Regulation of the Transcriptional Activity of c-Fos by ERK

A NOVEL ROLE FOR THE PROLYL ISOMERASE PIN1*

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The activation of the activating protein-1 (AP-1) family of transcription factors, including c-Fos and c-Jun family members, is one of the earliest nuclear events induced by growth factors that stimulate extracellular signal-regulated kinases (ERKs). In the case of c-Fos, the activation of ERK leads to an increased expression of c-fos mRNA. In turn, we have recently shown that ERK phosphorylates multiple residues within the carboxy-terminal transactivation domain (TAD) of c-Fos, thus resulting in its increased transcriptional activity. However, how ERK-dependent phosphorylation regulates c-fos function is still poorly understood. In this regard, it has been recently observed that the prolyl isomerase Pin1 can interact with proteins phosphorylated on serine or threonine residues that precede prolines (pS/T-P), such as the transcription factors p53 and c-Jun, thereby controlling their activity by promoting the cis-trans isomerization of these pS/T-P bonds. Here, we found that Pin1 binds c-Fos through specific pS/T-P sites within the c-Fos TAD, and that this interaction results in an enhanced transcriptional response of c-Fos to polypeptide growth factors that stimulate ERK. Our findings suggest that c-Fos represents a novel target for the isomerizing activity of Pin1 and support a role for Pin1 in the mechanism by which c-Jun and c-Fos can cooperate to regulate AP-1-dependent gene transcription upon phosphorylation by mitogen-activated kinase (MAPK) family members.

The transcription complex AP-1* (activating protein-1) is a dimer composed of Jun and Fos family members whose expression and activity is tightly regulated by the mitogen-activated protein kinase (MAPK) family of serine-threonine kinases (reviewed in Refs. 1–3). These kinases control the expression of up to 20% of the human genome (4). The expression vectors for c-Fos and c-Fos-m (aa 1–380) in pCEFL containing up to 2 µg of total plasmid DNA together with the Lipo-ACCELERATED PUBLICATION

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Pin1 regulates c-Fos activity upon ERK phosphorylation

**Preparation of Nuclear Extracts**—Cells were incubated in lysis buffer (10 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 0.5% Nonidet P-40) and centrifuged at low speed. The resulting nuclei (pellet) were disrupted with extraction buffer (20 mM HEPES, pH 7.9, 0.5 M NaCl, 1 mM EDTA, 1 mM GEMA, 1 mM DTT). Cell debris was separated by low speed centrifugation and nuclei aliquots (50 μg) were subject to GST pull-down assays.

**Reporter Assays**—Cells were transfected with different expression vectors together with 0.1 μg of each luciferase reporter and 0.01 μg of pRL-null (a plasmid encoding the luciferase gene from *Renilla reniformis*) that served as an internal control for transfection efficiency. The total amount of transfected DNA was normalized with pcDNAIII-β-gal, an expression vector for the enzyme β-galactosidase. Cells were lysed in passive lysis buffer (Promega) 24 h post-transfection. Cell lysates (50 μl/well) were transferred to a 96-well plate and firefly and *Renilla* luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega). Light emission was quantified using a Microliter Plate luminometer as specified by the manufacturer (Dynex Tech, Chantilly, VA).

**GST Pull-down Assays**—Cells were lysed in 25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM sodium vanadate, 1% Nonidet P-40, 1 mM PMSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Precleared cell lysates were incubated with fresh GST or GST-Pin2 beads (2 μg of protein) for 1 h at 4 °C. The beads were pelleted by centrifugation, washed three times with lysis buffer, denatured in SDS-loading buffer (400 μM Tris/HCl, pH 6.8, 10% SDS, 50% glycerol, 500 mM DTT, 2 μg/ml bromphenol blue), and analyzed by Western blot. To prepare the GST beads, *Escherichia coli* BL-21 Lys cells (Promega) were transformed with the vectors pGEX-4T3 or pGEX-4T3-Pin1 encoding for GST and GST-Pin1 proteins, respectively. Protein expression and purification were performed essentially as described previously (8).

**In Vitro Phosphorylation of Hisc-Fos TAD Fusion Proteins**—Hisc-tagged proteins were isolated using nickel-nitriilotriacetic acid magnetic agarose beads (Qiagen) as described previously (8). The kinase reactions were performed in 125 mM MOPS, pH 7.5, 125 mM glycerophosphate, 7.5 mM MgCl, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate, containing 20 μM unlabeled ATP, 1 mM DTT, and 1 μM substrate. The reactions were initiated by the addition of 1 μl of purified active ERK2 and allowed to proceed for 30 min at 30 °C before assaying the products in GST pull-down experiments.

