Phosphorylation of BATF regulates DNA binding: a novel mechanism for AP-1 (activator protein-1) regulation

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BATF is a member of the AP-1 (activator protein-1) family of bZIP (basic leucine zipper) transcription factors that form transcriptionally inhibitory, DNA binding heterodimers with Jun proteins. In the present study, we demonstrate that BATF is phosphorylated *in vivo* on multiple serine and threonine residues and at least one tyrosine residue. Reverse-polarity PAGE revealed that serine-43 and threonine-48 within the DNA binding domain of BATF are phosphorylated. To model phosphorylation of the BATF DNA binding domain, serine-43 was replaced by an aspartate residue. BATF(S43D) retains the ability to dimerize with Jun proteins *in vitro* and *in vivo*, and the BATF(S43D):Jun heterodimer localizes properly to the nucleus of cells. Interestingly, BATF(S43D) functions like wild-type BATF to reduce AP-1-

mediated gene transcription, despite the observed inability of the BATF(S43D):Jun heterodimer to bind DNA. These data demonstrate that phosphorylation of serine-43 converts BATF from a DNA binding into a non-DNA binding inhibitor of AP-1 activity. Given that 40 % of mammalian bZIP transcription factors contain a residue analogous to serine-43 of BATF in their DNA binding domains, the phosphorylation event described here represents a mechanism that is potentially applicable to the regulation of many bZIP proteins.

Key words: activator protein-1 (AP-1), basic leucine zipper, DNA binding domain, phosphorylation, transcription factor.

INTRODUCTION

AP-1 (activator protein-1) is a dimeric transcription factor composed primarily of the Jun (c-Jun, JunD, JunB) and Fos (c-Fos, FosB, Fra1, Fra2) classes of proteins [1]. AP-1 factors contain a bZIP (basic leucine zipper) motif that mediates protein dimerization and the subsequent binding of the dimer to a specific seven-nucleotide DNA sequence referred to as the TRE ['TPA' (PMA) responsive element] or AP-1 binding site [2,3]. Depending on the composition of the AP-1 dimer, binding to a target promoter can result in the activation or the inhibition of gene expression [4]. The Jun and Fos proteins display broad tissue distributions and have been shown to function in pathways regulating cell proliferation, transformation, differentiation and apoptosis [5,6].

BATF is a bZIP transcription factor that dimerizes with Jun-class factors to bind AP-1 DNA sites [7,8]. BATF does not form homodimers, nor does it dimerize with Fos-class factors [7]. Expression analysis of BATF mRNA and protein has revealed that the highest levels of expression are in haematopoietic tissues and cell lines [8,9]. Functional analysis of BATF in cell culture systems and in transgenic mice has demonstrated that BATF is an effective inhibitor of AP-1-mediated gene expression [8,9], and that cellular transformation by oncogenes that rely on robust AP-1 activity is blocked by co-expression of BATF [8]. Additional studies have described the induction of BATF following the viral infection of human B and T cells [10–12], by the activation of nuclear factor κ B in mouse pre-B cells [13], as a nuclear event triggered by signalling through the interleukin-6/gp130 receptor [14] and following the stimulation of CD4⁺ T cells [9]. Unfortunately, the precise genetic targets

regulated by the induction of BATF in these systems remain unknown.

Post-translational modifications that influence bZIP protein function are well documented [15]. For example, JNK (c-Jun N-terminal kinase) phosphorylates c-Jun and, in so doing, increases c-Jun protein stability and the transactivation potential of AP-1 [16]. Other bZIP factors, including c-Fos, ATF2 (activating transcription factor 2) and CREB (cAMP response element binding protein), are affected similarly by discrete phosphorylation events [17–19]. In addition to phosphorylation, modification by ubiquitination, sumoylation, O-linked glycosylation and reduction/oxidation (redox) have an impact on the activity of bZIP factors [20–23]. In the case of redox, the DBDs (DNA binding domains) of approx. 55 % of mammalian bZIP factors contain a conserved cysteine residue that is redox-regulated *in vivo*. DNA binding is inhibited by the oxidation of this cysteine in both proteins of the bZIP dimer [24,25].

In the present study, we demonstrate that BATF is phosphorylated on several residues *in vitro* and *in vivo*. In particular, the DBD of BATF contains a phosphorylated serine at position 43, which is in an analogous position to the aforementioned redox-regulated cysteine in other bZIP proteins, including c-Jun and c-Fos. Substitution of an acidic residue for serine-43 generates a protein that dimerizes with Jun to form an AP-1 complex that localizes properly to the nucleus and retains the ability to inhibit AP-1-mediated gene expression, yet is unable to bind AP-1 target DNA. This finding is significant, since 90 % of the bZIP proteins that are not potentially regulated by the reduction/oxidation of a cysteine within their DBDs possess either a serine or a tyrosine at the analogous position, and thus may be subject to regulation through phosphorylation.

