Actin filament associated protein mediates c-Src related SRE/AP-1 transcriptional activation

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Abstract

AFAP is an adaptor protein involved in cytoskeletal organization and intracellular signaling. AFAP binds and activates c-Src; however, the downstream signals of this interaction remain unknown. Here we show that co-expression of AFAP and c-Src induce transcriptional activation of SRE and AP-1 in a c-Src activity dependent fashion. Structural-functional studies suggest that the proline-rich motif in the N-terminus of AFAP is critical for c-Src activation, and subsequent SRE/AP-1 transactivation and the actin-binding domain in the AFAP C-terminus is negatively involved in the regulation of AFAP/c-Src mediated SRE/AP-1 transactivation. Selective deletion of this domain enhances transactivation of SRE. We conclude that in addition to its role in the regulation of cytoskeletal structures, AFAP may also be involved in the c-Src related transcriptional activities.

1. Introduction

Actin filament-associated protein (AFAP), originally cloned from chicken embryonic fibroblasts as a binding partner and a substrate of v-Src kinase, is an adaptor protein highly associated with actin filaments [1]. AFAP contains one Src Homology 3 (SH3) domain binding motif and two SH2 domain binding motifs in its N-terminus, two pleckstrin homology (PH) domains and another SH2 domain binding motif in the middle, and a leucine zipper motif and an actin binding domain (ABD) in its C-terminus [1,2]. This adaptor protein is involved in the regulation of actin filament integrity [3–8], podosome formation [9,10], and function of focal contacts [11]. AFAP is over-expressed in prostate cancer cells and may contribute to tumorigenic growth [11].

Previously, we have found that mechanical stretch rapidly activated c-Src in fetal rat lung cells with increased binding between c-Src and AFAP [12–14]. To determine the role of AFAP in mechanotransduction, we cloned rat and human AFAP genes [2,15], and demonstrated that AFAP can bind c-Src through its SH3 domain binding motif and its putative tyrosine phosphorylation sites in the N-terminus. A single amino acid mutation from proline to alanine at position 71 (AFAP71A) effectively blocked mechanical force-induced deformation of cytoskeletal structure can convert physical forces into biochemical reactions for signaling via specific protein–protein interactions [16]. Flynn and co-workers demonstrated that AFAP is involved in PKC-activation-induced c-Src activation [10,17]. Src family tyrosine kinases can mediate signals which control functions such as cell proliferation, cell death, differentiation, adhesion, migration, invasion, and cell cycle progression [18]. However, the down-stream signals of the AFAP/c-Src interaction have not been determined.

Several adaptors, such as CAS [19], Sin [20] and XB130 [21], can mediate Src kinase related SRE and AP-1 transcriptional activation [19,20,22]. SRE is located within the promoter region of many genes, such as c-fos [23,24]; it selectively binds the transcriptional factor serum response factor. AP-1 is a basic leucine zipper transcription factor that is composed of a homodimer or heterodimer of proteins from the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), or ATF (AP-1 transcription factor) family members [25,26]. SRE [24] and AP-1 [26] promoter sites are critical regulatory elements for transcription of genes responsive to mitogenic, transforming [23], proliferative [25,27], and cell survival signals. Therefore, we hypothesized that AFAP-induced Src activation

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leads to SRE/AP-1 transcriptional activation. The roles of the N-terminal SH3 domain binding motif and the functional domains/motifs in the C-terminus of AFAP in c-Src activation and SRE/AP-1 signaling were determined in the present study.

2. Materials and methods

2.1. Reagents, constructs and antibodies

Src (clone GD11) monoclonal antibody (mAb), phosphotyrosine (4G10) mAb, and horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Src phospho-Tyr416 and phospho-Tyr527 polyclonal antibodies were from Biosource International (Camarillo, CA). AFAP (F1) polyclonal antibody and the pCMV expression vectors for c-Src, wild type (WT) AFAP and its mutants were from Dr. Daniel C. Flynn (West Virginia University). SrcKD (kinase deficient) construct was from Dr. Andras Kapus (University of Toronto). SRE-Luc and AP-1-Luc plasmids were from Dr. Konstantina Alexandropoulos (Columbia University) [20]. β-Gal expression vector was from Invitrogen (Carlsbad, CA). All other reagents unless otherwise indicated, were purchased from Sigma (St. Louis, MO).

