Src Phosphorylation of Alix/AIP1 Modulates Its Interaction with Binding Partners and Antagonizes Its Activities*[S]

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Alix/AIP1 is an adaptor protein involved in regulating the function of receptor and cytoskeleton-associated tyrosine kinases. Here, we investigated its interaction with and regulation by Src. Tyr[319] of Alix bound the isolated Src homology-2 (SH2) domain and was necessary for interaction with intact Src. A proline-rich region in the C terminus of Alix bound the Src SH3 domain, but this interaction was dependent on the release of the Src SH2 domain from its Src internal ligand either by interaction with Alix Tyr[319] or by mutation of Src Tyr[527]. Src phosphorylated Alix at a C-terminal region rich in tyrosines, an activity that was stimulated by the presence of the Alix binding partner SETA/CIN85. Phosphorylation of Alix by Src caused it to translocate from the membrane and cytoskeleton to the cytoplasm and reduced its interaction with binding partners SETA/CIN85, epidermal growth factor receptor, and Pyk2. As a consequence of this, Src antagonized the negative regulation of receptor tyrosine kinase internalization and cell adhesion by Alix. We propose a model whereby Src antagonizes the effects of Alix by phosphorylation of its C terminus, leading to the disruption of interactions with target proteins.

Alix/AIP1 (referred to here as Alix to avoid confusion with other proteins termed AIP1) is an adaptor protein that has been identified as a binding partner of various proteins and accordingly implicated in diverse cellular processes. Initially, it was isolated as a binding partner of ALG-2 (apoptosis-linked gene-2) by two groups and named ALG-2-interacting protein X/ALG-2-interacting protein-1 (1, 2). ALG-2 is a small calcium-binding protein whose expression is required for apoptosis in lymphocytes in response to a variety of stimuli (3), and so Alix was initially implicated in the regulation of apoptosis (2). Shortly thereafter, Alix was isolated as a binding partner of SETA (SH3 domain encoding, expressed in tumorigenic astrocytes)/CIN85 (Cbl-interacting protein of 85 kDa)/Ruk (4), itself

an adaptor protein (5). More recently, Alix has also been shown to be involved in the endosomal sorting processes (6–8) and human immunodeficiency virus budding (9).

The interaction of Alix with SETA/CIN85/Ruk, which is involved in the negative regulation of receptor tyrosine kinases as part of a complex with Cbl ubiquitin ligases and endophilins (10, 11) as well as in the negative regulation of phosphatidylinositol 3-kinase (12), recently led us to explore Alix function in relationship to kinases. We observed that Alix and SETA are found in focal adhesions of astrocytes and that Alix can bind the tyrosine kinases Pyk2 (phosphotyrosine kinase-2) and focal adhesion kinase in adherent cells. These interactions negatively regulate cell adhesion and focal adhesion kinase activity (13). Since Alix can also interact with endophilins (14), we investigated the direct impact of Alix on receptor tyrosine kinases. The results show that Alix binds the epidermal growth factor receptor (EGFR)[1] constitutively and independently of SETA/CIN85 and, despite its ability to recruit endophilins, negatively regulates receptor internalization mediated by the Cbl-SETA/CIN85 complex by reducing phosphorylation levels of Cbl ligases and ubiquitination of its target proteins (15).

In this study, the focus is on the regulation of Alix function by the Src kinase. It was shown previously that the *Xenopus* ortholog of Alix, Xp95, can be phosphorylated by Src and Fyn (16), making a good case for a potential interaction of mammalian Alix with these kinases. Furthermore, as the Src family enzymes are associated with receptor tyrosine kinases and focal adhesion kinases, among many other signaling entities (17, 18), it is possible that they encounter Alix in situations where it is exerting its function. Here, we show that Alix interacts with both the Src SH2 and SH3 domains and mapped the relevant regions in Alix. Our data support a model in which the interaction with the SH2 domain is required for binding of Src to Alix, which can then be stabilized by binding of the Src SH3 domain to the proline-rich region of Alix. Alix was phosphorylated at Tyr[319] in the presence of normal levels of Src, and hyperphosphorylated by elevated levels of Src at its tyrosine-rich C terminus. This hyperphosphorylation resulted in a cellular redistribution of the protein away from the cytoskeleton and membrane. Furthermore, Alix hyperphosphorylation reduced the association of Alix with SETA/CIN85, receptor tyrosine kinases, and Pyk2 and antagonized the negative regulation of receptor tyrosine kinase internalization and cell

[1] The abbreviations used are: EGFR, epidermal growth factor receptor; SH, Src homology; GFP, green fluorescent protein; HEK293, human embryonic kidney 293; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; siRNA, small interfering RNA; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; Src-ca, constitutively active Src; Src-ki, kinase-inactive Src; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine.

adhesion by Alix. We propose a model whereby Src modulates Alix functions by phosphorylation of its C terminus, leading to the disruption of interactions with target proteins.

EXPERIMENTAL PROCEDURES

Constructs and Antibodies—Transfection experiments were performed using the following gene expression plasmid constructs. Full-length SETA (123cc) and LacZ were cloned into pcDNA6 (Invitrogen) as described previously (4, 5). C-terminally FLAG-tagged Alix (provided by Dr. Luciano D'Adamio) (2) and Alix mutants were cloned into pcDNA3. Alix mutants were made with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Various Src family kinase constructs were obtained from Drs. Mike Cox, Bruce Mayer, Tony Pawson, Ton Schumacher, Pamela L. Schwartzberg, and Marius Sudol. SETA refers to the rat cDNA and derived constructs, whereas CIN85 designates the human cDNA and derived constructs. CIN85 constructs were as described previously (10, 19). In all transfection experiments, the total amount of DNA was kept constant for each transfection using green fluorescent protein (GFP)- or LacZ-encoding control plasmid in place of SETA/CIN85-, Alix-, or Src-encoding plasmids where appropriate.

For detection of proteins in Western blotting and immunoprecipitations, we used the following antibodies. Rabbit anti-Src (SRC 2) and goat anti-EGFR (1005) polyclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-FLAG monoclonal antibody M2 and anti-phosphoserine antibody (PSR-45) were obtained from Sigma. Anti-V5 monoclonal antibody was from Invitrogen. Mouse anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate, Inc. Anti-Alix (49) and anti-Pyk2 (11) monoclonal antibodies were from BD Biosciences. Anti-SETA (4) and anti-CIN85 (20) polyclonal antibodies were made and used as described previously.

Cell Lines and Cell Transfection—Primary rat cortical astrocytes were isolated and used as described (4, 21) and cultured in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal calf serum. Human embryonic kidney 293 (HEK293) cells, HeLa cells, or mouse embryonic SYF (<u>S</u>rc/<u>Y</u>es/<u>F</u>yn triple knockout) fibroblasts (22) were cultured under standard conditions in the same medium. Chinese hamster ovary cells stably expressing the EGFR were cultured in F12K medium supplemented as described above. Cells were transfected with plasmids by a modified calcium phosphate procedure. The day prior to transfection, 2×10^6 cells were plated in 10-cm tissue culture dishes, transfected the next day, and harvested after 48 h. Alternatively, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's guidelines.

Immunoprecipitation and Glutathione S-Transferase (GST) Pull-down Assays—Cells were washed two times with ice-cold phosphate-buffered saline (PBS) and lysed on ice for 30 min in modified radioimmune precipitation assay buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Igepal CA-630 (Sigma), 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 4 mM sodium azide, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, a protease inhibitor mixture (2 μg/ml each aprotinin and leupeptin, 10 μg/ml each E-64 and trypsin inhibitor, and 1 μg/ml pepstatin A), and a phosphatase inhibitor mixture (2 mM each sodium vanadate and sodium fluoride, 5 mM sodium molybdate, and 15 mM *p*-nitrophenyl phosphate)). Following lysis, the cell suspension was sheared 10 times through an 18-gauge 1.5-inch needle and 10 times through an IM 1-inch needle and incubated on ice for another 30 min. The cell solution was then cleared by centrifugation at 20,000 $\times$ *g* at 4 °C. This supernatant was used for immunoprecipitation studies or GST pull-down assays. For immunoprecipitations, appropriate concentrations of primary antibody were added, and the solution was rotated at 4 °C for at least 1 h. Antibody-protein complexes were precipitated overnight with 50 μl of protein A-agarose solution (Roche Applied Science) by rotation at 4 °C. Agarose beads were collected by centrifugation at 12,000 $\times$ *g* for 5 min at 4 °C and washed seven times with radioimmunoprecipitation assay buffer on ice.

For GST pull-down assays, GST-tagged proteins were freshly isolated from *Escherichia coli* BL21 cells according to standard procedures (23). The amount of isolated GST fusion protein was determined by SDS-PAGE and Coomassie Brilliant Blue staining. In each pull-down experiment, equal amounts of GST-fused protein were added to the cell lysate, which had been precleared by GST coupled to glutathione-Sepharose 4B beads (Amersham Biosciences) for 30 min. The suspension was then rotated at 4 °C for 2 h, and the beads were collected by centrifugation at 12,000 $\times$ *g*. Subsequently, the GST-coupled beads were vigorously washed five times with radioimmune precipitation assay buffer.

Sediments achieved by immunoprecipitation or GST pull-down were boiled for 5 min at 95 °C in 2$\times$ NuPAGE® lithium dodecyl sulfate sample buffer (Invitrogen) containing 20% β-mercaptoethanol and transferred to ice immediately. The solution was cleared of insoluble particles by centrifugation and stored at -80 °C until further analysis by protein electrophoresis.