Abbreviations used: AP-1, activator protein 1; ATF, activating transcription factor; BiFC, bimolecular fluorescence complementation; bJun, c-Jun-(257–318); bFos, c-Fos-(118–210); bZIP, basic leucine zipper; CBB, Coomassie Brilliant Blue; C/EBP, CCAAT/enhancer binding protein; CIAP, calf intestinal alkaline phosphatase; CMV, cytomegalovirus; DAPI, 4',6'-diamidino-2-phenylindole; DBD, DNA binding domain; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HA, haemagglutinin; IEF, isoelectric focusing; IPG, immobilized pH gradient; MT-BATF, Myc-tagged BATF; TBS, Tris-buffered saline; YFP, yellow fluorescent protein; YC, YFP-(155–238); YN, YFP-(1–154).

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EXPERIMENTAL

Cell lines and constructs

Jurkat T cells and EL-4 mouse thymoma cells were maintained in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum, 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. C3H10T1/2 and HeLa cells were maintained and transfected in basal minimal essential medium supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. All media and supplements were obtained from Gibco/Invitrogen (Carlsbad, CA, U.S.A.).

The CMV (cytomegalovirus) promoter construct containing the coding sequence of human BATF fused to six Myc epitope tags at the 5' end (pCS2MT-BATF) was described previously [12]. CMV c-Jun HA (haemagglutinin antigen) was a gift from S. Rhodes (IUPUI, Indianapolis, IN, U.S.A.). pEMSVscribe α 2 constructs expressing c-Jun, c-Fos and BATF (pEM c-Jun, pEM c-Fos and pEM BATF respectively) and the AP-1 reporter gene (3xTRE-Lux) were described previously [8]. pCS2MT-BATF and pEM BATF variants containing various mutations in BATF (S43A, S43D, T48A and S43A/T48A) were generated using the Quik-Change mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The pGEX-2T1 plasmid for the bacterial expression of full-length or truncated BATF fused to GST (glutathione S-transferase) was described previously [7]. pCDNA3-OS-9 contains the full-length human OS-9 cDNA subcloned into pcDNA3 (Invitrogen) using *Hind*III and *Kpn*I. The bacterial expression plasmids for His₆-tagged c-Jun and His₆-tagged c-Fos (pDS56 c-Jun/c-Fos) were obtained from T. Curran (St. Jude Children's Research Hospital, Memphis, TN, U.S.A.). For bacterial expression of His₆-tagged BATF or His₆-tagged BATF(S43D), cDNAs were subcloned into the *Bam*HI/*Hind*III sites of the pET30A plasmid (Novagen, Madison, WI, U.S.A.) in-frame with the N-terminal His₆ tag to generate pET30A BATF and pET30A BATF(S43D).

Transfections and luciferase assays

For EL-4 mouse thymoma cells, 2.5×10^6 cells were electroporated (300 V, 900 μ F) with 15 μ g of plasmid DNA. For HeLa and C3H10T1/2 cells, 1×10^5 cells were seeded in six-well plates and after 24 h were transfected using calcium phosphate DNA precipitation as described previously [8] with 0.5 μ g of pRL0-null, 1 μ g of AP-1 reporter and 1 μ g each of pEM c-Jun, pEM c-Fos, the indicated BATF plasmid or vector DNA. After 5 h, the medium was replaced and the cells were harvested for luciferase assays at 36 h. Luciferase activity was measured and normalized using the dual-activity luciferase kit (Promega, Madison, WI, U.S.A.). All activities are expressed relative to activation by c-Fos and c-Jun, which was set at 100, and represent the means of at least three independent transfections. Error bars indicate S.D.

Metabolic labelling

Metabolic labelling was performed as described in [26]. Prior to labelling, mouse EL-4 thymoma cells were electroporated with pCS2MT-BATF. At 4 h after electroporation, cells were transferred to 1 ml of phosphate-free RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum. After an additional 1 h, 2 mCi of [³²P]P_i (8500 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) was added to the cultures and incorporation proceeded for 4 h before treatment with 1 μ M okadaic acid (Acros, Hanover Park, IL, U.S.A.) for

30 min at 37 °C. Cells were washed twice in ice-cold TBS (Tris-buffered saline) and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 % sodium deoxycholate, 1 % Triton X-100 and 0.1 % SDS) supplemented with 0.1 % phosphatase inhibitor cocktail 1, 0.1 % phosphatase inhibitor cocktail 2 and 0.1 % general protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, U.S.A.). Insoluble material was cleared by centrifugation at 14 000 g for 10 min at 4 °C.