2.2. AFAPΔABD expression vector

pEGFP-c3-AFAPΔABD construct was generated from chicken full length pEGFP-c3-AFAP by deleting 2062–2112 bp (actin binding domain), with QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) [21]. The forward primer is 5′-CGT GCC ATT GAA GTC GAA GAG GAA TGT AAG ACG-3′ and the reverse primer is 5′-CGT CTT ACA TTC TTC TTC AAC TTC AGC AGC ACG-3′.

2.3. Cell culture and biochemical studies

COS7 cells were maintained in low glucose Dulbecco's modified Eagle's medium (GIBCO, Rockville, MD) with 10% fetal bovine serum. Human embryonic kidney 293 cells (HEK293 cells) were cultured in high glucose Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal bovine serum. Culture medium also contained penicillin (1 mg/ml) and streptomycin (1 mg/ml) (GIBCO) with 10% fetal bovine serum. Culture medium also contained penicillin (1 mg/ml) and streptomycin (1 mg/ml) (GIBCO) with 10% fetal bovine serum. Culture medium also contained penicillin (1 mg/ml) and streptomycin (1 mg/ml) (GIBCO) with 10% fetal bovine serum.

2.4. Statistics

Each experiment was performed at least three times. The quantification of each value represents the mean ± standard deviation (S.D.). Significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis or by Student's t-test where appropriate. P values less than 0.05 were considered as significant.

3. Results

3.1. AFAP/c-Src interaction-induced SRE/AP-1 transcriptional activation

To determine whether the interaction between AFAP and c-Src induces transcriptional activation of SRE and AP-1, we transiently transfected HEK293 cells with plasmids expressing AFAP, c-Src or Src kinase deficient (KD) mutant, together with an SRE or AP-1 binding site driven luciferase reporter (SRE-Luc or AP-1-Luc). The co-expression of AFAP and c-Src significantly increased the activities of SRE-Luc and AP-1-Luc, in comparison with either AFAP or c-Src alone. In contrast, cells co-transfected with AFAP and SrcKDK did not show such effect (Fig 1A). Phosphorylation of Src Tyrosine 416 (SrcY416) in the activation loop is a critical step for the full activation of Src kinase [30]. When COS7 cells were transfected with AFAP and c-Src (but not with SrcKDK), phosphorylation of SrcY416 was dramatically enhanced (Fig 1B). Consistent with previous findings [15], we also observed that total Src protein level was higher in cells co-transfected with AFAP and SrcKDK. It is possible that inactivated Src is not subject to proteasome-dependent degradation [31], and therefore accumulates to higher levels than wild-type Src.

A proline-rich region in the N-terminus of AFAP, PPQMPLPEIP (Fig 2A), shares high similarity with the consensus sequence of SH3 domain binding motif, a stretch of seven amino acid residues: RPLPXXP [32,33]. Mutation from proline to alanine at position 71 (AFAP71A) significantly decreased total protein tyrosine phosphorylation and phosphorylation of SrcY416 in COS7 cells [15]. As compared to co-expression of AFAP with c-Src, AFAP71A failed to increase SRE or AP-1 transcriptional activities (Fig 2B). Co-expression of AFAP71A and c-Src also reduced co-immunoprecipitation of these two proteins, as well as SrcY416 phosphorylation (Fig 2C). The presence of AFAP in the anti-Src immunoprecipitate was quantified by densitometry and expressed as ratio of AFAP over c-Src. A
significantly decreased interaction between AFAP71A and c-Src was observed in comparison with co-expression of AFAP and c-Src (Fig 2D). Taken together, these results suggest that AFAP through its SH3 domain binding motif can bind to c-Src and enhance its activity, which subsequently induces transcriptional activation of SRE and AP-1 in a c-Src kinase activity dependent manner.