Western Blotting—Protein samples were analyzed by SDS-PAGE using an XCell SureLock mini-cell (Invitrogen) in combination with precast NuPAGE 4–12% or 10% BisTris gels (1 mm) at 200 V according to the manufacturer's guidelines. Following electrophoresis, proteins were blotted onto a polyvinylidene difluoride membrane, stained reversibly with Ponceau S in the case of GST-pull down experiments, and photographed and then incubated for at least 1 h in blocking buffer (5% bovine serum albumin and 1% Tween 20 in Tris-buffered saline). Membranes were incubated overnight with the appropriate dilutions of primary antibody in blocking buffer. The next day, membranes were washed and incubated for 1 h with alkaline phosphatase-conjugated secondary antibody solution (1:3000 anti-mouse antibody, 1:5000 anti-rabbit antibody, and 1:15,000 anti-goat antibody; Sigma) in blocking buffer. After additional washing steps, antibody complexes were visualized on film using Immun-Star-AP substrate (Bio-Rad).

Small Interfering RNA (siRNA) Silencing—Oligonucleotide-based (5′-GAT CCC GCC GCT GGT GAA GTT CAT CTT CAA GAG AGA TGA ACT TCA CCA GCG GCT TTT TGG AAA-3′ (sense) and 5′-AGC TTT TCC AAA AAG CCG CTG GTG AAG TTC ATC TCT CTT GAA GAT GAA CTT CAC CAG CGG CGG-3′ (antisense); Qiagen Inc.) silencing of Alix was achieved as described (24) and previously optimized (15). In brief, 1 μl of a 20 μM stock solution of an Alix siRNA duplex was transfected into cells, in each well of a 24-well plate using Lipofectamine 2000 to achieve ~90% knockdown efficiency.

Platelet-derived Growth Factor (PDGF) Receptor Down-regulation—HeLa cells were transfected with Alix and GFP or Src or were alternatively treated with Alix siRNA. After 48 h, cells were serum-deprived for 30 min at 4 °C in F12K medium plus 0.1% bovine serum albumin and 10 mM HEPES. The cells were then incubated in medium containing 50 ng/ml PDGF at 37 °C for the indicated times and rapidly moved back to wet ice. The cells were acid-washed with PBS plus 0.1% bovine serum albumin (pH 3.4) to remove surface-bound PDGF and incubated for 1.5 h with 1 ng/ml ^{125}I-PDGF at 4 °C to quantify the membrane PDGF receptor (PDGFR). Cells were washed five times with medium, and surface-bound ^{125}I-PDGF was quantified after cell lysis using a WIZARD 1470 γ-counter (PerkinElmer Life Sciences). Values were evaluated as percent of PDGFR on the surface of non-stimulated cells.

Cell Attachment Assays—Cell attachment assays were performed as described previously (25, 26). In brief, HEK293 cells were transfected and harvested after 48 h. Cells (3×10^5) were then seeded on extracellular matrix-coated or uncoated 6-well plates and incubated at 37 °C for 30 min or 1 h, respectively. Extracellular matrix-coated dishes were prepared by coating suspension dishes (Greiner Bio-One) with 10 μg/ml fibronectin or collagen (Upstate, Inc.) or with 100 μg/ml poly-L-lysine (Sigma) in PBS overnight. Before use, plates were washed with PBS, blocked with 1% bovine serum albumin in PBS for 2 h, and washed again with PBS.

Cell Fractionation—SYF cells were transfected with Alix and GFP or Src and lysed after 48 h in detergent-free hypotonic buffer (50 mM HEPES (pH 7.5) and 1 mM MgCl$_2$). NaCl (300 mM) and a protease/phosphatase inhibitor mixture (5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 2 μg/ml each aprotinin and leupeptin, 10 μg/ml each E-64 and trypsin inhibitor, 1 μg/ml pepstatin A, 2 mM each sodium vanadate and sodium fluoride, 5 mM sodium molybdate, and 15 mM *p*-nitrophenyl phosphate) were added, and lysates were centrifuged at 100,000 $\times$ *g* for 1 h to separate the S100 soluble fraction containing cytosolic proteins from the P100 pellets. P100 sediments were incubated with buffer containing 1% Triton X-100 to extract membrane-embedded proteins. After a second centrifugation step at 100,000 $\times$ *g*, the sediments were incubated with buffer containing additional 1% SDS to extract cytoskeletal proteins. The remaining insoluble material was removed by high speed centrifugation. Aliquots of each fraction were analyzed by immunoblotting.

RESULTS

The *Xenopus* ortholog of Alix, Xp95, is phosphorylated by Src at a consensus tyrosine phosphorylation site: 312KkdnD-fi.Y319 (uppercase letters designate invariant residues in the consensus, and the period identifies the tyrosine residue that is phosphorylated; PROSITE Database) (16, 27). This sequence is conserved between Xp95 and all p95 orthologs in mammals

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(Alix/AIP1 and Hp95), suggesting a potential evolutionarily conserved function (Supplemental Fig. S1) (28, 29). We were therefore interested in investigating the ability of Src to interact with and phosphorylate Alix and the impact of this on previously reported activities of Alix, including the modulation of cell adhesion (13) and the regulation of receptor tyrosine kinase internalization by Cbl-SETA/CIN85 complexes (15).

To map the domains involved in the interaction of Src with Alix, confrontation experiments with isolated regions of the Src protein and Alix mutants were performed (see the schematic of the Alix protein sequence in Fig. 1*A*). In these experiments, bacterially generated GST-Src domain fusion proteins were used to recover Alix proteins from the lysates of transfected HEK293 cells. We first determined whether the isolated Src SH2 domain was able to bind to Alix (Fig. 1*B*). The GST-Src SH2 domain recovered similar amounts of all Alix proteins tested, with the exception of the Alix-Y319F point mutant, which lacks the canonical tyrosine kinase target sequence (Fig. 1*A*; see Supplemental Fig. S1 for corresponding sequences). This suggests that the major binding site for the Src SH2 domain is at Tyr^{319} and not in the tyrosine-rich region of the C terminus, which is deleted in Alix-784Stop.

In addition to the consensus tyrosine phosphorylation site at Tyr^{319}, Alix also has a proline-rich C terminus, known to interact with SH3 domain-containing proteins such as SETA/CIN85 (4, 19) and endophilins (14), and so could interact with the Src SH3 domain. The isolated GST-Src SH3 domain was able to recover Alix protein from lysates of transfected cells with similar efficiency compared with SETA/CIN85 SH3 domains A and B (Fig. 1*C*), which mediate the binding of SETA/CIN85 to Alix. To determine which region of Alix the Src SH3 domain bound, pull-down assays were performed with the GST-Src SH3 domain and Alix mutant or deletion forms. The Src SH3 domain interacted with comparable efficiency with all Alix proteins, except Alix-(Δ717–784) (Fig. 1, *A* and *D*), which has a deletion of the N-terminal half of the proline-rich region of Alix (for details, see Supplemental Fig. S1). This mutant lacks both the endophilin-binding site, which is PPAKPQP-PARPPPP^{761} (14) and the SETA/CIN85-binding motif, which is PTPAPR^{745} (19). As expected from the preference of the Src SH3 domain for canonical P*XX*P-based motifs (30–32), the Alix-R745G point mutation of the conserved arginine in the binding motif of SETA/CIN85, which prefers a divergent consensus (19), did not appear to compromise interaction with the Src SH3 domain (Fig. 1*D*). This also provides evidence that interaction with SETA/CIN85 is not required for Alix binding to Src. There is one evident Src SH3 domain-binding consensus sequence (30, 33) in Alix between positions 717 and 784: the type II peptide sequence ^{752}PQPPAR^{757}. Therefore, we analyzed the interaction of an additional point mutant, Alix-P749A/P752A/P755A, with the Src SH3 domain (Fig. 1*E*). This analysis revealed that mutation of this motif resulted in a >5-fold reduction in recovery of Alix by the Src SH3 domain compared with the control point mutant Alix-P833A/P836A, supporting the notion that this sequence is an important mediator of binding to the Src SH3 domain.

The importance of the interactions between isolated Src domains and Alix depends on demonstrating that these interactions occur between endogenous proteins and that Alix is tyrosine-phosphorylated. Immunoprecipitation of Alix from untransfected HeLa cells led to the recovery of Src protein and vice versa (Fig. 2*A*), supporting the conclusion that the endogenous proteins bind. In both instances, Src and Alix were also detected as being phosphorylated at tyrosine. Interestingly, the level of phosphorylation of the Alix protein in the Src immunoprecipitates appeared to be higher than that recovered directly

with anti-Alix antibodies, suggesting that Src-associated Alix is more highly phosphorylated and that Src is responsible. Therefore, the interaction between these proteins is potentially physiologically relevant. The Alix/Src interaction has been assigned BIND Id 183522 in the Biomolecular Interaction Network Database (available at www.bind.ca).

The experiments with the isolated Src domains (Fig. 1) may not accurately reflect binding of the full-length Src protein, in which they are not necessarily accessible, depending on the overall activation state of the kinase (34, 35). Therefore, we studied the interaction of full-length and mutant proteins in transfected cells. Immunoprecipitation of Src allowed recovery of wild-type Alix as well as Alix-784Stop and Alix-(Δ717–784), but not Alix-Y319F (Fig. 2*B*), suggesting that the interaction of the Src SH2 domain with Alix-Y319F is necessary for binding in this assay, but that the proline-rich region of Alix that bound the Src SH3 domain (Fig. 1, *D* and *E*) and that is missing in Alix-(Δ717–784) is not. Furthermore, the recovery of the Alix-(Δ717–784) mutant suggests that the Alix-Y319F/Src SH2 domain interaction may be sufficient for binding to occur.

