Src Phosphorylates Ezrin at Tyrosine 477 and Induces a Phosphospecific Association between Ezrin and a Kelch-Repeat Protein Family Member*

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Ezrin, a linker between plasma membrane and actin cytoskeleton possesses morphogenetic properties and can promote dissemination of tumor cells. Ezrin is phosphorylated on tyrosine, but a detailed picture of the signaling pathways involved in this modification is lacking. The transforming tyrosine kinase Src has various cytoskeletal substrates and is involved in regulation of cellular adhesion. We studied the role of Src in tyrosine phosphorylation of ezrin in adherent cells. We show that ezrin is phosphorylated in human embryonic kidney 293 cells in a Src family-dependent way. In SYF cells lacking Src, Yes, and Fyn, ezrin was not tyrosine-phosphorylated but reintroduction of wild-type Src followed by Src activation or introduction of active Src restored phosphorylation. Mapping of the Src-catalyzed tyrosine in vitro and in vivo by site-directed mutagenesis demonstrated Tyr477 as the primary target residue. We generated a pTyr477-phosphospecific antibody, which confirmed that Tyr477 becomes phosphorylated in cells in a Src-dependent manner. Tyr477 phosphorylation did not affect ezrin head-to-tail association or phosphorylation of ezrin on threonine 566, indicating that the function of Tyr477 phosphorylation is not related to the intramolecular regulation of ezrin. A modified yeast two-hybrid screen in which ezrin bait was phosphorylated by Src identified a novel interaction with a kelch-repeat protein family member, KBTBD2 (Kelch-repeat and BTB/POZ domain containing 2). The Src dependence of the interaction was further verified by affinity precipitation assays. Identification of a functional interplay with Src opens novel avenues for further characterization of the biological activities of ezrin.

The Src family of transforming non-receptor tyrosine kinases is involved in cell adhesion, invasion, migration, cell cycle progression, and survival and is coupled to various receptor signaling pathways (reviewed in Refs. 1–3). Src also has a role in control of the actin cytoskeleton. Activation of Src by a complex mechanism leads to its translocation to plasma membrane and to association with detergent-insoluble protein fractions (4). Src has various cytoskeletal substrates, and its activation induces rapid formation of lamellipodia or membrane ruffles.

One of the linker proteins between cell surface receptors, especially adhesion molecules, and the actin cytoskeleton is ezrin, a member of the ERM (ezrin-radixin-moesin) protein family (reviewed in Refs. 5–7). Ezrin is typically localized to specialized membrane areas such as microvilli, membrane ruffles, or the uropods of polarized lymphocytes, and it possesses morphogenetic functions in a variety of cell types. In addition to membrane proteins and F-actin, ezrin interacts with cellular signaling proteins of the phosphatidylinositol 3-kinase, protein kinase A and Rho signaling pathways (8) and is thereby involved in the mechanisms of targeted signaling in cells.

Several mechanisms regulate the functional activity and molecular interactions of ERM proteins. An intramolecular association between the N terminus (N-ERMAD, N-terminal association domain) and C terminus (C-ERMAD, C-terminal association domain) keeps the proteins inactive and masks their interaction sites for other molecules (9–11). A key regulatory switch appears to be phosphorylation of a conserved threonine in the C-terminal domain. Phosphorylation of this residue results in dissociation of the intramolecular interaction and formation of monomers with morphogenic activity (12).

The consequences of tyrosine phosphorylation of ezrin are less well known. Ezrin is a major protein phosphorylated on tyrosine after epidermal growth factor (EGF) stimulation in A431 cells (13, 14), and tyrosine phosphorylation of ezrin is also induced by platelet-derived growth factor and hepatocyte growth factor (15, 16). The major phosphorylated sites by the EGF and hepatocyte growth factor pathways have been mapped to Tyr145 in the N-terminal domain and Tyr353 in the o-helical region (15, 17). Phosphorylation of Tyr145, which is not conserved in other ERM proteins, is required for the binding of the regulatory p85 subunit of phosphatidylinositol 3-kinase to ezrin and for the activation of the phosphatidylinositol 3-kinase/protein kinase Akt signaling pathway in LLC-PK1 cells (18). A defect in this signaling pathway due to a point mutation of Tyr353 to phenylalanine sensitizes cells to apoptosis. In addition, mutagenesis of both Tyr145 and Tyr353 in ezrin has been shown to disturb a morphogenic response to hepatocyte growth factor (15). Early findings showed that ezrin tyrosine phosphorylation was increased in v-Src-induced cells (19). We have shown that the Src family kinase (SKF) Lck catalyzes