**Western Blots and Immunoprecipitations**—For co-immunoprecipitations, cellular lysates were incubated with for 1 h at 14 °C with the specific antibody against AUS. Immunocomplexes were recovered with the aid of γ-bind Sepharose beads (Amer sham Biosciences). Immunoprecipitates or lysates were combined with SDS loading buffer, boiled for 5 min, and resolved by 10% SDS-PAGE. Fractionated proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). The membranes were incubated with 5% nonfat-dried milk in PBS containing 0.05% Tween 20 (PBS-T) and then with dilutions of the respective primary and horse-radish peroxidase-conjugated secondary antibody (ICN-Cappel) in PBS-T, 1% bovine serum albumin. Immunoreactive proteins were visualized by enhanced chemiluminescence detection (ECL-Plus System, Amersham Biosciences).

**RESULTS AND DISCUSSION**

**Pin1 Potentiates AP-1 Activity by Increasing the Transcriptional Response of c-Fos and c-Jun to Growth Factors: a Novel Interaction between Pin1 and c-Fos**—To explore the possibility that Pin1-directed prolyl isomerization of AP-1 factors controls the response of AP-1 to mitogenic signals, we examined whether Pin1 could affect the expression of an AP-1-driven luciferase reporter plasmid in response to PDGF in NIH 3T3 cells. As shown in Fig. 1A, PDGF significantly induced AP-1 transcription in these cells, and increasing concentrations of ectopically expressed Pin1 enhanced the basal AP-1 activity as well as the transcriptional response to this growth factor in a dose-dependent manner. To begin addressing the mechanism whereby Pin1 stimulates AP-1, we next examined the effect of Pin1 on the transactivating function of c-Jun and c-Fos. As shown in Fig. 1B, expression of c-Jun or c-Fos increased AP-1-dependent transcription and potentiated the AP-1 transcriptional response provoked by PDGF. This was enhanced by the co-expression of Pin1, which elevated the basal AP-1 stimulating activity of c-Jun and c-Fos, as well as increased dramatically their response to PDGF. Thus, Pin1 appears to regulate the transactivating activity of both members of the AP-1 family of transcription factors. Based on these observations, we then asked whether Pin1 may interact with c-Fos. For these experiments, we first generated a bacterial expression vector encoding a GST fusion protein of Pin1 and then used purified GST-Pin1 coupled to glutathione-Sepharose beads to pull down c-Fos from nuclear extracts of NIH 3T3 cells stimulated with PDGF. As expected, PDGF promoted the expression of c-Fos (Fig. 1C). Furthermore, the results from the pull-down assays indicated that Pin1 effectively binds to newly synthesized c-Fos proteins, thus providing a molecular explanation to the ability of Pin1 to stimulate the activation of AP-1 by c-Fos.

**The Interaction of c-Fos and Pin1 Depends on c-Fos Phosphorylation**—As the ability of Pin1 to associate to its substrates involves the recognition of specific phosphorylated motifs (14, 15), we next examined whether the interaction of c-Fos with Pin1 was dependent on the status of c-Fos phosphorylation. Of interest, in a previous study we have observed that exposure of NIH 3T3 cells to PDGF or serum leads to c-Fos phosphorylation by ERK in specific

