB130 siRNA. Reducing the expression of XB130 also altered the distribution of cells in different phases of cell cycle. It has been reported that GSK-3 activity suppresses cell proliferation (53, 54). We speculate that the inhibition of XB130 with siRNA may reduce Akt activity and subsequently reduce phosphorylation of Ser9 in GSK-3β, which may further inhibit cell cycle progression. Of course, this hypothesis merits further investigation.

In conclusion, we have identified a novel c-Src-interacting adaptor protein, XB130. Using c-Src as a model protein, we demonstrated that XB130 could interact with other proteins via specific functional motifs. XB130 may have dual role as both a Ssrc activator and effector in signal transduction. Finally, using siRNA technique we demonstrated that endogenously expressed XB130 is involved in EGF-related signal transduction and cellular functions.

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