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1 The abbreviations used are: ERM, ezrin-radixin-moesin; WT, wild type; BTB/POZ domain, Broad complex, Tramtrack, and Bric-a-brack/poxvirus and zinc-finger domain; KBTBD2, Kelch-repeat and BTB/POZ domain containing 2; N-ERMAD, N-terminal association domain; C-ERMAD, C-terminal association domain; EGF, epidermal growth factor; SFK, Src family kinase; ICAM-1, intercellular adhesion molecule-1; HEK, human embryonic kidney; GST, glutathione S-transferase; CIP, calf-intestinal phosphatase.
phosphorylation of Tyr\textsuperscript{145} in Jurkat T-cells (20). Cross-linking of intercellular adhesion molecule-1 (ICAM-1) activates Src kinases and leads to tyrosine phosphorylation of ezrin (21). Also, cross-linking of ICAM-2 induces tyrosine phosphorylation of ezrin and activation of the PKB/Akt pathway (22), which is blocked by herbimycin A, a potent inhibitor of Src and pp62\textsuperscript{302}. Recently, it was reported that Src and ezrin act cooperatively in regulation of cell-cell contacts and scattering of mammary carcinoma cells (23). However, the relation of Src and ezrin is not known at molecular level and the Src target site in ezrin has not been characterized.

We have studied the role of Src kinase in tyrosine phosphorylation of ezrin in adherent cells and characterized a novel Src-dependent phosphorylation site, Tyr\textsuperscript{477}. We show that, on one hand, phosphorylation at Tyr\textsuperscript{477} does not affect ezrin head-to-tail association and that, on the other hand, it is independent of ezrin threonine phosphorylation, which has been linked to conformational opening of the molecule. Moreover, we show that phosphorylation of ezrin by Src induces an association with a member of the kelch-repeat superfamily protein, KBTBD2 (Kelch-repeat and BTB/POZ domain containing 2).

**MATERIALS AND METHODS**

**Cells and Antibodies**—Human embryonic kidney 293 (HEK293, ATCC, Manassas, VA) cells were maintained in RPMI 1640 medium including 10% fetal calf serum and antibiotics. A431 epidermoid carcinoma cells (ECACC, Salisbury, United Kingdom) SYF mouse embryonic fibroblasts from the triple Src knockout mouse lacking Src, Yes, and Fyn SFKs (24) and the add-back versions of the SYF cells, which have been introduced either with the wild-type c-Src gene or the constitutively active Y527F mutant Src (25) kindly provided by Dr. J. A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) were maintained in Dulbecco’s modified Eagle’s medium including 10% fetal calf serum and antibiotics. GST fusion constructs encoding N-terminal (amino acids 1–309) and short C-terminal (amino acids 309–585) ezrin fusion proteins have been described previously (26). The wild-type ezrin construct was a kind gift of Dr. M. Arpin (Institut Curie, Paris, France). The cDNAs of the constructs were introduced with the wild-type c-Src gene or the constitutively active Y527F mutant Src (25) kindly provided by Dr. J. A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) were maintained in Dulbecco’s modified Eagle’s medium including 10% fetal calf serum and antibiotics.

Phosphotyrosine was detected with the monoclonal anti-phosphotyrosine monoclonal antibody clone PT-66 (Sigma). The polyclonal rabbit antibody E29 against ezrin has been described previously (26). The VSVG-tag monoclonal antibody was from Roche Diagnostics (clone F5D4, Mannheim, Germany). Mouse IgG1 (Dako Cytometon, Glostrup, Denmark) or rabbit preimmune serum were used as controls. Rabbit- and phospho-specific antibodies against Srcp(Y418) and against ezrin pThr\textsuperscript{266} (Tyr\textsuperscript{555} on moesin) were from BioSource International (Camarillo, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Rabbit polyclonal antibody recognizing human ezrin phosphorylated on Tyr\textsuperscript{477} was produced by BioSource International by immunization of a 6-mer synthetic phosphopeptide PV(pY)EPV and pp62\textsuperscript{302} (27). The recombinant GST fusion proteins were phosphorylated in 20 μl of Src buffer in the presence of 200 μM ATP (Sigma). Two samples were incubated with 1 unit of recombinant human Src, whereas one sample was incubated with no Src for 30 min at 30 °C. Kinase reaction was stopped by boiling in reducing sample buffer. The samples were resolved on SDS-PAGE, the gel was fixed and stained with Coomassie Blue, and dried and subjected to autoradiography.