Interaction between the Src SH2 domain and the endogenous phosphorylated Tyr^{527} of Src stabilizes the inactive conformation of the kinase, and the unlatching of this residue is thought to be an important event in its adopting an active conformation (34, 35). To test whether the inability of the Alix-Y319F mutant to compete for this internal SH2 ligand underlies its lack of interaction with Src, we next confronted Alix with a constitutively active form of Src (Src-ca) with the Y527F mutation (36, 37). These experiments were performed in SYF cells ($Src^{-/-}$/ $Yes^{-/-}$/$Fyn^{-/-}$ mouse embryo fibroblasts) to prevent any interference from endogenous Src family kinases. Alix was recovered in association with both Src and Src-ca as expected (Fig. 2*C*, *lanes 1* and *2*). As before, Alix-Y319F was not found to associate with wild-type Src; however, it was recovered in immunoprecipitates of Src-ca, albeit less efficiently than wild-type Alix (*lanes 3* and *4*). Therefore, when the Src SH2 domain is unbound and the kinase is in an open configuration, the requirement for interaction with Alix Tyr^{319} is removed.

As an independent test of whether the activation state of Src plays a role in the Alix/Src interaction, Src was treated with the Src inhibitor PP2, or kinase-inactive Src (Src-ki) was cotransfected with Alix proteins in HEK293 cells (Fig. 2*D*). As before, Src immunoprecipitates showed similar levels of Src interaction with Alix, Alix-(Δ717–784), and Alix-784Stop (Fig. 2*D*, *lanes 1–3*). Treatment of cells with 20 μM PP2 reduced both the level of tyrosine phosphorylation of Src and the amount of Alix recovered in Src immunoprecipitates (*lanes 4–6*). Consistent with this, no interaction between Src-ki and any of the Alix proteins was detected. Cotransfection of Src-ki reduced Alix levels in these cells, and so the lack of interaction was confirmed upon longer exposures (data not shown). Together, these data suggest that, in the interaction between Alix and Src, the binding of Alix Tyr^{319} to the SH2 domain necessarily precedes the binding of the Src SH3 domain to the proline-rich region of Alix and that, although each interaction alone can mediate binding (SH2 alone, Fig. 2*B*, *lane 2*; and SH3 alone, Fig. 2*C*, *lane 4*), binding is stronger when both are in place and also when Src is active.

Next, we investigated the Src-stimulated phosphorylation of Alix and Alix mutants. Under normal growth conditions and in the absence of transfected Src, tyrosine phosphorylation could be detected when Alix was overexpressed (Fig. 3*A*, *lane 1*). Both Alix-(Δ717–784) (*lane 2*) and Alix-784Stop (*lane 3*) showed lower levels of tyrosine phosphorylation than full-length Alix, but these were still readily detectable. However, Alix-Y319F (*lane 4*) did not react with the anti-phosphotyrosine antibody,
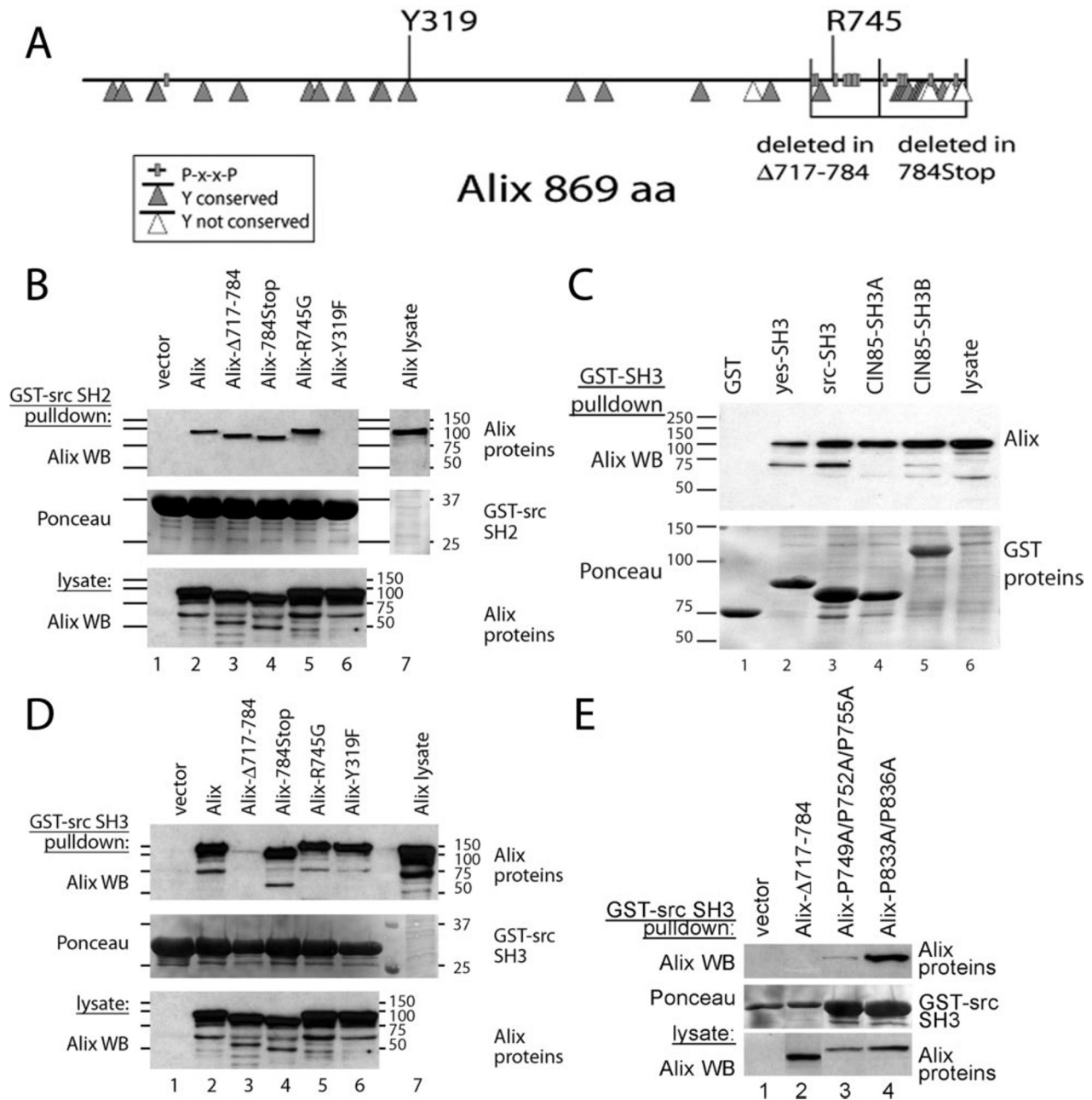
FIG. 1. **Src SH3 and SH2 domains bind Alix.** *A*, the schematic illustrates the distribution of P*XX*P motifs, the classical cognates of SH3 domains, and conserved and non-conserved tyrosines. Please note that all tyrosine residues are conserved between the two mammalian proteins and that non-conserved tyrosines are those that are not found in Xp95. The position of Tyr[319] and the regions deleted in two mutants used in this study are indicated. For additional details, please see Supplemental Fig. S1. *aa*, amino acids. *B*, the GST-Src SH2 domain was confronted with lysates from HEK293 cells transfected with Alix or Alix mutants as indicated, and the recovered proteins were analyzed by anti-Alix Western blotting (*WB*). In this experiment, all forms of Alix with the exception of the Alix-Y319F mutant were recovered by binding to the Src SH2 domain. Alix lysate is included as a positive control. (The section between *lanes 6* and *7* was removed, as it contained pen markings on the marker lane.) Ponceau S staining of the membrane prior to blotting confirmed equal recovery of the GST-Src SH2 domain, and Western blotting of the lysate confirmed similar levels of expression of all Alix proteins. Data are representative of two experiments. *C*, GST fusion proteins of isolated SH3 domains from Yes and Src and SETA/CIN85 SH3 domains A and B were confronted with lysate from HEK293 cells transfected with Alix. The *upper panel* shows the Western blot analysis of the recovered material with an antibody against Alix, whereas the *lower panel* shows Ponceau S staining that reveals the presence of the bacterial GST proteins. The negative control (*lane 1*) did not recover any Alix protein, the mobility of which is indicated by the positive control of the cell lysate (*lane 6*). The Src and human CIN85 SH3 domains recovered Alix, whereas the Yes SH3 domain did so less efficiently. Data are representative of three experiments. *D*, the GST-Src SH3 domain was confronted with the same lysates from HEK293 cells transfected with Alix or Alix mutants as described for *B* as indicated. The *upper panel* shows the Alix protein recovered after GST-mediated pull-down and demonstrates that all Alix proteins except Alix-(Δ717–784) (*lane 3*) were recovered with high efficiency. Alix-containing lysate is also included to provide a positive control for Western blotting (*lane 7*). The *middle panel* is a Ponceau S stain confirming the equal presence of the GST-Src SH3 domain in each lane. The *lower panel* demonstrates that similar amounts of Alix proteins were present in each lysate. Data are representative of two experiments. *E*, pull-down of Alix-P749A/P752A/P755A and Alix-P833A/P836A with the GST-Src SH3 domain showed that mutation of the prolines in the Src-binding consensus sequence caused a reduction in the recovery of Alix, whereas mutation of proline residues outside of this region showed no effect. Upon densitometry and normalization to levels in lysates, the intensity of the Alix-P749A/P752A/P755A band was <20% of that of the Alix-P833A/P836A band. Alix-(Δ717–784) served as a negative control. Data are representative of two experiments.

