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Cooperative interaction of Zhangfei and ATF4 in transactivation of the cyclic AMP response element

Melissa R. Hogan, Gregory P. Cockram, Rui Lu*

Department of Molecular and Cellular Biology, University of Guelph, Axelrod Building, Room 334, 50 Stone Road East, Guelph, Ont., Canada N1G 2W1

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Abstract Zhangfei (ZF) is a basic region-leucine zipper protein that has been implicated in herpesvirus infection cycle and related cellular processes. Here we show both in vivo and in vitro data demonstrating that ZF is a novel cellular binding partner of activating transcription factor 4 (ATF4) (or CREB2). We found that ZF competed with ATF4 to form ATF4-ZF heterodimeric complexes through the bZIP regions. ZF enhanced ATF4 binding to the cAMP response element (CRE), and augmented activation of a CRE reporter by ATF4, in response to MEK1 activation. These results suggest an important role of ZF in the MEK1-ATF4 signaling pathway.

Keywords: Activating transcription factor 4; Cyclic AMP response element; Leucine zipper; Mitogen-activated protein/extracellular signal-regulated kinase kinase; Protein–protein interaction; Zhangfei

1. Introduction

Zhangfei (ZF) [1] is a basic region-leucine zipper (bZIP) protein identified through its association with the Herpes Simplex Virus-1 related host cell factor protein-1, HCF-1 (or C1) [2,3]. HCF-1 has been shown to be involved in transcriptional regulation during herpesvirus latency as well as and cellular processes such as cell cycle progression (reviewed in [4]). It has been shown that ZF is mimicked by the viral protein VP16 in its ability to bind HCF-1. This observation has since led to the hypothesis that VP16 may be involved in cellular signaling events associated with HSV-1 latency/re-activation and related cellular signaling events [1].

ZF possesses a bZIP region that exhibits significant sequence homology with other members of the CREB/ATF bZIP family. These bZIP transcription factors form homo- and heterodimeric complexes through pairing of their leucine-zipper motifs, creating a DNA contact surface of their adjacent basic regions. Like another bZIP cellular factor CHOP/GADD153 however, ZF can homodimerize but is unable to bind DNA as a homodimer [1]. We have thus postulated that ZF need heterodimerize with other factors in order to bind target promoters and regulate the downstream genes.

Here we present both in vitro and in vivo data demonstrating that ZF interacts with activating transcription factor 4 (ATF4) (or CREB2) [5–7] through their bZIP regions. We found that ZF enhanced ATF4 binding to the cAMP response element (CRE), and augmented the transactivation potential of ATF4 in response to activation of the mitogen-activated protein kinases signaling pathway by MEK1.

2. Materials and methods

Clone construction and site directed mutagenesis. The ATF4 cDNA was amplified by PCR and cloned into pcDNA3.1 (Invitrogen) and pGEX-KG to make pcATF4 and pGEX-ATF4. The plasmids pFLAG-ZF and pHA-C-AE1 are based on pcDNA3.1 which expresses ZF with *N*-terminal FLAG epitope tag, or HA and FLAG tags on both ends. The pRL-SV40 plasmid [8] has the Renilla (*Renilla reniformis*) luciferase gene under the control of the SV40 immediate early promoter. ZFΔbZIP and ATF4ΔbZIP were generated by site-directed mutagenesis using the QuikChange II system (Stratagene).

Cell culture and calcium phosphate transfection. HEK 293, HEK 293-T7, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (high glucose; Sigma) containing 10% (v/v) fetal bovine serum and 1% penicillin and streptomycin at 37 °C in a 5% CO_2 atmosphere. Cell cultures were grown to approximately 50–65% confluency prior to transfection using the calcium phosphate method [9,10].