**Affinity Pull-down Experiments**—2 μg of the C-terminal GST-fusion ezrin antibody (amino acids 309–585) were phosphorylated in 20 μl of Src buffer in the presence of 200 μM ATP (Sigma). Two samples were incubated with 1 unit of recombinant human Src, whereas one sample was incubated with no Src for 30 min at 30 °C. Kinase reaction was stopped by boiling in reducing sample buffer. The samples were resolved on SDS-PAGE, and purified according to the manufacturer’s instructions (Amersham Biosciences). The GST portion of some of the fusion proteins was cleaved off by thrombin (Sigma). 2 μg of polypeptides were used for each reaction. The phosphorylation reaction was carried out in Src buffer (50 mM Tris-HCl, 10 mM MgCl\textsubscript{2}, 3 mM MnCl\textsubscript{2}, pH 7.4) including 10 μM of [γ\textsuperscript{32}P]ATP (PerkinElmer Life Sciences) and 1 unit of recombinant human Src (Upstate Biotechnology) for 15 min at 30 °C. Phosphorylation analysis involving a trace amount of [γ\textsuperscript{32}P]ATP, the phosphorylation level of Src-phosphorylated samples was ~12% total protein (data not shown). After reaction, 200 μl of 50 mM Tris-HCl, 10 mM EDTA was added to the mixtures together with glutathione-Sepharose beads. The mixtures were incubated on a shaker for 1 h followed by extensive washes of the beads, first in 50 mM Tris-HCl, 10 mM EDTA and then in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 1 mM MgCl\textsubscript{2}, 1 mM EDTA). In all of the washing steps during the experiments, 1 mM orthovanadate was included with the exception of the washes of the samples from the phosphotyrosylated samples where orthovanadate was omitted in steps preceding the CIP treatment. The CIP reaction was performed in 30 μl of the CIP buffer with 5 units of CIP (Finzymes Oy, Espoo, Finland) for 30 min at 37 °C. Other samples were incubated in the buffer with no added enzyme. Following ligation, the beads were extensively washed, once in the CIP buffer and three times in binding buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40).

Transfected cells were lysed in binding buffer, and the lysates were added on beads and incubated on a shaker for 2 h at 25 °C. Beads were washed in lysis buffer and boiled in 30 μl of Laemmli buffer, 10 μl of each sample was analyzed by SDS-PAGE and immunoblotting with appropriate antibodies. GST-KBTBD2 was bound to glutathione-Sepharose beads, incubated with lysates of cells transfected with wild-type full-length or C-terminal ezrin (amino acids 306–585) treated or untreated with pervanadate, washed, and analyzed as described above.

**Yeast Two-hybrid Screening and β-Galactosidase Filter Assay**—The C-terminal ezrin cDNA encoding for amino acids 309–585 was introduced by PCR and conventional cloning to a modified yeast two-hybrid bait plasmid pBTM116Src, which also encodes for active Src from a separate transcript (28). The bait was used in screening of a human fetal brain library in pACT2 (Clontech). To verify the Src dependence of the interaction, each prey clone was tested in a mating assay (29) against C-terminal ezrin bait in pBTM116Src vector or, as a control, in BTM116 without Src. The mated yeast cells expressing the bait and the prey plasmids were transferred to nitrocellulose filters and quick-frozen in liquid nitrogen, and the filter was incubated for 30 min at 37 °C on a wet Whatman filter moistened with Z-buffer (60 mM Na\textsubscript{2}HPO\textsubscript{4}, 40 mM NaH\textsubscript{2}PO\textsubscript{4}, 10 mM KCl, 1 mM Mg\textsubscript{2}SO\textsubscript{4} containing 0.7 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).
also in adherent cells, we investigated the phosphorylation status of ezrin in HEK293 cells in which SFK activity was stimulated by pervanadate. Following stimulation, endogenous ezrin was immunoprecipitated and its phosphorylation status was analyzed by immunoblotting with an anti-phosphotyrosine antibody (left panel). Src is activated during pervanadate treatment but not after preincubation with PP2 as shown by immunoblotting of whole cell lysates with an anti-Src-pY418 antibody (right panel). Ezrin is phosphorylated on tyrosine after pervanadate treatment, and phosphorylation is inhibited by preincubation with PP2. WCL, immunoblot of whole cell lysate; WB, Western Blot; pre-imm, immunoprecipitation (IP) with pre-immune serum.