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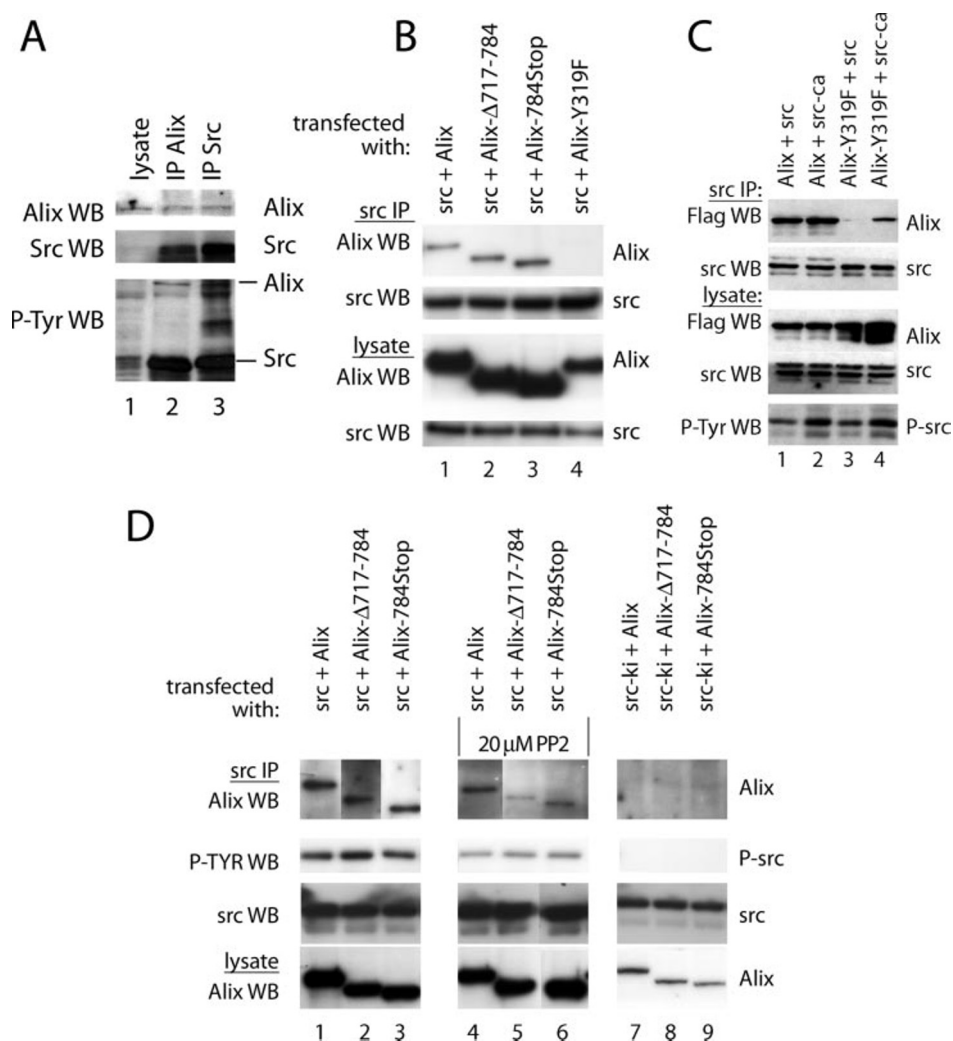
Fig. 2. **The Alix/Src interaction is dependent on the SH2 domain interaction and on Src activity.** *A*, lysates from untransfected HeLa cells were either Western-blotted directly (*lane 1*) or subjected to immunoprecipitation (*IP*) with anti-Alix antibodies (*lane 2*) or anti-Src antibodies (*lane 3*) and then analyzed by Western blotting (*WB*) with anti-Alix, anti-Src, and anti-phosphotyrosine antibodies as indicated. Please note that the anti-Alix antibodies did not work as efficiently as the anti-Src antibodies in immunoprecipitation or Western blotting. *B*, HEK293 cells were transfected with Src and different forms of Alix as indicated, and their lysates were subjected to Src immunoprecipitation, followed by Western blot analysis with antibodies directed against Alix or Src. Alix, Alix-(Δ717–784), and Alix-784Stop bound to Src efficiently, but Alix-Y319F did not. Data are representative of three experiments. *C*, Alix or Alix-Y319F was cotransfected into SYF cells, and Src was immunoprecipitated. As shown in *A*, Alix (but not Alix-Y319F) was able to associate with Src. However, Alix-Y319F was able to bind to Src-ca, which has a nonfunctional internal SH2 ligand due to a point mutation at Tyr^{527}. Less Alix-Y319F than Alix was recovered in association with Src-ca, suggesting that the loss of the SH2 domain interaction between Alix and Src reduces the affinity of these proteins for each other. Data are representative of two experiments. *D*, HEK293 cells were transfected with Src or Src-ki and Alix proteins as indicated. Some cells were also treated with the Src inhibitor PP2 at 20 μM. Following Src immunoprecipitation, Alix was detected by Western blotting, and the presence and phosphorylation levels of Src were also measured. The expression of Alix proteins in the lysates was also demonstrated. Attenuation of Src activity with an inhibitor reduced the recovery of Alix proteins, and Src-ki was not able to bind Alix proteins at all. All lanes shown in a row came from the same film, but were rearranged after photography for clearer presentation, in some instances. Longer exposures failed to show any Alix in the *upper panel* of *lanes 7–9*. Data are representative of two experiments.

suggesting that this residue represents the most important point of phosphorylation under these conditions and that lack of interaction with Src (Fig. 2) prevents it from attaining a detectable level of phosphorylation. The phosphotyrosine level in Alix was increased by cotransfection of wild-type Src, but not Src-ki, compared with transfection with vector alone (Fig. 3*B*). No differences in the low levels of phosphoserine were observed in this experiment. Therefore, the cotransfection of Src and Alix leads to an elevated level of Alix phosphorylation that will be referred to here as hyperphosphorylation to distinguish it from the basal level of phosphorylation seen at Tyr^{319} (Figs. 2*A* and 3*A*). That other members of the Src family are also capable of phosphorylating Alix is demonstrated by the ability of the Src relative Fyn to do so (Supplemental Fig. S2).

There are a total of 28 conserved tyrosine residues through-out Alix, and nearly one-third of these are found between positions 804 and 847 (Supplemental Fig. S1). Therefore, the two mutants with deletions in the Alix C terminus were further examined for their ability to be hyperphosphorylated in response to elevated Src (Fig. 3*C*). Deletion of the region containing many tyrosines (Alix-784Stop) resulted in a marked reduction in the tyrosine phosphorylation level of Alix, whereas deletion of the adjacent proline-rich region that has only one conserved tyrosine residue (Alix-(Δ717–784)) did not have a major impact. It is important to note that proteins were recovered using an anti-phosphotyrosine immunoprecipitation in this experiment, and so Alix-784Stop was recovered by virtue of its interaction with Src (Fig. 2*B*), the major tyrosine-phosphorylated protein in these Src-transfected cells (Fig. 3*C*), or other tyrosine-phosphorylated proteins. These data are consistent
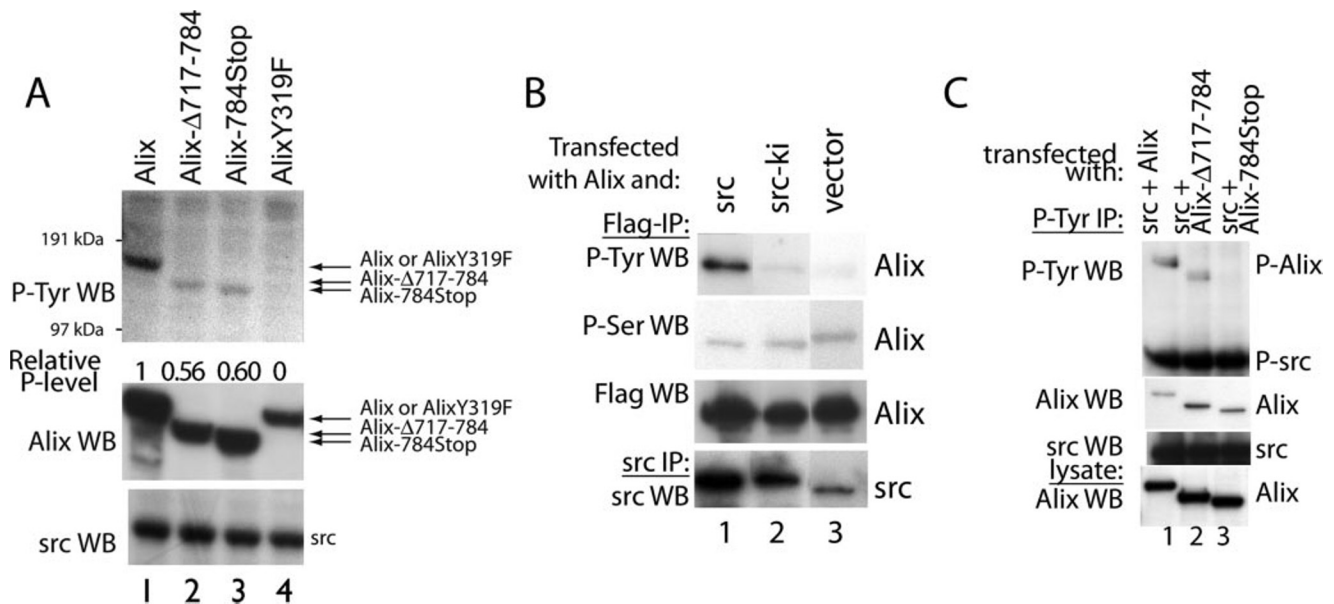
Fig. 3. **Alix is phosphorylated at its C terminus by Src.** *A*, lysates of HEK293 cells were transfected with Alix proteins and analyzed by Western blotting (*WB*) with antibodies to phosphotyrosine, Alix, and Src. Alix, AlixΔ717–784, and Alix-784Stop were detected as faint bands in the anti-phosphotyrosine blots (*lanes 1–3*), but Alix-Y319F was not (*lane 4*). These data suggest that Tyr[319] is an important site for basal levels of Alix phosphorylation. Data are representative of three experiments. *B*, Alix and Src or Src-ki were cotransfected into HEK293 cells, and Alix was immunoprecipitated (*IP*) via its FLAG epitope tag. Western blotting with anti-phosphotyrosine and anti-phosphoserine antibodies was performed. Anti-Src immunoprecipitation and Western blotting confirmed the expression of transfected and lower levels of endogenous Src in these experiments. Alix was phosphorylated by Src, but not by Src-ki. Data are representative of two experiments. *C*, Alix or the two deletion mutants Alix-(Δ717–784) and Alix-784Stop were cotransfected with Src into HEK293 cells, and the lysates were subjected to immunoprecipitation with anti-tyrosine antibody. The resulting proteins were then analyzed by Western blotting with anti-phosphotyrosine antibody. The major tyrosine-phosphorylated protein recovered was Src, and Alix proteins were also retrieved by this approach. In the case of Alix and Alix-(Δ717–784), the recovered protein was tyrosine-phosphorylated. In contrast, the recovered Alix-784Stop did not show any signs of phosphorylation. Data are representative of two experiments.