Immunoprecipitation

MT-BATF (Myc-tagged BATF) was immunoprecipitated by incubating whole-cell lysates with the anti-Myc monoclonal antibody 9E10 (A.T.C.C., Manassas, VA, U.S.A.) bound to Protein A-Sepharose (Amersham Pharmacia Biotech) for 90 min at 4 °C. The precipitates were washed extensively with RIPA buffer supplemented with 0.1 % phosphatase inhibitor cocktail 1, 0.1 % phosphatase inhibitor cocktail 2 and 0.1 % general protease inhibitor cocktail, and proteins were eluted by boiling in 1.5 $\times$ SDS sample buffer for 5 min. Proteins were resolved by SDS/12.5 %-PAGE, transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.) and visualized by autoradiography or immunoblot.

Two-dimensional electrophoresis

Transgenic mice expressing HA-tagged BATF in the thymus have been described previously [9]. Transgenic thymus tissue was homogenized in RIPA buffer and the protein concentration measured (Bio-Rad). A sample of 100 μ g of protein was diluted 4-fold with restriction buffer 4 (New England Biolabs, Beverly, MA, U.S.A.) and incubated at 37 °C for 90 min, with or without 5 units of CIAP (calf intestinal alkaline phosphatase; Calbiochem, San Diego, CA, U.S.A.). For two-dimensional PAGE, 13.2 μ g of each protein sample was diluted to a final volume of 125 μ l in IPG (immobilized pH gradient) gel buffer containing 8 M urea, 2 % (v/v) CHAPS, 10 mM dithiothreitol and 0.2 % (w/v) Bio-Lytes, pH 3–10 (Bio-Rad). Proteins were focused using the Protean IEF (isoelectric focusing) Cell (Bio-Rad) and Ready-Strip IPG strips (7 cm), pH 5–8 (Bio-Rad). After IEF, proteins in the IPG strips were separated by SDS/12.5 %-PAGE and blotted to a PVDF membrane (Bio-Rad) for immunoblot analysis.

Immunoblot analysis

Immunoprecipitates or whole-cell lysates resolved by SDS/12.5 %-PAGE were transferred to nitrocellulose or PVDF membranes and immunoblotted as described in [27]. As primary monoclonal antibodies, anti-Myc (9E10), anti-HA (12CA5; Babco, Richmond, CA, U.S.A.) and anti-Flag (M2; Sigma) were used at a dilution of 1:1000. Secondary antibodies conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, U.S.A.) were used at a dilution of 1:5000. Signals were visualized using chemiluminescence super signal reagent (Pierce, Rockford, IL, U.S.A.) and autoradiography.

Phosphopeptide mapping

Phosphopeptide mapping was performed as described [26,28]. Following digestion of radiolabelled MT-BATF with 1 μ g of endoproteinase Asp-N (Acros) in 50 mM NH₄HCO₃ for 4 h at 37 °C, phosphopeptides were resolved by alkaline 40 %-PAGE for 14 h at 12 mA and visualized by autoradiography.

Reverse-polarity PAGE was performed on Asp-N-digested MT-BATF as described in [29].

Purification of proteins from bacteria

Full-length and truncated GST-BATF fusion proteins were expressed in *Escherichia coli* BL21 (DE3) and batch-purified using glutathione affinity chromatography as described [7]. Recombinant His₆-c-Jun, His₆-c-Fos, His₆-BATF and His₆-BATF(S43D) were expressed in *E. coli* M15 or BL21 and purified by nickel chelate chromatography as described in [24]. The purified proteins were analysed by SDS/PAGE and visualized by CBB (Coomassie Brilliant Blue) staining.

Solid-phase kinase assays

Solid-phase kinase assays were performed using a modification of a protocol described previously [16]. Briefly, 2×10^6 Jurkat T cells were lysed by rocking at 4 °C for 20 min in 100 μ l of kinase buffer (50 mM Hepes, pH 7.5, 300 mM NaCl, 20 μ M EDTA, 0.1 % Triton X-100, 0.1 % phosphatase inhibitor cocktail 1, 0.1 % phosphatase inhibitor cocktail 2 and 0.1 % protease inhibitor cocktail). Lysates were cleared by centrifugation at 14 000 g at 4 °C for 10 min. A sample of 1 μ g of each GST fusion protein bound to glutathione-agarose resin was incubated for 30 min at 30 °C with 20 μ l of lysate, 10 μ M ATP, 10 μ M MgCl₂ and 5 μ Ci of [γ -³²P]ATP (6000 Ci/mmol; PE/DuPont/NEN, Boston, MA, U.S.A.). The resin was washed five times with kinase buffer, boiled in 1.5 $\times$ SDS sample buffer and resolved by SDS/12.5 % PAGE. Equal protein loading was confirmed by CBB staining. The gel was dried and radiolabelled proteins were visualized by autoradiography.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described in [30].