To provide further evidence for the involvement of Src kinase in ezrin phosphorylation, we used SYF embryonic fibroblast cells from the triple Src knock-out mouse lacking Src, Yes, and Fyn SFKs (24) and the add-back versions of SYF cells reconstituted with the wild-type c-Src gene or the constitutively activated Y527F mutant Src (25). When endogenous ezrin was immunoprecipitated from SYF or SYF/SrcWT cells, no phosphorylation was detected with the anti-phosphotyrosine antibody (Fig. 2A). On the contrary, in the SYF cells, which express the constitutively active mutant Src (SYF/Y527FSrc), ezrin was phosphorylated on tyrosine (Fig. 2A). Immunoblotting of the whole cell lysates with the antibody recognizing active pY418/Src confirmed that only SYF/Y527FSrc cells contained active Src (Fig. 2A, right panel), whereas SYF/SrcWT expressed low levels of wild-type Src, which is not active in basal cell culture conditions (25). When SYF/SrcWT cells were treated with pervanadate, ezrin became tyrosine-phosphorylated, whereas pervanadate had no effect on SYF cells (Fig. 2B). These results confirm that Src kinase activity is explicitly involved in phosphorylation of ezrin.

Mapping of the Phosphotyrosine Residue in Vitro—To map the tyrosine residue(s) phosphorylated by Src, we generated recombinant ezrin fragments fused with GST, produced them in E. coli, purified them, and subjected to in vitro phosphorylation reaction with recombinant Src kinase and [γ-32P]ATP. Previously identified ezrin phosphorylation sites, Tyr145 and Tyr353 (17), were mutated to phenylalanine in some of the fusion peptides to directly analyze whether they are the target sites for Src. Autoradiographs of the reaction mixtures separated in SDS-PAGE showed that the C-terminal half of ezrin (amino acids 309–585) and a short C-terminal construct (amino acids 477–585) had incorporated 32P (Fig. 3A). The fusion pro-
teins in which the previously identified phosphotyrosines were mutated did not differ from the corresponding wild-type fusion proteins. Interestingly, a C-terminal construct of merlin (amino acids 492–595), homologous to the short ezrin C-terminal construct, was not phosphorylated in a parallel experiment.

Analysis of the amino acid sequence of the short C-terminal construct revealed altogether four tyrosines, each of which was mutated to phenylalanine one by one and introduced into the GST fusion construct containing the C-terminal half of ezrin (amino acids 309–585). The upper right panel shows Coomassie Blue-stained gel of the peptide fragments either after the removal of GST (first two lanes) or as GST fusion peptides, and the bottom panel shows an autoradiograph of the same gel. Autophosphorylation of Src kinase is also seen (arrow). Only the C-terminal half (amino acids 309–585) and the very C-terminal end (amino acids 477–585) of ezrin incorporate radioactivity significantly. B, each of the four tyrosines at ezrin C terminus was substituted with phenylalanine and introduced in GST-ezrin-(309–585). Purified GST peptides were subjected to in vitro phosphorylation. Left panel, Coomassie Blue-stained gel. Right panel, an autoradiograph of the same gel. Substitution of Tyr477 but not other tyrosines to phenylalanine abolishes the incorporation of label into the fusion peptide. MW, molecular weight; C-term, C-terminal.

pTyr antibody. The expressed wild-type protein was readily phosphorylated after pervanadate treatment (Fig. 4). Interestingly, also the Y477F ezrin showed a low level of pTyr reactivity (<10% wild-type ezrin) after pervanadate treatment. To further characterize this remaining pTyr reactivity, we introduced a second mutation to the same expression construct, namely Y145F, because tyrosine 145 has been shown to be a phosphorylation target in ezrin (15, 17, 20). The double mutant Y145F/Y477F showed similar pTyr reactivity as ezrin-Y477F when the cells were stimulated with pervanadate (Fig. 4). The result indicated that Tyr477 is the major site of phosphorylation in ezrin during pervanadate treatment.