with those in Fig. 2*B* showing interaction between Src and Alix-784Stop and provide evidence that hyperphosphorylation of the C terminus of Alix is the major consequence of its association with Src, but that this is not required for binding of Alix to Src.

Src is a non-receptor tyrosine kinase that is localized in the cytoplasm. In contrast, Alix is associated with the cytoskeleton (13), receptor tyrosine kinases (15), and the ESCRT complex of the endosome pathway (6–8). To test whether the association between Alix and Src alters the cellular distribution of Alix, SYF cells were transfected with Src, Src-ki, and Alix and fractionated to generate soluble cytoplasmic, detergent-soluble membrane, and pelleted cytoskeletal portions. These were then subjected to Western blotting to detect Alix (Fig. 4). In the absence of Src, Alix existed predominantly in the pelleted fraction, representing the cytoskeleton and associated proteins, although ~12% was found associated with membranes or in the cytoplasm. In the presence of Src, the majority of Alix moved to the soluble cytoplasmic fraction at the expense of both membrane-associated and cytoskeletal pools. Transfection of Src-ki produced a much smaller shift of the cytoskeleton-associated Alix to the cytoplasm, although it was able to move membrane-associated Alix (Fig. 4). Therefore, association with Src causes Alix to relocate in the cell, suggesting that this might induce a loss of its normal associations.

Next, we studied the impact of Src-mediated hyperphosphorylation of Alix on its association with various binding partners. The first protein interaction we examined was between Alix and SETA/CIN85 (Fig. 5). In immunoprecipitates of Alix via its FLAG epitope, the amount of SETA/CIN85 recovered was dramatically reduced when Src was cotransfected (Fig. 5*A*, compare *lanes 4* and *6*). Although less Alix was recovered in *lane 6* than in *lane 4*, the proportional decrease in SETA/CIN85 present in the complex was much greater: we essentially could

not detect SETA/CIN85 in the FLAG immunoprecipitate when Src was also present (*lane 6*), whereas it was readily detectable in the absence of Src (*lane 4*). Similarly, when SETA/CIN85 was immunoprecipitated, the amount of Alix recovered was reduced by the presence of Src, and this could be seen in the case of both endogenous and transfected SETA/CIN85. In the case of Alix recovered by endogenous SETA/CIN85 (compare *lanes 1* and *5*), an almost 2-fold reduction in recovered Alix was detected by densitometry when normalized to the level of SETA/CIN85 recovered. (Endogenous SETA/CIN85 can be seen in the darker exposure of the anti-SETA/CIN85 Western blot.) When additional SETA/CIN85 was cotransfected (compare *lanes 4* and *6*), a proportionally more modest attenuation (of ~25%) in the association with Alix was detected by densitometry after normalization to the level of SETA/CIN85 recovered. In these experiments, a consistent reduction in the amount of SETA/CIN85 in lysates of cells that were transfected with both Alix and Src was observed (*lane 6*). To test whether inhibition of the proteasomal and lysosomal degradation could counteract this effect, cells were transfected with Alix or Alix-784Stop, which was not effectively phosphorylated by Src (Fig. 3*C*), but can bind CIN85 (15). Only when wild-type Alix and Src were present was a reduction in the level of CIN85 observed (Fig. 5*B*, *lane 5*), and this was effectively counteracted by inhibitors of protein degradation (*lane 11*). This demonstrates that the reduction in CIN85 seen in Fig. 5*A* is a consequence of Alix and Src activity and not an experimental artifact.

In addition, SETA/CIN85 was capable of modulating the level of Alix hyperphosphorylation by Src. Various SETA/CIN85 constructs were cotransfected with Alix and either Src or Src-ki. Alix was then recovered by FLAG immunoprecipitation, and the level of phosphorylation was assessed by anti-phosphotyrosine Western blotting (Fig. 5*C*). The forms of SETA/CIN85 used in this experiment encode the isolated second and third SH3 domains
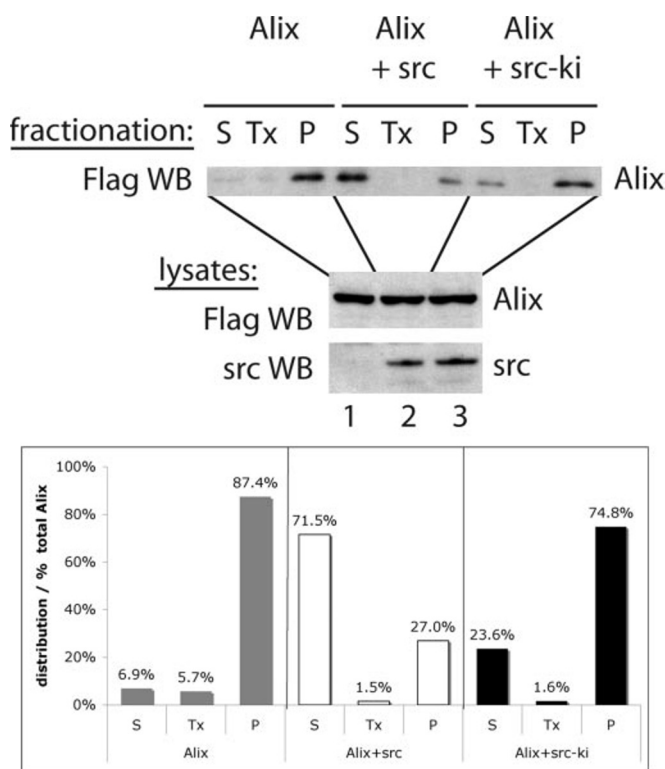
FIG. 4. **Src causes Alix to relocate to the cytoplasm.** Alix and Src or Src-ki were transfected into SYF cells, and the lysates were fractionated as described under "Experimental Procedures" into a soluble (*S*) fraction representing the cytoplasm, a Triton X-100-soluble (*Tx*) fraction representing membrane-embedded proteins, and an SDS-soluble pellet (*P*) representing cytoskeleton-associated proteins. These were analyzed by anti-Alix Western blotting (*WB*; *upper panel*), and the data were quantified by densitometry and are presented graphically (*lower panel*). Cotransfection of Src resulted in a significant relocation of Alix to the cytoplasmic fraction. Src-ki had a much less profound effect. Data are representative of two experiments.

(SH3-2 and SH3-3, of which SH3-2 primarily interacts with Alix) (4); both the second and third SH3 domains and the intervening sequence (SH3-23); from the second SH3 domain to the C terminus, including the coiled-coil domain (23cc); and the full-length SETA/CIN85 molecule, including all three SH3 domains and the coiled-coil domain (123cc). In each case, the presence of Src elevated the level of phosphotyrosine found in Alix (compare *even-* and *odd-numbered lanes*). However, the degree of hyperphosphorylation was stimulated by the presence of 123cc (*lane 12*) and, to a lesser extent, by 23cc (*lane 10*) and SH3-23 (*lane 8*). The presence of isolated SH3 domains of SETA/CIN85 did not have a profound effect on the hyperphosphorylation of Alix by Src, suggesting that other sequences in SETA/CIN85 or structural aspects of the SETA/Alix interaction that are obtained only with larger SETA/CIN85 molecules play a role in this effect. Based on the observation that SETA/CIN85 bound to region 717–784 of Alix, which is adjacent to a major site of Src hyperphosphorylation between positions 784 and 869, it is possible that it stabilizes the Alix/Src interaction, although this is not likely to involve direct stable SETA/Src binding, as no evidence for such an interaction was found in immunoprecipitations (data not shown).

Alix also interacts with the EGFR complex, which it stabilizes against Cbl-mediated degradation (15). Cotransfection of Src dramatically reduced the amount of Alix or Alix-(Δ717–784) recovered in an EGFR immunoprecipitate, but did not affect the recovery of Alix-784Stop (Fig. 6A). Therefore, the impact of Src on the Alix/EGFR interaction mirrored its ability to hyperphosphorylate Alix proteins (Fig. 3C), suggesting that

high levels of phosphorylation of the C terminus of Alix interfere with its interaction with the EGFR signaling complex. A testable hypothesis arising from these results is that EGFR-associated Alix is not strongly phosphorylated. Direct evidence for this came from analysis of EGFR-associated Alix and total Alix by anti-phosphotyrosine Western blotting (Fig. 6B). Although the hyperphosphorylation level of the total pool of Alix or Alix-(Δ717–784) was readily detectable following an epitope tag-mediated immunoprecipitation, the phosphorylation level of EGFR-associated Alix or Alix-(Δ717–784) was not detectable (Fig. 6B), suggesting that a less phosphorylated pool of Alix binds to this signaling complex and that increasing the phosphorylation status of Alix reduces its ability to interact with the EGFR.