Protein interaction assays

In vitro transcription/translation reactions were performed using the SP6 TNT quick-coupled *in vitro* transcription/translation system (Promega). For each protein, 1 μ g of DNA was added to 20 μ l of reaction mixture and incubated for 60 min at 30 °C in the presence of [³⁵S]methionine (1175 Ci/mmol; ICN, Irvine, CA, U.S.A.). An aliquot of 10 μ l of each reaction mixture was incubated with rocking for 90 min at 4 °C with 1 μ g of His₆-c-Jun bound to nickel-agarose resin in 500 μ l of RIPA buffer supplemented with 0.1 % general protease inhibitor cocktail. The resin was washed five times with 500 μ l of RIPA buffer and protein was eluted by boiling in 15 μ l of 1.5 $\times$ SDS sample buffer for 5 min. Samples were resolved by SDS/12.5 % PAGE and visualized by autoradiography.

EMSA (electrophoretic mobility shift assay)

EMSAs were performed using the gel-shift assay system (Promega). A double-stranded AP-1 oligonucleotide was radio-labelled using [γ -³²P]ATP (6000 Ci/mmol; Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England Biolabs). Unincorporated radioactivity was removed using a Sephadex G-50 spin column (Sigma). Bacterially purified His₆-c-Jun, His₆-c-Fos, His₆-BATF and His₆-BATF(S43D) proteins were allowed to dimerize in the absence of DNA for 10 min at room temperature in

4 % (v/v) glycerol, 1 mM MgCl₂, 500 μ M EDTA, 500 μ M dithiothreitol, 50 mM NaCl, 10 mM Tris/HCl, pH 7.5, and 50 μ g/ml poly(dI-dC) (Amersham Pharmacia Biotech). A 2 μ l aliquot of DNA was added to the proteins and incubated for an additional 20 min. Complexes were resolved using a non-denaturing 4 % (w/v) acrylamide gel (acrylamide/bisacrylamide, 30:1) and visualized by autoradiography.

Indirect immunofluorescence

HeLa cells were plated at 2×10^5 cells per 60 mm dish and, after 24 h, were transiently transfected as described in [12] with 5 μ g of the indicated plasmid DNA. At 24 h post-transfection, the cells were fixed for 20 min at room temperature with 3.7 % formaldehyde and permeabilized for 5 min at room temperature with 0.5 % Triton X-100. The cells were incubated for 1 h at room temperature with a 1:50 dilution of anti-HA or anti-Flag antibody in TBS containing 1 % (w/v) BSA. After extensive washes with TBS, the cells were incubated for 30 min at room temperature with a 1:250 dilution of Texas Red-conjugated anti-mouse IgG (TI-2000; Vector Laboratories). DAPI (4,6-diamidino-2-phenylindole; Sigma) was applied to stain DNA. The cells were viewed using a fluorescence microscope.

BiFC (bimolecular fluorescence complementation)

BiFC was performed in HeLa cells as described in [31]. The pBiFC-bJunYN155 and pBiFC-bFosYC155 vectors were obtained from T. Kerrpola (University of Michigan, Ann Arbor, MI, U.S.A.) and have been described previously [31]. Using the YC155 vector, expression plasmids were generated in which wild-type and variant BATF proteins are expressed as HA-tagged fusion proteins with YC [YFP-(155–238)], the C-terminal peptide of YFP (yellow fluorescent protein). A construct also was generated (BATF-YN) in which BATF is expressed as a Flag-tagged fusion protein with YN [YFP-(1–154)], the N-terminal portion of YFP. The indicated combinations of plasmids were transfected into HeLa cells. At 24 h following transfection, dimerization of the expressed proteins was visualized in living cells using fluorescence microscopy and a YFP filter set.

RESULTS

In vivo phosphorylation of BATF

Previously, our laboratory generated and characterized transgenic mice overexpressing HA-tagged BATF in the thymus [9]. Analysis by SDS/PAGE of the HA-BATF protein expressed in these animals revealed three distinct forms of the protein [9]. Since phosphorylation events have been shown to alter the mobility of some proteins, this result suggested the possibility that BATF is phosphorylated *in vivo*. A whole-cell lysate from transgenic mouse thymus was incubated with or without CIAP, resolved by SDS/PAGE and immunoblotted for HA-BATF. As shown in Figure 1(A), CIAP treatment abolished the BATF form with lower mobility, while the faster-migrating forms remained unaffected. These results indicate that BATF is indeed phosphorylated *in vivo*.

