Alix Facilitates the Interaction between c-Cbl and Platelet-derived Growth Factor β-Receptor and Thereby Modulates Receptor Down-regulation

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Alix (ALG-2-interacting protein X) is an adaptor protein involved in down-regulation and sorting of cell surface receptors through the endosomal compartments toward the lysosome. In this study, we show that Alix interacts with the C-terminal region of the platelet-derived growth factor (PDGF) β-receptor (PDGFRβ) and becomes transiently tyrosine-phosphorylated in response to PDGF-BB stimulation. Increased expression levels of Alix resulted in a reduced rate of PDGFRβ removal from the cell surface following receptor activation, and this was associated with decreased receptor degradation. Furthermore, Alix was found to co-immunoprecipitate with the ubiquitin ligase c-Cbl, and elevated Alix levels increased the interaction between c-Cbl and PDGFRβ. Interestingly, Alix interacted constitutively with both c-Cbl and PDGFRβ. Moreover, c-Cbl was found to be hyperphosphorylated in cells engineered to overexpress Alix compared with control cells. The increased c-Cbl phosphorylation correlated with enhanced proteasomal degradation of c-Cbl, which in turn correlated with a decreased ubiquitination of PDGFRβ. Our data suggest that Alix inhibits down-regulation of PDGFRβ by modulating the interaction between c-Cbl and the receptor, thereby affecting the ubiquitination of the receptor.

Platelet-derived growth factor (PDGF) is a family of signaling proteins that regulates the proliferation, survival, and migration of cells of mesenchymal origin. The PDGF family consists of four protein chains that form five biologically active dimers (PDGF-AA, -AB, -BB, -CC, and -DD) (1). PDGF isoforms exert their cellular effects via binding to two tyrosine kinases through the endosomal compartments toward the lysosome. The PDGF family consists of four protein chains that form five biologically active dimers (PDGF-AA, -AB, -BB, -CC, and -DD) (1). PDGF isoforms exert their cellular effects via binding to two tyrosine kinase receptors: PDGFRα, which binds PDGF-A, -B, and -C chains; and PDGFRβ, which binds PDGF-B and -D chains. Ligand binding induces dimerization and autophosphorylation of the receptors. The phosphorylated tyrosine residues have key roles in coupling to downstream signaling pathways as they mediate interaction with Src homology 2 domain (SH2)-containing molecules.

Alix (ALG-2-interacting protein X; also referred to as AIP1 or HP95) is a protein that was identified as a binding partner for ALG-2 (apoptosis-linked gene 2) (2). Apoptosis induced by various stimuli requires ALG-2, and several studies have indicated a role for Alix in regulation of cell death (3–6). Alix possesses an N-terminal Bro1 domain and a C-terminal region rich in proline and tyrosine residues, suggesting an adaptor/scaffold function. Recently, Alix has been implicated in receptor endocytosis and trafficking (7–9). Work by Schmidt et al. (8) demonstrates that Alix antagonizes epidermal growth factor receptor (EGFR) down-regulation by the c-Cbl-CIN85 complex, resulting in reduced ubiquitination and internalization (8). The same group also showed that Alix interacts with and is phosphorylated by Src family kinases and that this phosphorylation reduces the interaction between Alix and EGFR, CIN85, or Pyk2, thereby releasing the inhibitory function of Alix on endocytosis (9). Another role that has emerged for Alix is in the organization of endosomes and formation of multivesicular bodies required for transportation of proteins from the early endosome to the lysosome (10, 11). Furthermore, it has been demonstrated that Alix interacts with several cytoskeletal proteins, e.g. actin and tubulin, and with tyrosine kinases such as FAK and Pyk2, thereby negatively regulating cell adhesion (12). Overexpression of Alix in NIH3T3 cells promotes flat cell morphology and reduced proliferation (13), and Alix overexpression in HeLa cells leads to cell cycle arrest in G1 phase (14). In summary, Alix has been shown to be involved in numerous cellular events, such as modulation of cellular architecture, apoptosis, growth, and endocytosis; however, the precise function of Alix is not well understood.