To better study the phosphorylation of Tyr477 in vivo, we generated a phosphospecific ezrin-Tyr477 antibody. Its specificity was characterized with several methods. The antibody recognized recombinant C-terminal ezrin only when the peptide was phosphorylated in vitro by recombinant Src. The reactivity was lost if the ezrin preparation was subsequently dephosphorylated by CIP (Fig. 5A). The anti-ezrin-pTyr477 antibody recognized immunoprecipitated wild-type ezrin after pervanadate treatment, whereas ezrin mutants Y477F or Y145F/Y477F were not recognized under similar conditions (Fig. 5B). Immunoblotting of 293 cell lysates containing endogenous or trans-
fected wild-type ezrin demonstrated that the antibody recognized a band corresponding with the molecular size of ezrin, either after pervanadate treatment or in cells overexpressing Src. In contrast, when a kinase-dead dominant-negative mutant of Src was overexpressed, no reactivity was detected. The reactivity of the antibody was abolished by preincubation with

**FIG. 4.** Y477F substitution abrogates tyrosine phosphorylation of ezrin after pervanadate treatment. 293 cells were transfected with wild-type or mutant forms of VSVG-tagged ezrin. Cells were treated or untreated with pervanadate, ezrin was immunoprecipitated with an anti-VSVG antibody, and its phosphorylation was analyzed by immunoblotting with an anti-phosphotyrosine antibody (left panel). The filter was reprobed with the anti-VSVG antibody (right panel). The full-length wild-type ezrin polypeptide is readily phosphorylated on tyrosine during pervanadate treatment, whereas the Y477F and the Y145F/Y477F double mutant show only marginal reactivity. WCL, immunoblot of untreated whole cell lysate; WB, Western blot; IP, immunoprecipitation; contr, control.

**FIG. 5.** Reactivity of the anti-ezrin-pTyr477 antibody. A, GST-ezrin-(309–5850) was untreated, phosphorylated with recombinant Src or phosphorylated with Src, and further dephosphorylated with CIP. Anti-ezrin-pTyr477 antibody recognizes ezrin after Src-catalyzed phosphorylation, and phosphatase treatment abolishes the reactivity. B, VSVG-tagged ezrin was immunoprecipitated from 293 cells transfected with different expression constructs (wild type, Y477F, or Y145F/Y477F), and the precipitates were blotted with anti-ezrin-pTyr477 antibody. The antibody reacts only with the wild-type ezrin precipitated from pervanadate-treated cells. C, lysates from untransfected 293 cells or cells transfected with wild-type ezrin, Src, or kinase-dead (KD, dominant-negative) Src were probed with anti-ezrin-pTyr477 antibody. The antibody recognizes a band consistent with the molecular size of ezrin in untransfected and ezrin-overexpressing cells after pervanadate treatment. It also recognizes a similar band in cells overexpressing Src but not dominant-negative Src. Preincubation of the antibody with the immunogenic phosphopeptide abolished the reactivity with the lysates (right panel). D, lysates from cells transfected with various ezrin mutants were probed with anti-ezrin-pTyr477 antibody. The antibody recognizes a band consistent with ezrin in pervanadate-treated cells expressing ezrinY145F and ezrinY353F but not Y477F and Y145F/Y477F. E, ezrin was immunoprecipitated from SYF, SYF/SrcWT, and SYF/SrcY527F cells and analyzed by immunoblotting with the anti-pTyr477 antibody (left panel). The filter was reprobed with the anti-ezrin antibody (right panel). Ezrin is phosphorylated on Tyr477 in SYF/SrcY527F cells expressing constitutively active form of Src. WCL, immunoblot of untreated whole cell lysate; WB, Western blot; IP, immunoprecipitation; contr, control; untransf., untransfected.
the antigenic phosphopeptide (Fig. 5C). Furthermore, pervanadate-treated lysates containing transfected Y145F or Y353F ezrin mutants reacted with the anti-εzrin-pTyr477 antibody, whereas the lysates expressing Y477F or Y145F/Y477F did not react (Fig. 5D). In conclusion, these results indicate that the antibody is indeed specific for ezrin-Tyr477 when phosphorylated by Src.

**Tyr477 Is phosphorylated in SYF/SrcY527F Cells**—To see whether ezrin can be constitutively phosphorylated on Tyr477 in cells with up-regulated Src activity, we analyzed SYF cells and SYF/SrcWT and SYF/SrcY527F variants with anti-εzrin-pTyr477 antibody. In these experiments, the antibody reacted with ezrin in unstimulated SYF/SrcY527F cells (Fig. 5E). The result further verified that Tyr477 is a target for Src in these cells.