The association of Alix with receptor tyrosine kinases, such as EGFR and PDGFR, attenuates their internalization by the Cbl-SETA/CIN85 complex (15). The observations that Src reduced the association of Alix with SETA/CIN85 (Fig. 5) and the EGFR (Fig. 6) suggested the hypothesis that Src would antagonize the negative effect of Alix on receptor internalization. To test this directly, we measured the internalization of the endogenous PDGFR in transfected HeLa cells (Fig. 7A). The transfection of Src promoted the internalization of the PDGFR compared with the control. Furthermore, Src was very effective at neutralizing the inhibition of receptor internalization mediated by Alix and Alix-(Δ717–784), but noticeably less so for Alix-784Stop, which was not efficiently phosphorylated by Src: it reduced the levels of the remaining receptor in Alix- and Alix-(Δ717–784)-transfected cells to control levels, but reduced the levels in Alix-784Stop-transfected cells only partially (Fig. 3C). Alix-Y319F was equivalent to the control in this assay, suggesting that the presence of the phosphorylatable Tyr^{319} is important for Alix function. As demonstrated previously, reduction of endogenous Alix expression by siRNA promotes receptor internalization (15). Interestingly, in these experiments, reduction of Alix expression by siRNA prevented any further significant promotion of receptor internalization by Src, allowing the possibility that Src works primarily through Alix in this instance; alternatively, receptor internalization rates may already have reached a maximum.

Another activity of Alix that we previously reported involves a reduction in cell adhesion by association with Pyk2 (13). Therefore, we tested whether Src could also antagonize this aspect of Alix function by determining the proportion of HEK293 cells that attached to either collagen or fibronectin after transfection with Alix and Src (Fig. 8). Although Src itself had a mildly inhibitory effect on cell adhesion to both substrates, it very efficiently antagonized the strong negative effect of Alix and increased adhesion levels to control values (Fig. 8, *A* and *B*). Similarly, the weaker negative impact of Alix-(Δ717–784) was attenuated by Src. Alix-784Stop had an even weaker inhibitory effect, which was not modulated by Src; and again, Alix-Y319F was identical to the control. That these effects were dependent on activation of integrin complexes, which contain the focal adhesion kinases, is suggested by the lack of effect of Alix or Src when cells were plated on poly-L-lysine (Fig. 8C). The observation suggesting that Src-mediated antagonization of Alix function can increase cell adhesion is supported by the finding that reduction of Alix protein levels by siRNA could increase cell adhesion in different cells tested (Fig. 8D). The hypothesis that Alix mediates its negative impact on adhesion by binding to Pyk2 and attenuating its activity was previously proposed on the basis that the presence of Alix proteins in the Pyk2 complex correlates with their negative effect on adhesion (13). Therefore, we tested whether the presence of Src could reduce the amount of Alix
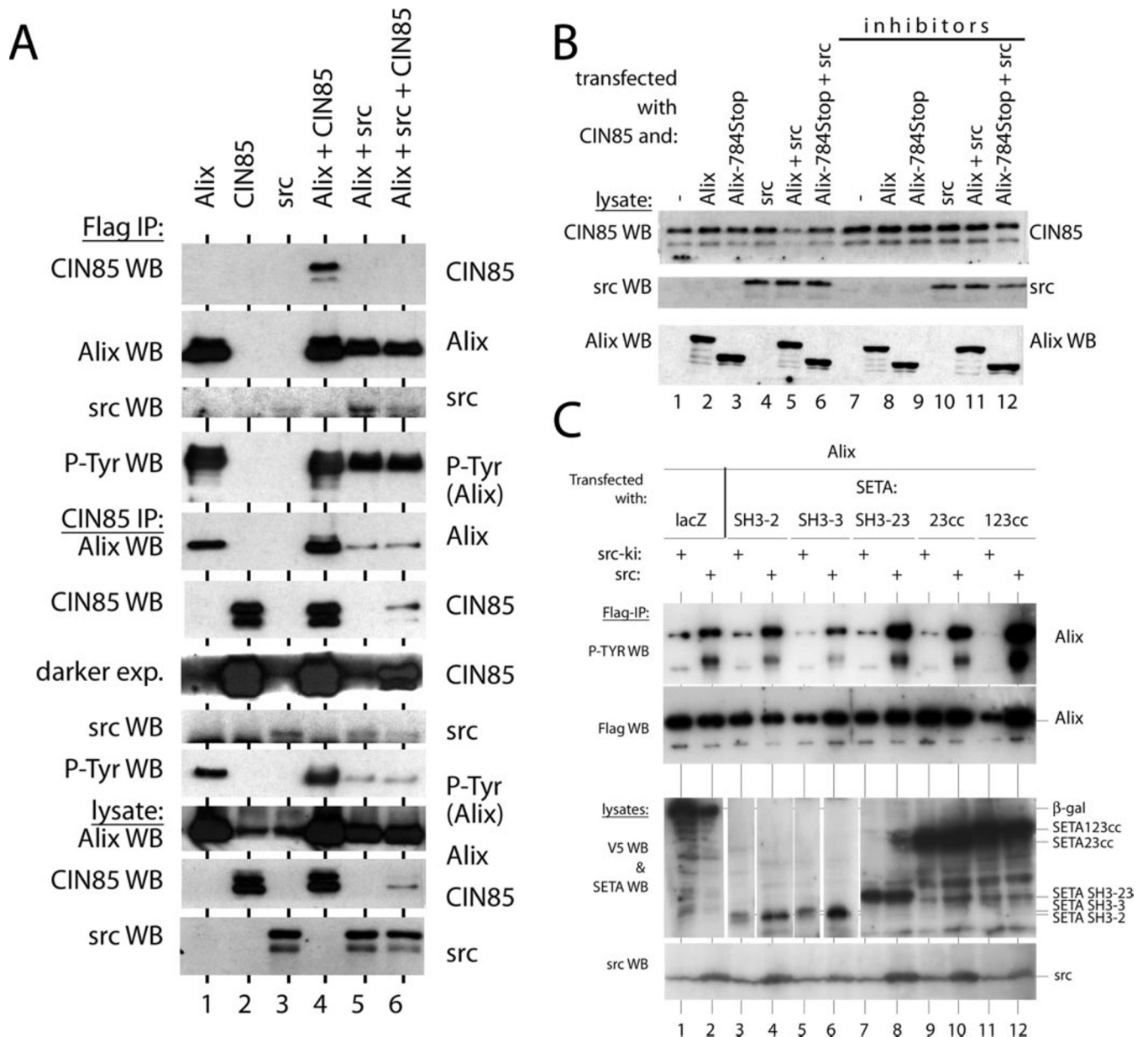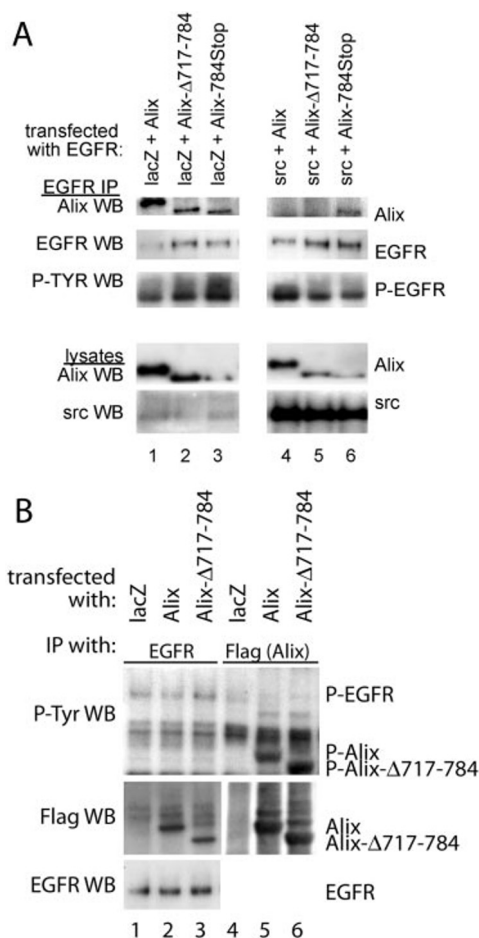
FIG. 5. **Phosphorylation of Alix by Src reduces its association with SETA/CIN85.** *A*, Alix, CIN85, and Src were transfected into HeLa cells as indicated, and the lysates were subjected to immunoprecipitation (*IP*) with anti-FLAG antibodies to recover Alix or with anti-CIN85 antibodies. Western blot (*WB*) analysis was performed to detect the components of the immunoprecipitated complexes as indicated. The presence of Src reduced the level of CIN85 recovered in the Alix immunoprecipitate dramatically. Similarly, the amount of Alix in immunoprecipitates of endogenous CIN85 was reduced by 2-fold (compare *lanes 1* and *5*), and the level in complexes formed following transfection of additional CIN85 was reduced by 25% (compare *lanes 4* and *6*) as assessed by densitometry. A darker exposure (*exp.*) of the anti-CIN85 Western blot following CIN85 immunoprecipitation is shown to allow the endogenous protein to be seen. Lower levels of CIN85 in cells transfected with Alix and Src (*lane 6*) were consistently observed. Data are representative of two experiments. *B*, direct analysis of the effect of inhibitors of proteasomal and lysosomal degradation on CIN85 stability was performed in cotransfected HeLa cells. Cells were transfected as indicated, and some were treated with inhibitors of protein degradation (20 μM MG132, 10 μM lactacystin, 10 mM NH_4Cl, and 50 μM chloroquine) for 8 h prior to lysis and analysis by Western blotting as indicated. The levels of CIN85 were reduced only when Src plus Alix (but not Alix-784Stop) were present (*lane 5*), and this was attenuated when protein degradation inhibitors were also present (*lane 11*). Data are representative of two experiments. *C*, cotransfection of Alix, Src or Src-ki, and various forms of SETA/CIN85 into primary rat cortical astrocytes was followed by recovery of Alix and assessment of its phosphorylation level. Relative to the amount of Alix recovered in each lane, the degree of tyrosine phosphorylation varied considerably. The full-length SETA form 123cc enhanced Alix phosphorylation considerably, whereas 23cc and SH3-23 had a smaller but noticeable effect. The isolated SETA SH3 domains had no impact. Control Western analysis of the lysates revealed the presence of the SETA and β-galactosidase proteins by anti-V5 Western blotting (*β-gal*, SH3-2, and SH3-3) or by anti-SETA Western blotting (SH3-23, 23cc, and 123cc) as well as the expression of Src and Src-ki. Data are representative of two experiments.