In the present report, we show that Alix interacts constitutively with PDGFRβ. It modulates the stability of the E3 ubiquitin ligase c-Cbl, thus affecting receptor ubiquitination and down-regulation.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant human PDGF-BB was generously provided by Amgen (Thousand Oaks, CA). The following antibodies were used for immunoprecipitation and immunoblotting: phosphotyrosine (sc-7020) and PDGFRβ (sc-339) antisera were from Santa Cruz Biotechnology (Santa Cruz, CA); the...
monoclonal PDGFRβ antibody recognizing the extracellular domain (GR14L) was from Oncogene Research Products (Cambridge, MA); monoclonal β-actin antibody was from Sigma (A5441); the monoclonal HA antibody (12CA5) was from Roche Applied Science; the monoclonal c-Cbl antibody (610442) was from BD Transduction Laboratories; and Ras-GAP antibodies were from Upstate Biotechnology (05-178). An antiserum against Alix was raised by immunizing a rabbit with the synthetic peptide CSYPFPQQPQSYPQQ conjugated to keyhole limpet hemocyanin. The antibodies were purified by chromatography over a column of the corresponding peptide immobilized on Sepharose beads. Purified antibodies were stored in 0.15 M NaCl, 20 mM Hepes, pH 7.4, and 50% glycerol at −70 °C. Rabbit antiserum against PLCγ was generated by immunizing rabbits with a peptide corresponding to the C terminus of bovine PLCγ (15).

Cell Culture and Transfection—Porcine aortic endothelial (PAE) and 293T cells were cultured in Ham's F-12 and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were transfected by lipofection according to the protocol provided by the manufacturer (Invitrogen). For transient transfections, cells were assayed 48 h after transfection. To establish stable transfectants, cells were selected 48 h after transfection by the presence of 400 μg/ml zeocin until only resistant cells were left.

Affinity Purification of Tyrosine-phosphorylated Proteins—Subconfluent PAE cells (total 10⁷ cells) expressing either PDGFRα or PDGFRβ were serum-starved overnight and then stimulated for 5 min with 100 ng/ml PDGF-BB and lysed in 0.5% Triton, 50 mM NaCl, 10 mM NaF, 30 mM tetrasodium pyrophosphate, 10% glycerol, 1 mM EDTA, 20 mM Tris, 1 mM Pefabloc, 1% Trasylol, and 1 mM sodium orthovanadate, pH 7.4. Extracts were cleared by centrifugation, and tyrosine-phosphorylated proteins were immunoprecipitated with 4G10 antibodies coupled to agarose beads (Upstate Biotechnology) for 2 h. The immunocomplex was washed three times in lysis buffer and then eluted twice with 100 mM phenylphosphate in 10 mM Hepes, pH 7.4, at 4 °C. Eluted proteins were precipitated using the Wessel-Flügge procedure (16) and resuspended in 100 μl of sample buffer containing 10 mM dithiothreitol. The samples were treated with 25 mM iodoacetamide for 15 min at room temperature before separation by SDS-PAGE and visualization by silver staining using an in-gel digestion compatible protocol (17).

Protein Identification—Excised protein bands were de-stained (18) and dried with acetonitrile. A solution of porcine sequence grade trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate, 10% acetonitrile was allowed to soak into the gel pieces on ice for 45 min. Approximately 100 ng trypsin/gel band was applied and incubated at 30 °C overnight. The digestion was stopped by making the samples 1% with respect to trifluoroacetic acid. After dilution in 0.1% trifluoroacetic acid, samples were concentrated and desalted on a μTip C18 column (Millipore Corp., Bedford, MA), and the digest mixture was eluted directly onto the matrix-assisted laser desorption/ionization (MALDI) target with 70% acetonitrile, containing about 8 mg/ml matrix α-cyano-4-hydroxycinnamic acid. Peptide mass fingerprinting was done by MALDI time-of-flight mass spectrometry (MALDI-TOF/MS) on a Bruker Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany). The instrument was operated in reflector mode and optimized for analytes up to 4000 Da. The spectra were internally calibrated using autolytic trypsin peptides. The peptide mass lists were used to scan protein sequence data banks for protein identities via the ProFound search engine. Significance of matches were judged after taking several parameters into account, such as “z-score,” error distribution, number of miscuts, and migration behavior.