To expand this observation, two-dimensional gel electrophoresis was performed to estimate the number of BATF phosphorylation events. Thymus extracts from transgenic mice were treated or not with CIAP and then resolved in the first dimension by IEF and in the second dimension by SDS/PAGE. HA-BATF proteins were detected by anti-HA immunoblot. With the control sample, HA-BATF was resolved as two molecular mass species

Figure 1 BATF is phosphorylated *in vivo*

(A) Thymus extracts prepared from wild-type (left lane) and BATF-transgenic (middle and right lanes) mice were treated with (+) or without (–) CIAP, resolved by 12.5 %–PAGE and immunoblotted with anti-HA monoclonal antibody (α -HA) to detect BATF. The migration of molecular mass standards (kDa) is indicated on the left of the panel. (B) Thymus tissue extracts from BATF transgenic mice were treated with (lower panel) or without (upper panel) CIAP and resolved in two dimensions using IEF (pH 5–8) and SDS/12.5 %–PAGE. The migration of molecular mass standards (kDa) is indicated on the left of each panel. BATF protein was detected by immunoblotting with anti-HA antibody (α -HA).

and around ten distinct isoelectric points, with species with nine isoelectric points migrating with a higher molecular mass (Figure 1B). Following CIAP treatment, only two molecular mass species, each with a different isoelectric point, were detected. These data demonstrate that the majority of the isoelectric points displayed by HA–BATF correspond to different phosphorylated forms of the protein. It has not been determined why there are two phosphatase-resistant forms of HA–BATF. However, the consistent observation of these two forms in our experiments suggests that BATF is targeted for additional, post-translational modification(s).

In vitro phosphorylation of BATF

To determine the specific regions of BATF that are phosphorylated, *in vitro* solid-phase kinase assays were performed (see the Experimental section for details). For these studies, Jurkat T cell lysates served as the source of kinase and full-length GST–BATF(1–125) or GST–BATF truncation mutants served as the substrates. Endogenous BATF is expressed in Jurkat T cells [12] and, therefore, these cells should contain the physiologically relevant kinases. BATF truncation mutants included N-terminal peptides containing (residues 1–49) or excluding (residues 1–31) the BATF DBD, and a C-terminal peptide containing the BATF leucine zipper (residues 49–125) (Figure 2A). The radiolabelled samples were resolved by SDS/PAGE and equal loading of substrates was confirmed by CBB staining (Figure 2B, upper panel). The extent to which each protein was phosphorylated was examined by autoradiography. Native GST showed no detectable phosphorylation and, as expected, GST–BATF(1–125) exhibited the greatest level of phosphorylation (Figure 2B, lower panel). Of the truncated proteins, GST–BATF(1–31)

Figure 2 The DBD of BATF is phosphorylated *in vitro*

(A) Schematic representation of GST–BATF fusion proteins, including the location of the DBD and leucine zipper (LZ) motifs. (B) Solid-phase kinase assays were performed using Jurkat T cell lysates as a source of kinase and GST–BATF proteins as substrates. CBB staining confirmed equal loading of substrates, and radiolabelled proteins were visualized by autoradiography (32 P). The migration of molecular mass standards (kDa) is indicated on the right of each panel. (C) Phosphoamino acid analysis of GST–BATF(1–125) phosphorylated by Jurkat T cell lysates. Arrows labelled S, T and Y indicate the migration of phosphoserine, phosphothreonine and phosphotyrosine respectively.

showed relatively low levels of phosphorylation when compared with GST–BATF(1–49) and GST–BATF(49–125). These data indicate that inclusion of the DBD in the fusion protein results in additional phosphorylation events.

BATF is a small protein with an amino acid composition in which serine, threonine and tyrosine constitute 22 % of residues. To establish a phosphoamino acid profile for BATF, *in vitro* phosphorylated GST–BATF(1–125) was subjected to phosphoamino acid analysis (Figure 2C). Phosphoserine was the most abundant phosphoamino acid; however, significant amounts of phosphothreonine and a detectable level of phosphotyrosine were also observed. The DBD of BATF contains a serine residue at position 43 and a threonine at position 48. Since appending the DBD to GST–BATF(1–31) increases phosphorylation of the resultant GST–BATF(1–49) protein (Figure 2B), it is likely that one, or both, of these residues are phosphorylated by T cell lysates.

In vivo phosphorylation of the BATF DBD

The *in vitro* phosphorylation data suggested that the DBD of BATF may be phosphorylated on serine-43 and/or threonine-48. To establish if the phosphorylation of these amino acids occurs *in vivo*, MT-BATF and MT-BATF variants in which serine-43 and/or threonine-48 were changed to alanine were expressed in EL-4 cells and metabolically labelled with [32 P]P_i (Figure 3A). The proteins were immunoprecipitated from cell extracts and subjected to phosphoamino acid analysis. Asp-N was the endo-proteinase chosen, since it is predicted to generate a set of BATF peptides with the DBD isolated on a single, positively charged peptide (Figure 3B).