Glutathione S-transferase (GST)-pulldown assay. Recombinant GST fusion proteins were produced in *Escherichia coli* BL21 (DE3) (Novagen) and purified using glutathione–Sepharose beads (Pharmacia). Proteins were ^{35}S-labeled using the TnT® Coupled Rabbit Reticulocyte Lysate System (Promega), and the pull down assays were performed as previously reported [11]. ZF and ATF4 proteins used in the competition assays were eluted from beads via thrombin cleavage. All protein sample concentrations were quantified by BioRad protein assay and by SDS–PAGE against bovine serum albumin standards using the Image Quant program (Amersham Biosciences).

Electrophoretic mobility shift assay (EMSA). Oligonucleotides representing the consensus cellular CRE sequence (5′-CCGGTGACGTCATCGCA) were annealed and end-labeled using [α-^{32}P]dCTP and the Klenow fragment of the *E. coli* DNA Polymerase (New England Biolabs). The assay was carried out using purified recombinant proteins described above, as reported previously [12].

Luciferase reporter assays. 40 h post-transfection, cell lysates were prepared and dual-luciferase® reporter assays were performed according to the manufacturer's protocol (Promega). Reporter activity was

*Corresponding author. Fax: +1 519 837 2075.
E-mail address: rlu@uoguelph.ca (R. Lu).

Abbreviations: ATF4, activating transcription factor 4; bZIP, basic region-leucine zipper; CRE, cyclic AMP response element; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; PERK, protein kinase-like endoplasmic reticulum kinase; UAS, upstream activation sequence; ZF, Zhangfei

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calculated as relative luciferase activity (firefly luciferase/*Renilla* luciferase) to correct for transfection efficiency. Assays were independently repeated at least three times and shown with standard errors.

Co-immunoprecipitation and immunoblotting. Cells were harvested and lysed 24 h post-transfection in the RIPA buffer for 20 min at 4 °C. Pre-cleared aliquots of lysate were incubated with specific immunoprecipitation antibodies for 12 h, followed by addition of protein G–Sepharose beads for 2 h at 4 °C. Proteins were detected by immunoblotting using ECL Plus (Amersham).

3. Results

3.1. ZF and ATF4 interact through their C-terminal bZIP domains

Recently, a potential association between the bZIP proteins ZF and ATF4 was suggested in a coiled-coil peptide array [13]; however, such high-throughput analyses are often prone to false positives and negatives. To assess if a *bona fide* interaction exists between these factors, we first chose to test it by competitive GST-pulldown assays, with GALAD-GFP and HCF-1 as controls (Fig. 1A). We found that radiolabeled ZF was able to bind ATF4 as well as itself (Fig. 1B). Addition of unbound ATF4 protein drastically reduced ZF binding to ATF4. Reciprocally, ^{35}S-labeled ATF4 could also interact with ZF and itself (result not shown). These results suggest that ZF and ATF4 interact through the same domain that is utilized in their homodimerization, i.e., the bZIP region.