Immunoprecipitation and Western Blotting—Subconfluent cells were starved in 0.1% fetal bovine serum overnight and incubated with 100 ng/ml PDGF-BB for the indicated periods of time. Cells were washed with ice-cold phosphate-buffered saline and lysed (0.5% Triton, 50 mM NaCl, 10 mM NaF, 30 mM tetrasodium pyrophosphate, 10% glycerol, 1 mM EDTA, 20 mM Tris, 1 mM Pefabloc, 1% Trasylol, and 1 mM sodium orthovanadate, pH 7.4). Extracts were clarified by centrifugation, and protein concentration was determined by the BCA protein assay system (Pierce). Equal amounts of lysates were incubated with 1 μg of antibody as indicated. The immunoprecipitates were collected on protein A-Sepharose beads, washed three times in lysis buffer, and then boiled in SDS sample buffer containing dithiothreitol. Immunoprecipitates or equal amounts of total cell lysates were analyzed by SDS-PAGE. For Western blotting, samples were electrotransferred to polyvinylidene difluoride membranes (Immobilon P), which were blocked in 5% bovine serum albumin in phosphate-buffered saline solution containing 0.1% Tween 20. Primary antibodies were used with the concentrations and buffers recommended by the suppliers and incubated overnight in the cold. After washing, the membranes were incubated with horseradish peroxidase-conjugated antimouse or anti-rabbit IgG antibodies (both from GE Healthcare/Amersham Biosciences), and proteins were visualized using ECL Western blotting detection systems from Roche Applied Science on a cooled charge-coupled device camera (CCD; Fuji, Minami-Ashigara, Japan). Before reprobing, the membranes were stripped with 0.4 M NaOH for 10 min at room temperature, blocked, and incubated with the corresponding antibody.

Biotinylation of Cell Surface Proteins—Cells were serum-starved overnight and stimulated with 20 ng/ml PDGF-BB for different periods of time at 37 °C. Next, cells were washed twice in ice-cold phosphate-buffered saline, pH 7.4, and incubated for 1 h on ice with 0.2 mg/ml sulfo-NHS-SS-biotin (Pierce) in phosphate-buffered saline. To inactivate unbound sulfo-NHS-SS-biotin, cells were treated for 5 min with 50 mM Tris, pH 8.0. Cells were lysed, and biotinylated proteins were precipitated with streptavidin-agarose (Sigma) for 1 h at 4 °C. The beads were washed three times in lysis buffer and boiled with SDS sample buffer containing dithiothreitol. Eluted proteins were separated using SDS-PAGE, transferred to Immobilon P membranes. The membranes were blocked with 5% bovine serum albumin in phosphate-buffered saline, and the amount of PDGFRβ was visualized and quantified by immunoblotting with PDGFRβ antibodies.
**RESULTS**

Identification of Tyrosine-phosphorylated Proteins after PDGF Stimulation—We were interested in identifying proteins that become tyrosine-phosphorylated after PDGF stimulation and hence are likely to participate in signal transduction downstream of the PDGFRs. To this end we used monoclonal anti-phosphotyrosine antibodies (4G10) immobilized on Sepharose beads to immunoprecipitate proteins from PDGF-BB-stimulated PAE cells transfected with either PDGFRα or PDGFRβ. This allowed us to investigate the tyrosine-phosphorylated proteins downstream of the two PDGFR isoforms, expressed at comparable levels in the same cellular background. Bound proteins were eluted, separated by SDS-PAGE, and visualized by silver staining (supplemental Fig. 1). Proteins tentatively phosphorylated after PDGF stimulation were excised and identified by mass spectroscopy as indicated in supplemental Fig. 1. Among the proteins identified, Alix was selected for further study in regard to PDGFRβ signal transduction. Interestingly, we observed that Alix was not significantly phosphorylated by PDGFRα, indicating that Alix is a preferred substrate for PDGFRβ.