associated with endogenous Pyk2 and observed a clear negative effect (Fig. 8*F*). Similar results were obtained with focal adhesion kinase (data not shown). These data suggest that Src can also antagonize the impact of Alix on cell adhesion and does so by reducing the ability of Alix to bind to the relevant target protein, Pyk2.

DISCUSSION

The adaptor protein Alix has been implicated in a variety of functions, including the regulation of kinases, such as the EGFR and focal adhesion kinases (13, 15), as well as membrane-associated transport processes mediated by the ESCRT complex (6–9). However, little is known about how the activity

Src Phosphorylates Alix and Antagonizes Its Function

FIG. 6. **Phosphorylation of Alix by Src reduces its association with the EGFR.** *A*, the EGFR was cotransfected with Src and Alix or deletion mutants of Alix as indicated into HEK293 cells (*upper panels*). Proteins were recovered by EGFR immunoprecipitation (*IP*) and analyzed by anti-Alix Western blotting (*WB*). This showed that the expression of Src reduced the amount of Alix or Alix-(Δ717–784) in EGFR complexes (compare *lanes 1* and *4* and *lanes 2* and *5*), but affected the amount of Alix-784Stop to a lesser degree (compare *lanes 3* and *6*). Controls demonstrating the presence of the EGFR and its activity state as measured by phosphotyrosine levels are also shown. The results from analysis of lysates showing expression of Alix proteins and Src are shown (*lower panels*). Data are representative of two experiments. *B*, HEK293 cell lysates cotransfected with *lacZ*, Alix, or Alix-(Δ717–784) were subjected to immunoprecipitation of either the endogenous EGFR (*lanes 1–3*) or Alix via its FLAG tag (*lanes 4–6*) and analysis by anti-phosphotyrosine Western blotting. Although both Alix and Alix-(Δ717–784) were readily recovered in association with the EGFR, neither protein was detectably phosphorylated at tyrosine. In contrast, when Alix was recovered directly from these same lysates via its FLAG tag, tyrosine phosphorylation was readily detected. This suggests that the pool of Alix associated with the EGFR is phosphorylated at tyrosine to a lesser degree than other pools of Alix protein. Data are representative of two experiments.

of Alix itself is regulated. Here, we report that Src binds to Alix and phosphorylates it and that this phosphorylation negatively regulates the ability of Alix to participate in molecular interactions and to exert its biological function.

The demonstration that endogenous Src and Alix in untransfected cells were associated and that Alix was phosphorylated at tyrosine under these circumstances suggests that the interaction between Src and Alix has physiological relevance. The primary sequence of the Alix protein reveals motifs that are candidate ligands for both of the Src SH2 and SH3 domains. Analysis of the interactions of these isolated domains of Src with Alix provided direct evidence that the SH2 domain binds to motif [312]KKDNDFIY[319] and that the SH3 domain preferen-
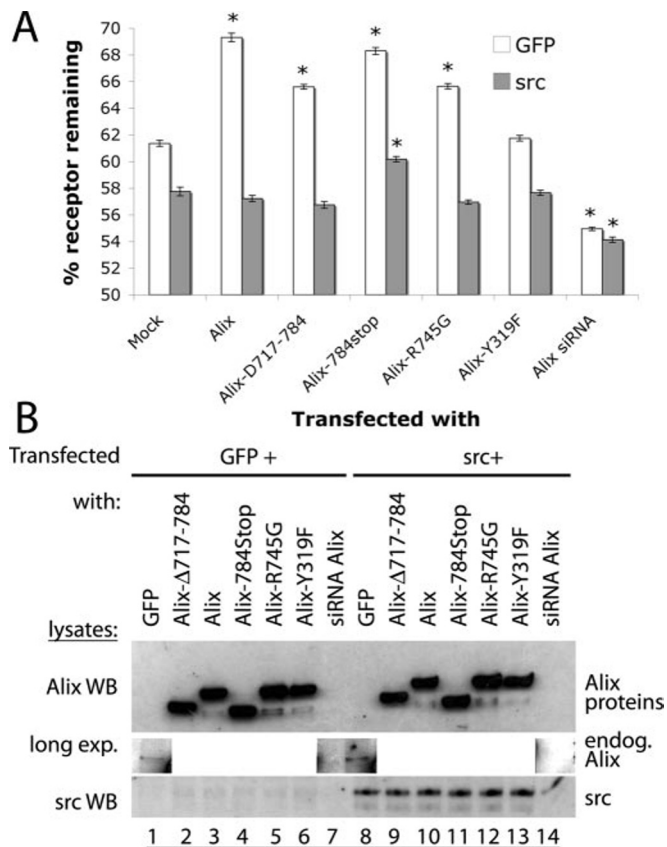
FIG. 7. **Src antagonizes the inhibition of PDGFR internalization mediated by Alix.** *A*, the proportion of the PDGFR remaining on the surface of HeLa cells transfected with GFP (*open bars*) or Src (*closed bars*) along with various Alix constructs or with Alix siRNA as indicated was measured using radiolabeled PDGF. As shown previously (15), Alix increased the level of receptor tyrosine kinase remaining at the cell surface. Src was able to promote down-regulation of the PDGFR in control cells. Furthermore, Src reduced the effect of Alix and Alix-(Δ717–784) to control levels. However, Src had a weaker effect on Alix-784Stop, which remained elevated relative to the control at a statistically significant level, suggesting that phosphorylation of the Alix C terminus is an important component of this effect. Furthermore, the acceleration of receptor down-regulation caused by the reduction of Alix levels by siRNA (15) was more pronounced than the effect engendered by Src and prevented further acceleration of this process by Src. *Asterisks* indicate statistically different values compared with respective mock controls at the $p < 0.005$ level as determined by Student's *t* test. *B*, the results from Western blot (*WB*) analysis of lysates of the cells used to obtain the results in *A* demonstrate expression of Alix proteins and Src. A long exposure (*exp.*) of the anti-Alix Western blot is also provided for the control GFP lanes (*lanes 1* and *8*) and the siRNA lanes (*lanes 7* and *14*) to show endogenous (*endog.*) Alix and its reduction by siRNA treatment; the other lanes were omitted for clarity, as their signals were too intense. Data are representative of two experiments.