3.2. C-terminus of AFAP regulates AFAP/Src interaction and activation

The C-terminus of AFAP contains several functional domains involved in self-association, direct binding, and cross-linking to actin filaments, which are related to and affected by Src activity [8]. We then investigated the role of the C-terminus of AFAP in c-Src binding and activation. A group of AFAP mutants with different truncations or deletion in its C-terminus (Fig 3A) were co-expressed with c-Src in COS7 cells. In comparison with AFAP or c-Src alone, co-expression of c-Src with WT AFAP increased total protein tyrosine phosphorylation and phosphorylation of SrcY416 (Fig 3B). Deletion of the last 115 amino acids of AFAP (AFAP \( D_{115} \)) significantly inhibited the co-expression induced total protein tyrosine phosphorylation, SrcY416 phosphorylation, and c-Src binding to AFAP (as determined by co-immunoprecipitation) (Fig 3B). Interestingly, with further truncations (AFAP \( D_{137} \) and AFAP \( D_{177} \)), the total protein tyrosine phosphorylation, SrcY416 phosphorylation, and AFAP/c-Src binding were gradually recovered (Fig 3B). Flynn and co-workers have shown that deletion of the leucine zipper motif between amino acids 554 and 594 of AFAP (Fig 3A) resulted in a dramatic increase in total protein tyrosine phosphorylation and cytoskeletal rearrangement [4,8]. In the present study, in addition to the total protein tyrosine phosphorylation, we found that the binding between AFAP\(\Delta\)lzip and c-Src was increased in
comparison with WT AFAP (Fig 3B and D). Western blotting of the whole cell lysates showed that AFAP and its mutants expressed at relatively similar levels. The ratio of SrcpY416/Src (Fig 3C), and the binding between AFAP (or its mutants) and c-Src were semi-quantified with a densitometric analysis (Fig 3D). These results suggested that different regions in the C-terminus of AFAP play distinct roles in affecting the interaction between AFAP and c-Src, and consequently influence c-Src activation.

3.3. AFAP C-terminal mutants mediate c-Src-induced SRE/AP-1 transcriptional activation with different efficiencies

Since these AFAP mutants resulted in different degrees of c-Src activation (Fig 3C), we sought to use these mutants to further determine whether AFAP/c-Src interaction affects SRE/AP-1 transcriptional activation in a c-Src activity dependent fashion. We examined SRE and AP-1 transcriptional activities by co-expression...
of c-Src with AFAP and its mutants in HEK293 cells. As expected, all mutants showed higher SRE/AP-1 transactivation in the presence of c-Src. To our surprise, although the AFAPΔ115 mutant was unable to enhance phosphorylation of Src at Y416 (Fig 3C) and was less capable of association with c-Src (Fig 3D), it retained full activity to enhance SRE-Luc and AP-1-Luc activities. More interestingly, the SRE and AP-1 transcriptional activities in cells co-transfected with c-Src and AFAPΔ137 were dramatically enhanced compared to that in cells co-transfected with c-Src and WT AFAP (Fig 4A). From Fig 3C, we can see that AFAPΔ137 was less capable to

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 4.** Deletion of actin binding domain in AFAP (AFAPΔABD) enhanced SRE transactivation. (A) Effects of C-terminal truncation of AFAP on c-Src related SRE/AP-1 transcriptional activation. HEK293 cells were transfected with constructs as indicated. AFAPΔ137/c-Src co-expression led to the highest SRE/AP-1 transcriptional activation. Results showed as the mean ± S.D. represent three independent experiments. **P < 0.01 compared with other groups. (B) Schematic representation of wild type as well as the actin binding domain deletion mutant (ΔABD) of AFAP. Izip: leucine zipper motif; ABD: actin binding domain. (C) Co-expression of AFAPΔABD and c-Src increased transcriptional activation of SRE. HEK293 cells were transfected with indicated plasmids and treated with or without EGF (10 ng/ml) for 12 h. Results represent three independent experiments showed as the mean ± S.D. Two way ANOVA: **P < 0.001 between EGF treated and untreated groups. *P < 0.05 than all other groups without EGF; #P < 0.05 than all other groups with EGF. (D) Proposed roles of AFAP in transcriptional activation of SRE/AP-1. AFAP associated actin filaments may effectively bind and activate c-Src. AFAP in the cytoplasm, free from cytoskeleton, may transmit signals from c-Src and/or other kinases to transcriptional activation of SRE/AP-1.
enhance c-Src activity as determined by phosphorylation of SrcY416. In contrast, although AFAPA177 or AFAPAΔlizp showed similar or higher ability to enhance c-Src-induced total protein tyrosine phosphorylation than WT AFAP did (Fig 3C), they did not further enhance SRE/AP-1 transactivation (Fig 4A). These over-expression studies suggest that AFAP may have two separate functions: (1) activation of c-Src via specific binding and (2) mediate c-Src activation induced downstream signals for SRE/AP-1 transcriptional activation.