Alix Is Phosphorylated in Response to PDGF and Interacts Constitutively with PDGFRβ—To verify the mass spectroscopy results, we immunoprecipitated Alix from cells stimulated with PDGF-BB and resolved the immunocomplexes by SDS-PAGE followed by transfer to an Immobilon P membrane. A phosphotyrosine immunoblot revealed bands with sizes corresponding to Alix (−95 kDa) and PDGFRβ (−180 kDa) in the Alix immunoprecipitate (Fig. 1A, *top left panel*). The 95- and 180-kDa phosphoproteins were confirmed to be Alix and PDGFRβ, respectively, by reprobing the membrane with Alix- or PDGFRβ-specific antibodies (Fig. 1A, *left panels*). Interestingly, we found that Alix interacted with PDGFRβ in a constitutive manner, although its tyrosine phosphorylation was ligand-independent. The interaction could also be detected in a reciprocal experiment (Fig. 1A, *right panels*). Next, we performed a kinetic analysis of Alix phosphorylation. We found that Alix was transiently phosphorylated in response to PDGF-BB (Fig. 1B, *top panel*) with kinetics similar to the overall receptor activation as determined by anti-phosphotyrosine immunoblotting of WGA-Sepharose-enriched PDGFRβ (Fig. 1B, *bottom panel*). Our results suggest that Alix is phosphorylated by PDGFRβ. It is possible that Alix is also phosphorylated indirectly by Src family kinases activated by PDGFRβ; however, such a contribution is likely to be minor, because Alix phosphorylation was only slightly inhibited by the low molecular weight inhibitor SU6656 (data not shown).

Alix Binds to the C-terminal Region of PDGFRβ—To determine the binding region for Alix in PDGFRβ, we decided to analyze different C-terminally truncated mutants of the receptor for their abilities to interact with Alix. By expressing deletion mutants of the receptor in 293T cells, we found a partial loss of interaction when the 75 most C-terminal amino acid residues of PDGFRβ were deleted and an almost complete loss of binding when the last 98 amino acid residues were deleted (Fig. 2A). Because Alix, despite its not being significantly phosphorylated by PDGFRα, was also found to interact with this receptor (data not shown), we compared the amino acid sequence of PDGFRα and -β in the region important for binding. We observed that the sequence surrounding Tyr-1021 in PDGFRβ is similar between both receptor isoforms (Fig. 2B). Furthermore, this region is rich in proline residues, which can interact with SH3 domain or WW domain-containing proteins in a constitutive manner. To clarify whether this region is responsible for the interaction with Alix, we synthesized peptides corresponding to the amino acid sequence between residues 1015 and 1030, with the addition of an N-terminal cysteine to facilitate coupling to a solid support (Fig. 2B), with Tyr-1021 either phosphorylated or not phosphorylated. Next we performed in vitro interaction assays using this peptide immobilized on beads followed by immunoblotting for target proteins. We found that Alix interacted with both peptides, independent of phosphorylation, in agreement with the constitutive binding observed in vivo (Fig. 2C). As a control, we analyzed the interaction between these peptides and its known interaction partner PLCγ1. Indeed, we could detect a phosphorylation-dependent interaction with PLCγ1 (Fig. 2C). As a negative control we used a peptide containing tyrosine 771 in the PDGFRβ, which is known to bind RasGAP. As seen in Fig. 2C, this peptide, when phosphorylated on tyrosine, could pull down RasGAP but not Alix. Thus, we concluded that Alix can interact with the PDGFRβ in the region between residues 1015 and 1030 in a receptor phosphorylation-independent manner. However, other regions in the PDGFRβ may contribute, because there was a weak residual binding also when the last 98 amino acids of the receptor were deleted.
Alix Inhibits PDGF-BB-induced Removal of PDGFRβ from the Cell Surface—To investigate whether the expression level of Alix influenced the rate of PDGFRβ cell surface clearance, we analyzed cells that were engineered to overexpress Alix (Fig. 3A). To measure the amount of receptors on the cell surface, we stimulated cells for different periods of time and biotinylated the cell surface proteins prior to lysis. Biotinylated proteins were purified using streptavidin-agarose, and the levels of PDGFRβ were then analyzed by immunoblotting (Fig. 3B). As can be seen, the PDGF-BB-induced clearance of PDGFRβ from the cell surface was reduced in Alix-overexpressing cells as compared with mock-transfected control cells.