**Phosphorylation of Tyr477 and Thr566 Are Independently Regulated**—Ezrin undergoes conformational regulation, which is dependent on the phosphorylation of Thr566. Previous studies have shown that T566D substitution renders ezrin in a constitutively active form (12, 31). We wanted to analyze whether Tyr477 phosphorylation affects the phosphorylation level of Thr566. HEK293 cells were transfected with VSVG-tagged wild-type and Y477F mutant ezrin, and lysates of transfected or untransfected cells treated with pervanadate were probed with a phosphospecific antibody against phosphorylated Thr566 ezrin. Both forms of ezrin reacted with anti-pThr566 antibody at a similar level (Fig. 6A). The result suggests that Tyr477 phosphorylation does not promote Thr566 phosphorylation.

**Phosphorylation of Tyr477 Does Not Affect Ezrin Head-to-Tail Interaction**—To find out the effect of ezrin Tyr477 phosphorylation on the intramolecular N-ERMAD to C-ERMAD association, we tested how tyrosine-phosphorylated ezrin self-associates in an affinity precipitation assay. The C-terminal ezrin GST fusion protein (amino acids 309–505) was either non-phosphorylated or phosphorylated in vitro with recombinant Src and bound to glutathione-Sepharose. One phosphorylated sample was further treated with CIP to verify the effect of phosphorylation on binding. Lysates of transfected cells expressing N-terminal ezrin-(1–309) protein were incubated with the treated C-erm GST beads. N-εzrin bound to beads was detected by immunoblotting of the washed and eluted samples separated on SDS-PAGE. The results show that non-phosphorylated, phosphorylated, or phosphorylated and CIP-treated C-terminal ezrin-GST bound N-εzrin in a similar manner (Fig. 6B). This indicates that the phosphorylation of Tyr477 does not affect the ability of ezrin to form an intramolecular or intermolecular association via N- and C-ERMADs.

**Tyr477 Is Not Phosphorylated after EGF Stimulation in A431 Cells**—Ezrin is a major cellular protein phosphorylated on tyrosine after EGF stimulation (14). To see whether EGF stimulates Tyr477 phosphorylation on ezrin, we stimulated A431 epidermoid carcinoma cells with EGF. After immunoprecipitation of endogenous ezrin, we probed the samples either with a general anti-pY antibody or with the anti-εzrin-pTyr477 antibody. The results shown in Fig. 7A indicate that, although EGF stimulates ezrin tyrosine phosphorylation, it does not activate Src and does not lead either directly or indirectly to the phosphorylation of Tyr477. In parallel experiments, pervanadate treatment of the cells resulted in Src activation and phosphorylation of ezrin at Tyr477.

**Tyr477 Is Not Phosphorylated in Jurkat T-cells**—We have shown earlier that the Src family kinase Lck phosphorylates ezrin at Tyr477 in Jurkat T-cells (20) and wanted to test whether Tyr477 was phosphorylated also in these non-adherent cells during pervanadate treatment. Although immunoblotting of the whole cell lysates showed that pervanadate induced a prominent phosphorylation on tyrosine as detected with anti-pY antibody, there was no signal when the lysates were probed with anti-εzrin-pTyr477 (Fig. 7B).

**Src-dependent Phosphorylation of Ezrin Induces Association with KBTBD2**—To identify Src phosphorylation-dependent binding partners for ezrin, we performed a yeast two-hybrid screen of a fetal brain library with bait encoding C-terminal ezrin together with active Src. The positive clones of the screen were further analyzed to verify the dependence of the interaction of Src activity by a filter β-galactosidase assay in which the interaction was detected by the blue color of the yeast colony. In this assay, the identified clones were tested against C-terminal ezrin in a vector encoding for Src or a similar vector without Src. This assay distinguished between interactions that occurred independent of Src (e.g. clone A) and others that required the presence of Src (Fig. 8A). Sequencing of the clones, whose interaction was Src-dependent followed by database searches, identified two independent clones encoding for KBTBD2. Both clones contained the sequence of the 3’ end of KBTBD2 cDNA and were missing ~560 nucleotides from the 5’ end of the open reading frame. To further verify the interaction and demonstrate its dependence on Src activity, full-length KBTBD2 cDNA was obtained and fused to GST and the fusion protein was used in an affinity precipitation assay. Lysates containing VSVG-tagged C-terminal ezrin or full-length ezrin from control cells or cells treated with pervanadate were allowed to bind to GST or GST-KBTBD2. The results showed significant binding between ezrin and KBTBD2 only under conditions in which ezrin was phosphorylated by Src (Fig. 8B).
DISCUSSION