![FIGURE 1. Pin1 binds to c-Fos and potentiates AP-1 by increasing the transcriptional response of c-Fos and c-Jun to PDGF. A, dose-dependent effect of Pin1 on the activation of AP-1 by PDGF. NIH 3T3 cells were transfected with pAP-1-Luc and pRNull, along with increasing concentrations of pCMV-Sport-Pin1 (Pin1). Cells were grown in serum-free conditions overnight and where indicated (+) were stimulated with PDGF (20 ng/ml) for 6 h before reading dual luciferase activities. The data represent firefly luciferase activity normalized by Renilla luciferase activity present in each sample, expressed as a percentage of control values. B, effect of Pin1 on the transcriptional activity of c-Fos and c-Jun. Cells were transfected with pAP-1-Luc and pRNull along with 0.2 μg of pCEFL-AUS-c-Fos (c-Fos), pCEFL-c-Jun (c-Jun), or pCDNAIII-β-gal (control (c)), in the absence or presence of 0.5 μg of pCMV-Sport-Pin1. Cells were processed as described for A, and data are represented as above. C, binding of Pin1 to c-Fos. Cells were grown overnight in the absence of serum and then stimulated with PDGF (20 ng/ml) for the indicated times. Nuclear fractions were collected and aliquots were processed for GST pull-down assays (below) and evaluation of c-Fos expression (above) as described under Experimental Procedures. WB, Western blot.
residues, Thr\(^{232}\), Thr\(^{235}\), Thr\(^{325}\), and Ser\(^{374}\), within the COOH-terminal c-Fos TAD (8). To explore this possibility, we transiently overexpressed c-Fos from a constitutively active promoter, EF1, in NIH 3T3 cells and performed GST-Pin1 pull-down assays in total lysates from cells subjected to conditions that promote c-Fos phosphorylation. In fact, as shown in Fig. 2A (left lanes) Pin1 was able to strongly interact with the phosphorylated forms of c-Fos (slower migrating bands) generated by the stimulation with serum or PDGF and much weaker with c-Fos proteins from non-stimulated cells. Moreover, the interaction Fox-Pin1 was reduced dramatically when using a mutant c-Fos protein where all sites for ERK phosphorylation were replaced by alanine residues (c-Fos-m) (Fig. 2A, right lanes). To confirm that c-Fos can interact with Pin in vivo, we expressed the AU5-tagged c-Fos and c-Fos-m together with a GFP-tagged Pin in HEK-293T cells. As shown in Fig. 2B, low levels of GFP-Pin1 were already detected in c-Fos immunoprecipitates in these cells, reflecting a partial phosphorylated status of c-Fos when overexpressed in HEK-293T cells. A brief exposure to EGF provoked a substantial increase in the level of Pin1 co-immunoprecipitated with c-Fos. In contrast, no co-immunoprecipitation of Pin1 was detectable in cells expressing c-Fos-m even after EGF stimulation. In this regard, we have previously shown that this mutant form of c-Fos cannot be phosphorylated by ERK and therefore is impaired in its transcriptional response to ERK-dependent signals (8). The availability of this mutant protein allowed us to determine the primary structure of the murine c-Fos TAD (aa 229–380) and the location of the four potential ERK phosphorylation sites (S/T-P). His\(^{6}\)-tagged constructs bearing none (c-Fos-m) or single phosphoacceptor sites for ERK (c-Fos-T232, T235, T331, and S374) were obtained by site-directed mutagenesis, as described previously (8). C, in vitro analysis of the interaction of Pin1 with the purified c-Fos TAD. Bacterially expressed His\(^{6}\)-tagged c-Fos TAD fusion proteins, corresponding to those depicted in B, were used as substrates for the phosphorylating activity of recombinant purified ERK2 (active) in vitro kinase assays (see "Experimental Procedures") and then subjected to pull-down assays using beads of GST (data not shown, no signal was observed) or GST-Pin1 (above). Total His\(^{6}\) protein assayed in each pull-down reaction was confirmed by Western blot (WB) (below); note the mobility shift experienced by c-Fos proteins after phosphorylation by ERK2.

FIGURE 2. PDGF, EGF, and serum stimulate the interaction of Pin1 with c-Fos. A, requirement of c-Fos phosphorylation for Pin1 binding. As indicated, NIH 3T3 cells were transfected with pCEFL-AUS c-Fos and pCEFL-AUS c-Fos-m (c-Fos-m) (1 ng/well, starved overnight, and then stimulated with PDGF or serum (10% FBS) for 30 min. Cells were lysed, and aliquots were used for GST pull-down assays (right panel) and for the evaluation of c-Fos expression (left panel). Control (c), control from untransfected cells. B, EGF enhances the association of c-Fos with Pin1 in vivo. HEK-293T cells were transfected with pCEFL-GFP-Pin1 together with pCEFL vector (control (c)), pCEFL-AUS c-Fos (c-Fos-wt), or pCEFL-AUS c-Fos-m (c-Fos-m) (1 ng/plate), starved overnight, and then stimulated with EGF for 30 min. Cells were lysed, and aliquots were immunoprecipitated (IP) with an anti-AU5 antibody. Pin1 associated with c-Fos was detected by anti-GFP Western blot (WB) analysis of the anti-AU5 immunoprecipitates. Total levels of GFP-Pin1 and c-Fos expression were evaluated in total cell lysates (TCL). C, role of c-Fos phosphorylation in the effect of Pin1 on the activation of AP-1 by c-Fos. Cells were transfected with pCEFL-AUS-Fos-Pin1 and pCEFL-AUS-Fos-m (0.2 ng/well), in the absence or presence of pCMV-Sport-Pin1 (Pin1). Cells were serum-starved overnight and then stimulated with PDGF for 5 h before reading dual luciferase activities. Reporter assay data represent firefly luciferase activity normalized by Renilla luciferase activity present in each sample, expressed as absolute counts.