Alix Overexpression Enhances Interaction between c-Cbl and PDGFRβ and Phosphorylation of c-Cbl—Ubiquitination of receptor tyrosine kinases is important for their internalization, and c-Cbl is a major ubiquitin ligase connected with PDGFRβ. Because we noticed a reduced rate of PDGFRβ down-regulation from the cell surface in cells overexpressing Alix (Fig. 3B), we investigated the effect of increased Alix levels on c-Cbl phosphorylation and association with the PDGFRβ after ligand stimulation. We found that elevated Alix levels resulted in increased interaction between c-Cbl and PDGFRβ (Fig. 4). In addition, we observed that Alix co-immunoprecipitated with c-Cbl. Interestingly, neither the interaction between c-Cbl and Alix nor that between c-Cbl and PDGFRβ was induced by PDGF stimulation, but it existed in a constitutive manner both in control and Alix-overexpressing cells. Moreover, PDGFR-induced c-Cbl phosphorylation was augmented in cells overexpressing Alix compared with control cells (Fig. 4).

Elevated Levels of Alix Lead to Reduced PDGFRβ Down-regulation but Increased c-Cbl Degradation in Response to PDGF-BB—Next, we wanted to determine whether the reduced rate of PDGFRβ removal from the cell surface in cells overexpressing Alix was associated with changes in receptor stability. We therefore stimulated cells with PDGF-BB for different periods of time and determined the amount of PDGFRβ by immunoblotting. Indeed, ligand-induced PDGFRβ degradation was reduced in Alix-overexpressing cells compared with mock-transfected cells (Fig. 5). In contrast, we could not observe any effects of Alix overexpression on PDGFRβ phosphorylation. However, c-Cbl was hyperphosphorylated in cells overexpressing Alix (Figs. 4 and 5). We found that the enhanced c-Cbl phosphorylation in response to PDGF-BB stimulation in cells with elevated levels of Alix correlated with increased c-Cbl degradation (Fig. 5). The enhanced rate of c-Cbl degradation was completely inhibited by MG132, suggesting that the degradation occurred in proteasomes (Fig. 6).

Elevated Alix Expression Decreases Ligand-induced PDGFRβ Ubiquitination—The observation that increased Alix expression resulted in increased proteasomal degradation of c-Cbl suggested that the level of PDGFRβ ubiquitination might also be affected. To investigate this possibility, we transfected Alix-overexpressing or mock-transfected cells with ubiquitin fused with an HA epitope and performed immunoprecipitation of PDGFRβ followed by immunoblotting with HA antibodies. Indeed, we found that the PDGFRβ was significantly less ubiquitinated in cells overexpressing Alix (Fig. 7). However, after shorter periods (5–15 min) of PDGF-BB stimulation, we could not demonstrate a significant difference in level of PDGFRβ ubiquitination between control and Alix-overexpressing cells (data not shown). This suggests that the bulk of c-Cbl
proteins must first be degraded before the reduction in ubiquitination can be observed. In summary, Alix overexpression stabilizes the PDGFRβ/H9252β, possibly through promoting enhanced proteasomal degradation of c-Cbl in response to PDGF-BB and thereby reducing c-Cbl-mediated ubiquitination of the PDGFRβ.