The oncogenic kinase Src is involved in multiple signal transduction cascades. Many of these pathways are coupled to cytoskeletal alterations, and various Src substrate proteins are indeed functionally linked to cytoskeleton. Cells transformed by Src display reduced actin bundling and fewer cellular contacts. On one hand, Src is activated by extracellular signals via cell surface receptors such as growth factor receptors or integrins. On the other hand, the activation of Src affects cell adhesion to extracellular matrix and other cells via focal adhesions and adherens junctions. In this study, we examined the connection between Src and the cytoskeleton-cell membrane linker ezrin and showed that Src phosphorylates ezrin at Tyr477. This is a previously unknown form of ezrin regulation and might have important functional consequences, because it connects ezrin to an oncogenic signaling pathway. Furthermore, we identified a novel molecular interaction, which is dependent on the Src-driven ezrin regulation.

Regulation of ezrin by Src is unique among ERM proteins, because Tyr477 is not conserved in other ERM members or merlin. Tyr477 resides in close proximity to a seven-proline stretch, which is also present in radixin sequence. This region is partly included in the crystal structure of the complexed FERM and tail domain of human moesin (32). Tyrosine at position 477 is located between the stretch of prolines and the C-ERMAD, which includes an actin binding site. An isolated radixin/H9251-helical domain can form a monomeric and stable helical rod structure, which is stabilized by extensive salt bridge interactions (33). Based on structural studies on other ERM proteins, it is possible that Tyr477, located in the vicinity of the end of the α-domain rod and the stretch of prolines, could be available for a kinase even when ezrin is in closed confor-
mation via the association of N- to C-ERMAD. Furthermore, the finding that pervanadate treatment of cells expressing wild-type or Y477F mutant ezrin does not affect phosphorylation of the conserved threonine 566 suggests that conformational opening of the molecule and phosphorylation of Tyr\(^{477}\) are independently regulated.

In addition to Src, stimulation by EGF, platelet-derived growth factor, or hepatocyte growth factor results in tyrosine phosphorylation of ezrin (14–16). However, the outcome in regard to target residues is dependent on the type of stimulation and, despite close sequence similarity, various ERM proteins display tight specificity for tyrosine phosphorylation. Both ezrin and radixin, but not moesin, are phosphorylated on tyrosine following platelet-derived growth factor receptor activation (16). After EGF stimulation, ezrin is phosphorylated at Tyr\(^{145}\) and Tyr\(^{353}\), whereas radixin or moesin is not (16, 17), although Tyr\(^{145}\) is conserved in all family members. In agreement with previous studies, we detected tyrosine phosphorylation of ezrin after 5 min of EGF stimulation in A431 cells but found no evidence of Src induction or phosphorylation of ezrin at Tyr\(^{477}\). Interestingly, also Krieg and Hunter (17) reported that the tryptic phosphopeptide map of an ezrin construct phosphorylated in \textit{vitro} by pp60-c-Src did not show similarity with the map derived from EGF-stimulated A431 cells (17). We conclude that EGF and Src stimulation differentially regulate ezrin.

In our study, we did not detect any change in Src-induced phosphorylation of ezrin \textit{in vivo} when Tyr\(^{145}\) or Tyr\(^{353}\) was mutated to a phenylalanine. Both \textit{in vitro} and \textit{in vivo} experiments demonstrated that Tyr\(^{477}\) is the major target residue for Src; however, a low level of tyrosine phosphorylation was present in lysates from cells expressing tagged Y477F mutant ezrin. This weak phosphorylation remained also in cells expressing the Y145F/Y477F double mutant. A plausible explanation for the result is that immunoprecipitation of transfected ezrin mutant coprecipitates phosphorylated endogenous ezrin because of the formation of homodimers or oligomers with transfected protein (12, 34). However, we cannot rule out the possibility of a second target site(s) for Src in \textit{in vivo}, although it seems unlikely that this site would be Tyr\(^{145}\) or Tyr\(^{353}\). Previously, we found that, in T-lymphocytes, the Src family kinase Lck catalyzes the phosphorylation of Tyr\(^{445}\) (20). In this study, we did not see any phosphorylation on Tyr\(^{477}\) in Jurkat cells after pervanadate treatment. The differences between adherent cells and cells growing in suspension may account for the distinct phosphorylation. The signaling pathways involving Src family kinases regulate not only adhesion but also various other cellular responses involving cytoskeletal changes, which are differentially regulated in circulating lymphatic cells and in adherent cells. The results in A431 and Jurkat cells suggest a specific role for ezrin-Tyr\(^{477}\) phosphorylation in cell signaling, because they point out to a cell type- and stimulation-dependent regulation.