Figure 3 The DBD of BATF is phosphorylated *in vivo*

(A) *In vivo* phosphorylation of MT-BATF, MT-BATF(S43A), MT-BATF(T48A) and MT-BATF(S43A, T48A) in transfected EL-4 mouse thymoma cells. Radiolabelled samples were immunoprecipitated, resolved by SDS/PAGE and visualized by autoradiography. (B) Predicted peptides and net charge of Asp-N-digested MT-BATF. M.W., molecular mass (Da). (C) Radiolabelled MT-BATF or variants digested with Asp-N were resolved using 40% polyacrylamide alkaline PAGE. Phosphopeptides were visualized by autoradiography. (D) Asp-N-digested, radiolabelled MT-BATF and BATF variants were resolved by reverse-polarity PAGE and peptides were visualized by autoradiography.

The peptide containing the DBD of BATF is difficult to resolve using conventional one-dimensional phosphopeptide mapping techniques [28] due to its net positive charge. Therefore, instead of using alkaline PAGE, which resolves peptides on the basis of size and net negative charge, we developed reverse-polarity PAGE [29] to take advantage of the net positive charge of the DBD and to separate this fragment from other BATF peptides. The technique has already been used successfully to establish that the BATF DBD is phosphorylated *in vivo* [29]. In the current experiments, the technique was used to examine the phosphorylation state of the BATF(S43A), BATF(T48A) and BATF(S43A, T48A) variants. As shown in Figure 3(C), the resolution of phosphorylated Asp-N-generated BATF peptides by 40% polyacrylamide alkaline PAGE produced a complex, but identical, banding pattern for all proteins. By reverse-polarity PAGE, however, the DBD was the only peptide resolved and was phosphorylated in all proteins except the BATF(S43A, T48A) double mutant (Figure 3D). These results clearly demonstrate that both serine-43 and threonine-48 within the DBD of BATF can be phosphorylated *in vivo*. The similar migration of the wild-type and mutant DBDs would suggest that BATF is phosphorylated on serine-43 or threonine-48, but not simultaneously on both residues. However, it remains formally possible that the resolution of this gel system is not sufficient to distinguish singly compared with doubly phosphorylated forms of the BATF DBD.

Phosphorylation of serine-43 prevents DNA binding and sequesters dimer partners away from target DNA

To model the effects of phosphorylation at serine-43 in BATF, the residue was changed to alanine or aspartate to mimic the unphosphorylated or phosphorylated states of BATF respectively (Figure 4A). *In vitro* protein binding assays were performed to examine the effects of the S43A and S43D mutations on protein dimerization with c-Jun. Wild-type MT-BATF, MT-BATF variants and control c-Fos and c-Jun proteins were ^{35}S -labelled by *in vitro* translation and then incubated with His₆-c-Jun protein bound to nickel-agarose. Bound proteins were resolved by SDS/PAGE and visualized by autoradiography (Figure 4B). MT-BATF, MT-BATF(S43A), MT-BATF(S43D) and the positive control c-Fos protein bound to His₆-c-Jun. Consistent with previous reports [7,24], c-Jun homodimer formation is much less efficient than c-Jun heterodimer formation with Fos, BATF or, as demonstrated here, the BATF(S43D) variant. These data suggest that substitution of a negatively charged residue at position 43 of BATF (S43D) does not affect heterodimer formation with c-Jun.

To establish that the BATF(S43D) protein binds to c-Jun *in vivo*, co-immunoprecipitation assays were performed. EL-4 cells were transfected with MT-BATF or MT-BATF(S43D) alone or in the presence of HA-tagged c-Jun. Jun protein complexes were immunoprecipitated using anti-HA antibody, resolved by SDS/PAGE and transferred to a PVDF membrane. The membrane was immunoblotted first with anti-HA to detect Jun and then stripped and reprobed with anti-Myc to detect BATF. As shown in Figure 4(C), MT-BATF and MT-BATF(S43D) were detected exclusively in extracts with HA-c-Jun. This is consistent with the *in vitro* binding studies and demonstrates that BATF(S43D) dimerizes efficiently with c-Jun *in vivo*.