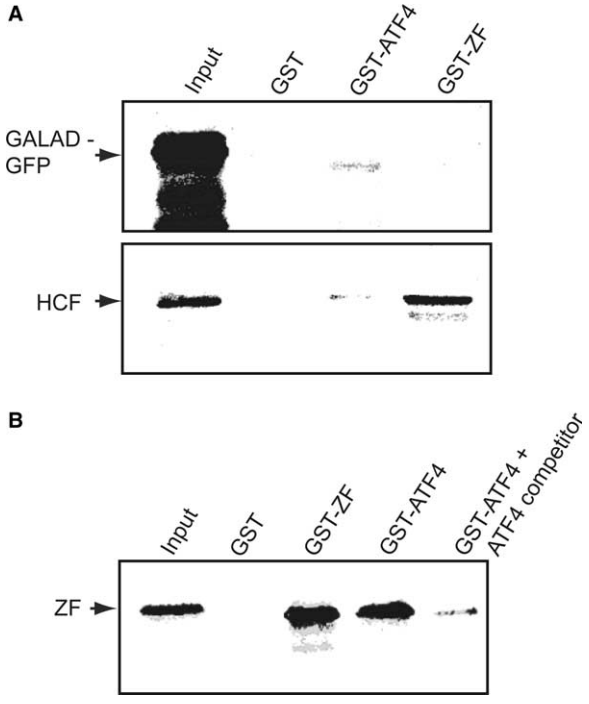

Fig. 1. ZF interacts with ATF4 in vitro as demonstrated by competitive GST-pulldown assays. GALAD-GFP and HCF-1 were used as negative or positive control respectively (A). In the competitive GST-pulldown assays (B) equal amount of bead-bound and eluted proteins were used as indicated. Bound proteins were eluted, resolved by SDS–PAGE and visualized using Typhoon 9400 phosphorimager. The "Input" lane represents 10% of the [^{35}S] methionine-labeled protein added to each respective assay.

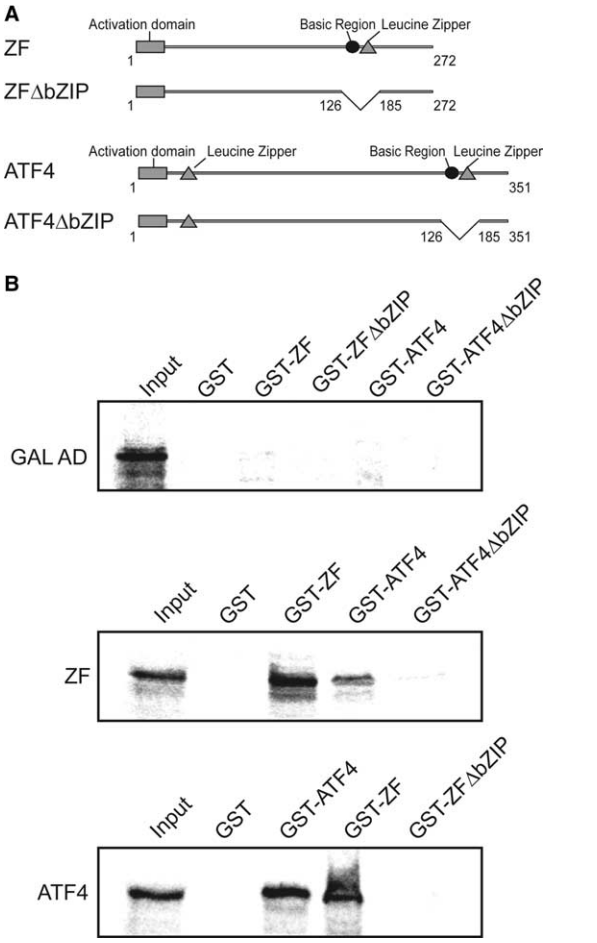

Fig. 2. ZF and ATF4 interact through their C-terminal bZIP domains. (A) Schematic illustration of ZF and ATF4 and their mutants. (B) GST-pulldown assays. Equivalent amount of bead-coupled proteins were used. Input lanes represent 10% of the [^{35}S]-labeled protein added to each pulldown assay.

To verify ZF and ATF4 indeed interact through their bZIP regions, mutants were generated in which the bZIP regions were removed (Fig. 2A). In a GST-pulldown experiment, we found that radiolabeled ZF and ATF4 could bind to the wild-type proteins, but not the bZIP deletion mutants (Fig. 2B). These observations indicate that ZF and ATF4 form classical bZIP dimers by interacting through their bZIP domains.

3.2. ZF associates with ATF4 in vivo

To examine whether ZF interacts with ATF4 within the cell, co-immunoprecipitation experiments were carried out. Endogenous ATF4 was induced in ZF-transfected HeLa cells using the endoplasmic reticulum (ER) stress-inducing agent tunicamycin [14]. We found that ZF could readily co-precipitate with ATF4, as detected by an ATF4 antibody (Fig. 3A). It was noticed that two prominent ATF4 bands were present in the ZF precipitate. These doublet bands likely represent two forms of ATF4 of different phosphorylation status, as reported previously [15]. Reciprocal immunoprecipitation in ATF4 and ZF-transfected cells by the ATF4 antibody also confirmed the result (Fig. 3B).