Src phosphorylates a variety of cytoskeletal proteins, but the consequences of phosphorylation are variable. The epithelial cell actin-binding protein villin is phosphorylated on several tyrosines by Src, and phosphorylation of villin plays an essential role in cell migration and actin organization (35). Phosphorylation decreases the binding affinity of villin for F-actin (36). It is also reported that tyrosine phosphorylation of villin releases an autoinhibited conformation (37). Another substrate for Src is vinculin (38), an actin-binding protein regulated by phosphatidylinositol 4,5-bisphosphate binding. Tyrosine phosphorylation of vinculin does not affect its actin binding but decreases the intramolecular binding of the vinculin head domain to the vinculin tail (39). In the case of ezrin, it was recently shown that phosphatidylinositol 4,5-bisphosphate binding precedes threonine phosphorylation and is needed for the targeting of ezrin to cell membrane (31). The sequence of events concerning tyrosine phosphorylation, phosphatidylinositol 4,5-bisphosphate binding, and threonine phosphorylation of ezrin and the functions of the different heteromeric and homomeric molecular forms of the ERM proteins have yet to be clarified. However, our results suggest that Tyr\(^{477}\) phosphorylation and opening of the molecule are separate events.

Another interesting cytoskeletal substrate for Src is cortactin (reviewed in Ref. 41). Although tyrosine phosphorylation does not affect F-actin binding of cortactin (42), it promotes cell migration (43). Interestingly, phosphorylation of platelet cortactin by Src induces proteolysis of cortactin by calpain (44). Ezrin is known as a calpain substrate (45, 46), and we have observed an increase in proteolysis of tyrosine-phosphorylated ezrin by calpain \textit{in vitro} (data not shown). Regulation of calpain-mediated proteolysis of ezrin could provide a mechanism to dynamically modulate cytoskeleton-membrane linkage during cell migration and invasion.

We identified a novel phosphospecific interaction with KBTBD2 protein as a functional consequence of Src-induced phosphorylation of ezrin. We used a modified yeast two-hybrid bait system containing active Src to find specific binding partners for tyrosine-phosphorylated ezrin. A similar approach has previously been successfully used to find interactions that depend on the phosphorylation of the bait protein by Src (28, 47). KBTBD2 has been discovered in screens of novel human cDNAs (48–50), and in databases, EST sequences of KBTBD2 are found ubiquitously in different tissues (UniGene, NCBI). However, its functions or molecular interactions have not been reported. KBTBD2 contains an N-terminal BTB/POZ domain and five kelch motifs. BTB/POZ domains (reviewed in Refs. 51 and 52) are present in several zinc-finger transcription factors and kelch-repeat proteins and are conserved in evolution. The BTB/POZ domains mediate homomeric or heteromeric dimerization. Kelch family proteins typically contain multiple (four to seven) Kelch motifs that form a kelch-repeat domain, which folds as a conserved tertiary structure, a β-propeller (reviewed in Refs. 53 and 54). Kelch-repeat superfamily members are widespread in evolution from viruses to mammals (54) and have versatile functions and cellular distribution. Interestingly, many actin-binding and actin-bundling proteins are found in this superfamily (55–63). Moreover, several kelch-repeat proteins are involved in the regulation of cell adhesion or cell morphology (64–66).

Our results open novel avenues for analysis of the biological functions of ezrin in normal and abnormal cells. Several recent studies have demonstrated that ezrin is up-regulated in malignant tumors as is Src (reviewed in Refs. 67–69) and that this up-regulation may play a role in tumor dissemination and metastasis (70–77). Recent generation of an ezrin knock-out mouse model (40) should provide tools to further study the role of Tyr\(^{477}\) in these processes and dissect the signaling pathways in which ezrin and Src cooperate. In addition, a detailed characteristic of KBTBD2 and the cellular complexes in which it is found is needed to understand how ezrin, Src, and KBTBD2 function in regulation of actin cytoskeleton, adhesion, or cellular signaling.

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