tially interacts with the sequence [752]PQPPAR[757]. A more complex picture emerged from examination of association between full-length Src proteins and Alix in the context of the multivalent interactions observed in transfected cells. In these experiments, a requirement for the interaction of Src with Alix Tyr[319] and its function to compete with the endogenous SH2 domain-binding sequence in Src at Tyr[527] became evident, as mutation of this residue restored binding of Src to Alix-Y319F. Furthermore, the ability of the Alix-(Δ717–784) mutant to bind to full-length Src suggests that the interaction of the SH2 domain with the Alix Tyr[319] motif is sufficient. These data, together with results showing the lack of binding of Alix to inactive Src, support a model whereby fully open and active Src binds to Alix at two points via its SH2 and SH3 domains. An intermediate step may involve the unlatching of Src by binding
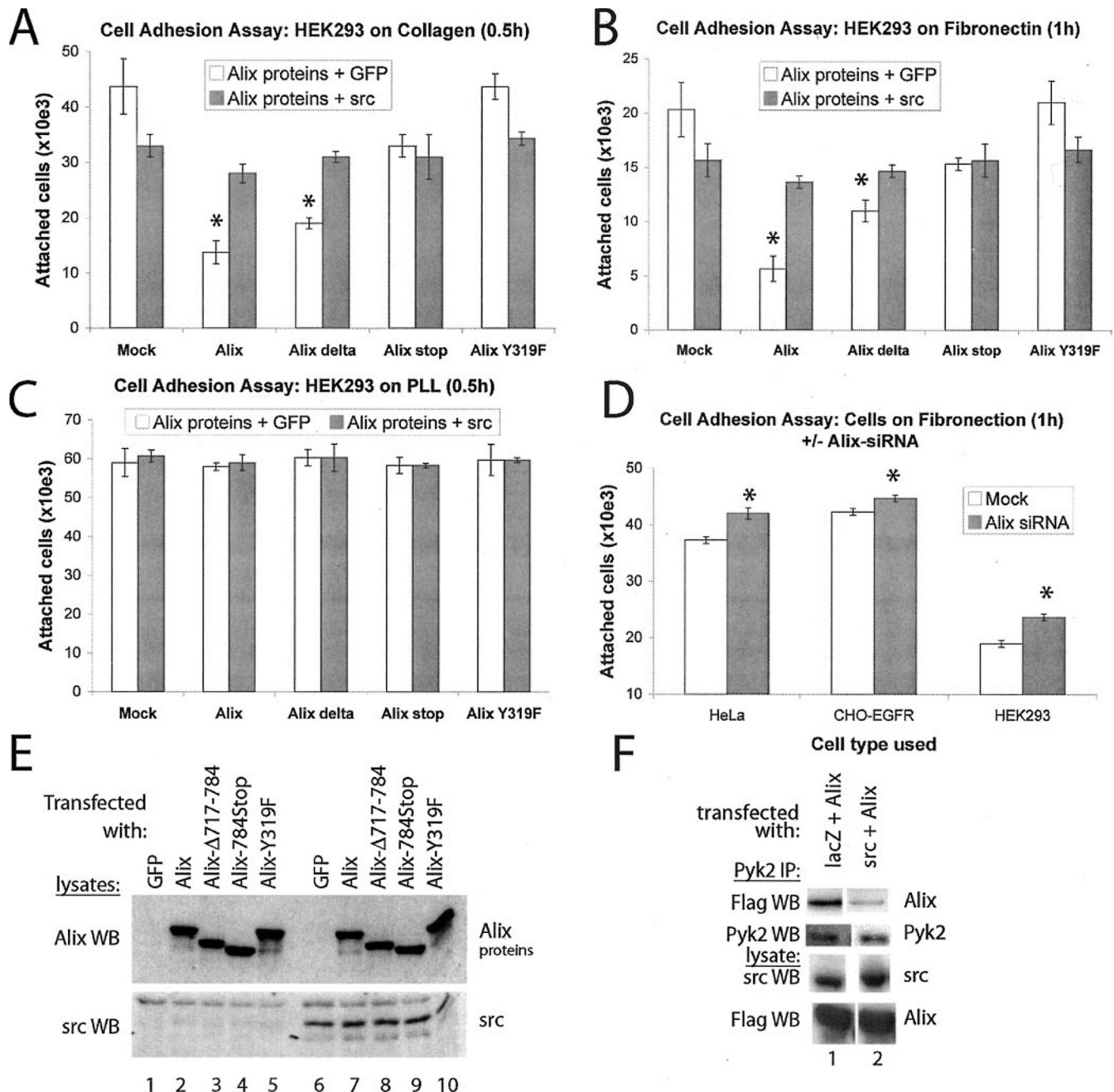
FIG. 8. **Src antagonizes the reduction in cell adhesion mediated by Alix.** *A–C*, HEK293 cells transfected with GFP or Src in combination with Alix proteins as indicated were assessed for adhesion to collagen, fibronectin, or poly-L-lysine (*PLL*), respectively, and the number of attached cells was determined. As shown previously (13), Alix, Alix-(Δ717–784) (*Alix delta*), and Alix-784Stop (*Alix stop*), but not Alix-Y319F, reduced cell adhesion. When Src was also transfected, this activity of Alix proteins was counteracted, with the exception of Alix-784Stop. On poly-L-lysine, no difference was observed, suggesting that engagement of integrins is essential for the observation of any effect of either Src or Alix. *Asterisks* indicate statistically different values from mock controls at the $p < 0.001$ level in *A* and at the $p < 0.05$ level in *B* as determined by Student's *t* test. When Src was cotransfected, no statistical differences were observed in comparison with the control, demonstrating that Src abrogates the impact of the Alix proteins capable of reducing cell adhesion. *D*, three different cell types were treated with Alix siRNA or mock-transfected, and adhesion was measured as described for *A–C*. In each instance, reduction of Alix levels resulted in a statistically significant increase in cell adhesion. *Asterisks* indicate statistically different values from the untreated cell controls at the $p < 0.01$ level as determined by Student's *t* test. *CHO*, Chinese hamster ovary. *E*, the results from Western blot (*WB*) analysis of the lysates of the cells used in *A* and *B* demonstrate the expression of Alix and Src proteins. For siRNA-mediated reduction in Alix expression, see Fig. 7. Data are representative of two experiments. *F*, the results from Western blot analysis of an endogenous Pyk2 immunoprecipitate (*IP*) of transfected HEK293 cells show that cotransfection of Src dramatically reduced the amount of Alix recovered. Data are representative of two experiments.

of Alix phospho-Tyr[319] to the Src SH2 domain, as has been shown to be the case for other proteins (reviewed recently in Ref. 38). Proteins that bind to Src are typically phosphorylated by it, and our experiments showed that Alix conforms to this. Analysis of deletion mutants of the Alix C terminus showed that the major site of Src-mediated hyperphosphorylation is in this region, where 8 of 28 conserved tyrosines are found. In

summary, it can be predicted from these findings that the tertiary structure of Alix is likely to accommodate simultaneous binding to the Src SH2 and SH3 domains while presenting the tyrosine-rich C terminus to the kinase domain.

Phosphorylation of proteins is generally believed to be a major regulatory signal in cells; and accordingly, Src-mediated phosphorylation of Alix modulated its ability to bind to its

effectors as well as to regulate cellular functions. Protein/protein interactions are regulated at the level of the conformation of the participating proteins as well as their spatial and temporal presentation. In the case of Alix, binding and activation of Src caused Alix to relocate in the cell from membrane-associated and cytoskeletal pools to the soluble cytoplasmic fraction. Interestingly, Src-ki, which did not bind strongly to Alix in immunoprecipitations, was able to subtly affect Alix localization, but not phosphorylation. This allows for the possibility that low affinity interactions between these proteins in the absence of phosphorylation can mediate some redistribution. Direct examination of the impact of Alix hyperphosphorylation by Src showed that this interfered with its binding to SETA/CIN85 and the EGFR, two proteins with which Alix complexes (4, 15). The interaction with SETA/CIN85 is direct and via a Pro-Arg motif in the Alix C terminus (19), suggesting that changing the charge and shape of residues in this region could easily perturb it. The molecular details of the interaction between Alix and the EGFR are not yet clear, and so it is not possible to say whether the same mechanism underlies the attenuation of both effects. Furthermore, in the absence of any structural information on Alix, it is difficult to predict precisely how phosphorylation of its C terminus will affect its shape. Analysis of Alix function at the cellular level provided further evidence that Src is capable of negatively regulating this protein. Consistent with the molecular data showing disruption of Alix interactions, analysis of receptor tyrosine kinase internalization and cell adhesion showed a reduction in the impact of Alix on these processes.

Following engagement of integrins, Src is recruited to focal adhesions, where it binds to and phosphorylates focal adhesion kinases and many other associated proteins, including adaptor proteins (Refs. 39 and 40; reviewed in Ref. 17). Although Src is not strictly required for cells to adhere to substrates, its recruitment and activation contribute to the formation of focal adhesions and their promotion of cell adhesion. In our experiments, Src did not promote cell adhesion in HEK293 cells when transfected alone, but was very effective at attenuating the negative effect of Alix on cell adhesion. This effect was dependent on the ability of Src to phosphorylate Alix effectively, as Alix mutants that did not bind Src or that were not phosphorylated by it were not affected. Although these experiments were performed with transfected Alix, allowing that the phenomena observed are not physiological, siRNA experiments support a role for Alix in cell adhesion. However, the effect of Alix on cell adhesion, whether overexpressed or underexpressed, was relatively modest, as would be expected from a complex process involving many proteins; and so we are not suggesting that Src acts in this cellular process primarily through modulation of Alix activity. However, our data support that attenuation of the negative impact of Alix on Pyk2 or focal adhesion kinase activity (13) by Src is one mechanism by which it affects focal adhesion function.

Src is recruited to receptor tyrosine kinases, and the interactions with the PDGFR were the first to be recognized and are the best described (17, 41), which is why we chose to study this receptor system here in addition to analyzing the EGFR, for which Alix interactions have been more thoroughly studied (15). Interaction with an active receptor tyrosine kinase typically leads to the phosphorylation and activation of Src, which can then cooperate in the transmission of its signal. A considerable amount of redundancy exists in the signals generated by the activation of growth factor receptors, but the activation of Src is generally considered to be growth-promoting and a step that amplifies the signal (17, 41). Src can further activate receptor tyrosine kinases (17, 41), including the PDGFR (42), which is consistent with our observation that Src promotes an increase in PDGFR internalization when transfected alone. Src was even more effective at attenuating Alix inhibition of PDGFR down-regulation when it was able to bind and phosphorylate this target. Interestingly, when endogenous levels of Alix were reduced below the threshold of detection by siRNA, this significantly eroded the ability of Src to further promote down-regulation of the PDGFR. It is possible that, under these circumstances, the rates of receptor turnover were maximal. Alternatively, it supports the hypothesis that Src affects receptor internalization primarily by neutralizing the ability of Alix to bind and so negatively impact Cbl-SETA/CIN85 complex function (15). According to this hypothesis, the impact of Src in cells transfected with Alix-784Stop, which was not phosphorylated by Src, would be mediated in part by targeting endogenous Alix; residual inhibition of PDGFR internalization in these cells would be due to the inability of Src to attenuate Alix-784Stop.

In summary, our data show that Src binding and phosphorylation of the adaptor protein Alix are an important node in many critical signal transduction pathways. The consequence of this modification is the negative regulation of Alix associations and functions, as exemplified by binding to receptor tyrosine kinases, focal adhesion kinases, and the adaptor molecule SETA/CIN85 and the consequent reduction in cell adhesion and receptor tyrosine kinase down-regulation.

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