**DISCUSSION**

Down-regulation of receptor tyrosine kinases is essential for proper response to ligand, and disturbances in this process may contribute to transformation (19). It has been shown that monoubiquitination of receptor tyrosine kinases is an important mechanism that regulates receptor internalization and intracellular trafficking (20). c-Cbl is a ubiquitin ligase targeting several receptor tyrosine kinases including the PDGFRβs. In this report, we present data on c-Cbl and the multifunctional adaptor protein Alix and on their involvement in PDGFRβ down-regulation. In general, our results are consistent with earlier work with the EGFR (8, 9).

We found that Alix forms a constitutive complex with PDGFRβ and is transiently phosphorylated in response to ligand stimulation. An activation-independent interaction has also been reported between Alix and the EGFR (8). We mapped the binding site of Alix to the C-terminal tail of PDGFRβ. However, contributions from other regions of the receptor in this interaction cannot be excluded. The C-terminal region contains a proline-rich sequence that may mediate the interaction with Alix. However, Alix does not contain any domain that is known to interact with proline-rich regions. Thus, it is possible the interaction between Alix and PDGFRβ is indirect, through an adaptor protein. In fact, Alix is believed to bind indirectly to the EGFR because the association cannot be observed using recombinant proteins (8). Moreover, the interaction between Alix and PDGFRβ was not affected by mutation of tyrosines 1009 and 1021 in the C-terminal tail of the receptor to phenylalanine (data not shown).

It was recently shown that overexpression of Alix results in a reduced EGFR internalization (8). We found that overexpression of Alix also reduced the rate of ligand-induced PDGFRβ removal from the cell surface compared with mock-transfected cells; this reduction was accompanied by a decreased rate of receptor degradation. Moreover, we observed c-Cbl hyperphosphorylation in response to PDGF-BB in Alix-overexpressing cells that was associated with enhanced proteasomal degradation of c-Cbl. These observations are in concurrence with a study from Bao et al. (21) showing that expression of active Src (or overexpression of normal Src) promotes c-Cbl hyperphosphorylation.
phorylation and, as a consequence, its degradation (21). However, the low molecular weight Src inhibitor SU6656 had no significant effect on PDGF-BB-induced c-Cbl phosphorylation in our cell model (data not shown).

Our results suggest that c-Cbl, Alix, and PDGFR interact constitutively, although our data could not distinguish whether a ternary complex or separate binary complexes are formed. However, elevated Alix expression enhances the interaction between c-Cbl and PDGFR. Notably, the constitutive interaction between c-Cbl and PDGFR was also detected in mock-transfected cells and hence is not an artifact of overexpression. The enhanced c-Cbl phosphorylation observed in cells overexpressing Alix may be explained by enhanced interaction between c-Cbl and the receptor, which may allow a more efficient phosphorylation.

Given that c-Cbl is a major ubiquitin ligase downstream of receptor tyrosine kinases (22), we anticipated that an increase in c-Cbl degradation would be reflected in a decreased level of receptor ubiquitination. Indeed, we observed that Alix overexpression correlated with a reduced level of PDGFR ubiquitination. It has been suggested that degradation of hyperphosphorylated c-Cbl allows the EGFR to recycle back to the plasma membrane instead of being sorted for lysosomal degradation (21), potentially because of a lack of sustained ubiquitination (23, 24). This is compatible with our results showing that Alix overexpression leads to increased c-Cbl phosphorylation and degradation, as well as to a reduced rate of PDGFR clearance from the cell surface. It remains to be determined whether this
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is because of decreased internalization or increased recycling. In conclusion, Alix may function as an adaptor/scaffold protein regulating PDGFRβ ubiquitination and consequently intracellular sorting by modulating the degradation of c-Cbl.

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