FIGURE 3. The phosphorylation of c-Fos by ERK is required for the binding of Pin1 and c-Fos. A, evaluation of the interaction between c-Fos and Pin after the phosphorylation of c-Fos by ERK in vivo. Cell lysates from c-Fos-wt or c-Fos-m overexpressing cells in the absence or presence of MEK EE ERK2 (0.2 ng each), as indicated, were subjected to GST pull-down assays (left) and analysis of c-Fos expression (right). Input, B, schematic representation of the c-Fos TAD used in this study, depicting the primary structure of the murine c-Fos TAD (aa 229–380) and the location of the four potential ERK phosphorylation sites (S/T-P). His\(^{6}\)-tagged constructs bearing none (c-Fos-m) or single phosphoacceptor sites for ERK (c-Fos-T232, T235, T331, and S374) were obtained by site-directed mutagenesis, as described previously (8). C, in vitro analysis of the interaction of Pin1 with the purified c-Fos TAD. Bacterially expressed His\(^{6}\)-tagged c-Fos TAD fusion proteins, corresponding to those depicted in B, were used as substrates for the phosphorylating activity of recombinant purified ERK2 (active) in vitro kinase assays (see "Experimental Procedures") and then subjected to pull-down assays using beads of GST (data not shown, no signal was observed) or GST-Pin1 (above). Total His\(^{6}\) protein assayed in each pull-down reaction was confirmed by Western blot (WB) (below); note the mobility shift experienced by c-Fos proteins after phosphorylation by ERK2.
The COOH-terminal TAD of c-Fos Mediates c-Fos Interaction with Pin: Role for the Phosphorylation of Specific S/T-P Sites—To confirm the requirement of c-Fos phosphorylation by ERK in the interaction between Pin1 and c-Fos, we forced the accumulation of phosphorylated species of c-Fos, which are readily detectable by SDS-PAGE as electrophoretically bands of apparent higher molecular weight, by transiently overexpressing c-Fos together with ERK2 and MEK EE, a constitutively active form of MEK1 that renders ERK2 permanently active. Fig. 3A shows that Pin1 preferentially binds to c-Fos when c-Fos is phosphorylated by ERK in vivo. Moreover, the interaction of c-Fos with Pin1 was decreased to basal levels when the c-Fos-m protein was assayed for its ability to interact with Pin under similar conditions (Fig. 3A).

As it is possible that c-Fos might bind to Pin1 indirectly, for example by binding to Jun proteins, we decided to explore whether Pin1 can bind to the c-Fos TAD in vitro. To this end, we performed GST-Pin pull-down assays using a purified preparation of bacterially expressed His$_x$-tagged forms of the c-Fos COOH-terminal TAD (Fig. 3B). Of interest, we first observed that the purified c-Fos TAD fusion protein was unable to interact with Pin1. However, a stable interaction between these two proteins was readily detectable when the c-Fos TAD was phosphorylated by activated ERK2 in vitro prior to assaying for its ability to bind Pin1 (Fig. 3C). Aligned with this finding, no detectable interaction was observed when an equivalent amount of c-Fos-TAD-m (c-Fos-m in Fig. 3B) was used as a Pin1 target, even in the presence of purified activated ERK2. These observations confirmed that Pin1 can bind directly to c-Fos and demonstrated that the COOH-terminal domain of c-Fos (aa 209–380) is insufficient to mediate this interaction, provided that it is first phosphorylated by ERK.

To study the contribution of each potential ERK phosphorylation site on the c-Fos TAD to the interaction between Pin1 and c-Fos, we generated mutant forms of the c-Fos TAD in which all ERK sites were mutated to alanine, and then each site was re-introduced individually (Table I), as depicted in Fig. 3B. By using this add-back approach, we have observed that each of these serines or threonines are independent in vivo targets for the enzymatic activity of ERK2 but that all are required for the transcriptional responsiveness of the c-Fos TAD to extracellular signals (8). The results from Fig. 3C indicate that the phosphorylation of individual sites by ERK was not sufficient to support its binding to Pin1, suggesting that the concerted phosphorylation of multiple sites on c-Fos are required to promote its interaction with Pin1.

Pin1 Stimulates c-Fos Activity Through Its Actions on the Phosphorylated c-Fos TAD—Based on these observations, we set out to investigate whether the TAD of c-Fos was the direct target of Pin1 function leading to changes in c-Fos-initiated transcription. For these experiments, we examined the effect of Pin1 on a Gal4 reporter system where the TAD of c-Fos is expressed as a fusion protein in frame with the DNA-binding domain (DBD) of the yeast transcription factor Gal4, and the activity of this chimera is assessed by its ability to stimulate transcription from a Gal4-regulated luciferase reporter plasmid (pGal4-Luc). Pin1 did not stimulate the basal activity of the Gal4-DBD at any of the concentrations tested (data not shown) but, as shown in Fig. 4A, synergistically cooperated with PDGF to further increase the transactivating function of the c-Fos TAD. Conversely, the fusion protein Gal4-c-Fos-TAD-m, containing alanine replacements in all potential ERK sites, failed to respond to Pin1 with an increase in transactivation after PDGF stimulation (Fig. 4B), all of which support the direct function of Pin1 on the activity of the c-Fos TAD upon its phosphorylation.

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