In comparison with AFAPAΔ115, AFAPA137 has an extra 22 amino acids truncated (from residual 594 to 616), which contains an actin binding domain (ABD) identified by Flynn and co-workers [8]. To determine the potential function of this domain, we selectively deleted it from AFAP and generated an AFAPAΔABD construct fused with GFP (Fig 4B). We transiently transfected HEK293 cells with c-Src and pEGFP-AFAP or pEGFP-AFAPAΔBD, and then stimulated cells without or with EGF (10 ng/ml for 12 h). As shown in Fig 4C, co-expression of c-Src/AFAPAΔABD significantly increased transcriptional activity of SRE compared to all other groups (P < 0.05). EGF stimulation further enhanced SRE transactivation in comparison with untreated cells (P < 0.01). Similar results were obtained with AP-1 transactivation (data not shown).

4. Discussion

AFAP, actin filament-associated protein, is important to modulate changes in actin filament integrity in different cell types, such as fibroblasts, epithelial cells, endothelial cells and cells in hematopoietic lineages [6]. AFAP plays an important role in the regulation of cytoskeletal structures [3,9,11]. AFAP has been attributed to tumorigenic growth by regulating focal contacts [11]. The roles of AFAP in podosome formation further emphasize the importance of this protein in cancer cell migration and invasion [9,10]. Recent studies demonstrated that AFAP is also an important mediator for intracellular signal transduction, involved in mechanical force [2,15,16] or PKC-induced c-Src activation [10,17]. A novel finding of the present study is that overexpression of AFAP may enhance c-Src activation and lead to SRE/AP-1 transcriptional activation. This may open a new avenue in the study of AFAP related functions, because SRE and AP-1 are critical regulatory elements for the expression of genes that are mediators of various mitogenic, transforming and survival signals [24,26].

AFAP harbors a leucine zipper motif, an actin binding domain and a tail with unidentified function in its C-terminus [1]. The fact that the AFAPAΔ115 mutant was unable to enhance phosphorylation of c-Src (at Y416) and was less able to associate with c-Src suggests that the presence of the C-terminus is important to maintain the configuration of AFAP for its function. Interestingly, further truncations in the C-terminus of AFAP resulted in recovery of c-Src binding and activation. In comparison with the AFAPAΔ115, the actin binding domain was deleted in AFAPAΔ137 and the leucine zipper motif was further deleted in the AFAPAΔ177. The roles of these functional structures in c-Src binding and activation, and in c-Src-related SRE/AP-1 transactivation were further determined in the present study.

The actin binding domain of AFAP contains two motifs with a partial overlap: VNA G R K Q TV L ID L Y L K. The letters in italic show similarity with a consensus actin-binding motif termed ABD1 (actin-binding domain 1), while the letters with underline demonstrate similarity to ABD3 [8,34]. In the present study we found that the AFAPA137 mutant showed the highest efficiency to mediate c-Src-activation induced transcriptional activation of SRE/AP-1. Since both the ABD and the C-terminal tail were deleted in AFAPA137, we generated a new construct with the selective deletion of ABD. Similar to AFAPA137, AFAPAΔABD also significantly enhanced c-Src-related SRE/AP-1 transactivation.

The leucine zipper motif consists of a periodic repetition of a leucine residue at every seventh position and can form an α-helical conformation, which facilitates dimerization and in some cases higher oligomerization of proteins [35]. Deletion of the leucine zipper motif in AFAP induced a phenotype which resembled v-Src transformed cells by repositioning long actin stress fibers into podosomes [7]. In the present study, we demonstrated that deletion of the leucine zipper motif (either AFAPAΔ177 or AFAPAΔlizp) increased AFAP interaction with c-Src. However, the c-Src-related SRE/AP-1 transcriptional activation was not enhanced by deletion of the leucine zipper motif. Therefore, even though the leucine zipper motif and the actin binding domain are next to each other, they have distinct regulatory effects on AFAP-function.

It is likely that the association of AFAP with the F-actin is a dynamic process; certain amount of AFAP is associated with actin filament, while others are present in the cytoplasm. We speculate that AFAP associated with F-actin may be more effective in c-Src binding and activation, while AFAP free from actin filaments may be more effective to mediate SRE/AP-1 activation initiated by c-Src and other signaling pathways (Fig 4D). In conclusion, our results indicate that AFAP is not only involved in the regulation of cytoskeletal structure, but also involved in the regulation of transcriptional activation. This new function merits further investigations.

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