The dimerization of AP-1 partner proteins is necessary to align adjacent DBDs for sequence-specific DNA binding. To address whether BATF(S43D):Jun heterodimers bind to DNA, EMSAs were performed. Equal amounts of bacterially purified His₆-tagged c-Jun, His₆-tagged c-Fos, His₆-tagged BATF and His₆-tagged BATF(S43D) proteins were incubated alone, or in the indicated combinations, with a ^{32}P -labelled AP-1 oligonucleotide probe. Protein-DNA complexes were resolved on non-denaturing polyacrylamide gels and visualized by autoradiography (Figure 4D). As predicted, since c-Fos and BATF are unable to form homodimers, they did not bind DNA. Consistent with previous reports [7,24], inefficient homodimer formation by c-Jun resulted in weak binding to AP-1 DNA. Importantly, both Fos:Jun and BATF:Jun heterodimers generated AP-1 shifts with distinct mobilities due to differences in the mass and/or charge of the complexes [7-9]. In contrast, no shift was observed for BATF(S43D) and Jun (Figure 4D). Based on the observed dimerization properties of these proteins *in vitro* and *in vivo* (Figures 4B and 4C), we conclude that the negatively charged residue at position 43 prevents the BATF(S43D):Jun complex from making stable contact with DNA.

BATF functions to negatively regulate AP-1 activity [8,9]. To examine the impact of the BATF(S43A) and BATF(S43D) variants on AP-1-mediated transcription, C3H10T1/2 mouse fibroblasts were transfected with an AP-1 luciferase reporter gene and the indicated combinations of vectors expressing c-Jun, c-Fos and BATF proteins. Cells were harvested for immunoblot analysis to assess equivalent expression of the BATF proteins (Figure 4E, lower panel) and for the measurement of luciferase activity (Figure 4E, upper panel). Activation of the reporter gene by Fos and Jun was set to 100. As expected, BATF and BATF(S43A) were able to compete effectively with Fos for Jun, bind to the reporter gene and inhibit luciferase expression. Interestingly, expression

Figure 4 Effect of phosphorylation of serine-43 on BATF function

(A) Serine-43 within the DBD of BATF was changed to alanine (A) or aspartate (D) to mimic an unphosphorylated or phosphorylated residue respectively. LZ refers to the leucine zipper motif. (B) His₆-c-Jun fusion protein bound to nickel-agarose beads was incubated with *in vitro*-synthesized, ³⁵S-labelled c-Fos, c-Jun, MT-BATF (BATF), MT-BATF(S43A) or MT-BATF(S43D). A sample of 0.1 vol. of each labelled protein used for the binding assays was resolved by SDS/PAGE (upper panel). Bound proteins were resolved by SDS/PAGE and visualized by autoradiography (lower panel). (C) MT-BATF (B) and MT-BATF(S43D) (D) were expressed in mouse EL-4 thymoma with (J) or without (-) HA-tagged c-Jun. Protein complexes were immunoprecipitated (IP) with anti-HA antibody (α-HA) and immunoblotted (IB) with α-HA to detect Jun (upper panel) and anti-Myc antibody (α-Myc) to detect BATF (lower panel). The migration of molecular mass standards (kDa) is indicated on the right of each panel. (D) EMSAs using ³²P-labelled AP-1 DNA as the probe and the indicated combinations of bacterially purified His₆-tagged c-Jun (J), His₆-tagged c-Fos (F), His₆-tagged BATF (B) and His₆-tagged BATF(S43D) (D) proteins. Arrows indicate the migration of Jun:Jun (J:J), Fos:Jun (F:J) and BATF:Jun (B:J) AP-1 DNA complexes. (E) C3H10T1/2 cells were transfected with the AP-1 luciferase reporter gene along with the indicated combinations of expression vectors for c-Jun (J), c-Fos (F), MT-BATF (B), MT-BATF(S43A) (A) or MT-BATF(S43D) (D). The activation by Fos and Jun is set to 100, and all other activities are expressed relative to that value. The data represent the means of three independent transfections per group; bars denote S.D. The lower panel shows the result of an anti-Myc immunoblot detecting each MT-BATF protein in the C3H10T1/2 cells extracts. The migration of molecular mass standards (kDa) is indicated on left of the panel.

of BATF(S43D) inhibited reporter gene expression to the same extent as the other BATF proteins, even though the BATF(S43D):Jun complex does not bind AP-1 DNA. These results suggest that the inhibition of a simple AP-1 reporter gene by BATF occurs independently of DNA binding. In the context of a complex promoter, however, inhibition of AP-1 by sequestering partners away from DNA may have a different impact on gene expression.

Phosphorylation of serine-43 does not alter the cellular location of BATF or BATF:Jun heterodimers

Previous reports have demonstrated that a stretch of basic amino acids within the DBD of Jun proteins functions as a nuclear

localization signal [32,33], and that a phosphorylation event adjacent to the nuclear localization signal in v-Jun shifts a fraction of the protein from the nucleus to the cytoplasm of the cell [33]. Therefore it was of interest to determine if phosphorylation of serine-43 changes the intracellular location of BATF, or if the juxtaposition of the BATF phosphorylation event with the Jun DBD alters the location of a BATF:Jun heterodimer in cells. To accomplish these objectives, we employed a recently described technique, BiFC [31]. In this technique, individual proteins are expressed as fusions with one of two complementary portions of YFP. Various combinations of these proteins are expressed in cells and, if a protein-protein interaction occurs, the YFP chromophore is reconstituted and fluorescence is observed. Vectors were

Figure 5 The subcellular location of BATF and BATF-containing AP-1 complexes is not altered following mutation of the BATF DBD

(A) Schematic representation of YFP fusion proteins used for these *in vivo* localization experiments (see the Experimental section for details). The BATF-YN and bJun-YN fusion proteins are expressed tagged with the Flag epitope (F). All YC fusion proteins are tagged with HA (H). (B) The indicated proteins were expressed in HeLa cells and, following fixation, localized using anti-Flag (α -Flag) or anti-HA (α -HA) as the primary antibody and Texas Red-conjugated IgG as the secondary antibody (IF). The cells were co-stained with DAPI to visualize nuclei. (C) The indicated combinations of plasmid DNAs were used to transfect HeLa cells: J, bJun; F, bFos; B, BATF. Protein extracts from the cells were analysed by immunoblot to confirm equivalent expression of Flag-tagged (YN) and HA-tagged (YC) proteins. The migration of molecular mass standards (kDa) is indicated for each panel. (D) The transfected cells from (C) not processed for immunoblot analysis were used for detecting protein dimerization in living cells using BiFC.

constructed to direct the expression of BATF proteins fused to N-terminal (YN) or C-terminal (YC) peptides and tagged with a Flag or HA epitope respectively (Figure 5A). Expression plasmids for bJun-YN [where bJun is c-Jun-(257–318)] and bFos-YC [where bFos is c-Fos-(118–210)] provided the controls. Following transfection of HeLa cells, the epitope tags were used to establish the intracellular location of individual proteins by indirect immunofluorescence. The results showed the appropriate nuclear location of Jun, Fos, BATF and both BATF(S43A) and BATF(S43D) variants (Figure 5B), demonstrating that modelling

phosphorylation with the DBD of BATF does not affect the nuclear localization of the protein. Next, HeLa cells were transfected with the indicated pairs of vectors and a portion of the cultures was harvested for immunoblot analysis to establish equivalent expression of the fusion proteins (Figure 5C). The remaining cells were visualized using fluorescence microscopy with a YFP filter set. The results mirrored the established dimerization properties of the individual proteins, with Jun binding to Fos and to all BATF proteins, and with no interaction observed between BATF and Fos (Figure 5D). Importantly, all fluorescent dimers were nuclear,

indicating that a negative charge juxtaposed to the Jun nuclear localization signal did not alter the location of this non-DNA binding heterodimer in cells.

DISCUSSION

The present study demonstrates that the DBD of BATF is phosphorylated on serine and threonine residues *in vitro* and *in vivo*. Using site-directed mutagenesis to model the phosphorylated and unphosphorylated states of serine-43 in the context of a full-length BATF protein, we observed that phosphorylation prevents DNA binding and alters the mechanism by which a BATF heterodimer inhibits AP-1-mediated gene transcription. The possibility that the presence of a negative charge within the DBD of BATF could influence the subcellular location of the protein was suggested by previous studies with v-Jun [33]. However, our results clearly show that BATF and the BATF(S43A) and BATF(S43D) variants localize to the nucleus and dimerize as expected with Jun. This discrepancy may be due to the size of BATF and its ability to enter the nucleus by diffusion and/or by virtue of its association with an efficiently transported dimer partner. Future experiments in which BiFC is used to localize the BATF(S43D):v-Jun heterodimer will address this issue.

In vitro evidence has been presented previously for a phosphorylation event occurring at positions analogous to serine-43 within the DBDs of v-Jun, C/EBP α (CCAAT/enhancer binding protein α) and C/EBP [32–35]. As mentioned above, the studies on v-Jun focused on changes in the subcellular localization of the modified protein, which our studies have shown do not apply to BATF. No DNA binding, dimerization or gene transactivation studies were performed. For C/EBP, previous work demonstrated that various protein kinase C isoforms were capable of phosphorylating the C/EBP DBD *in vitro* and that this modification abrogated DNA binding by C/EBP homodimers [34]. Interestingly, we also demonstrated that serine-43 of BATF is phosphorylated by protein kinase C *in vitro* (C. D. Deppmann and E. J. Taparowsky, unpublished work), although, as with the C/EBP study [34], the *in vivo* link between kinase and substrate has not been determined. Our studies are the first to generate evidence that the DBD of BATF, and most probably other bZIP factors, is phosphorylated *in vivo* and that this modification does not exert a significant impact on protein dimerization or nuclear localization, but does have a profound effect on DNA binding that could have significant consequences for target gene transcription, depending on the role of the affected bZIP protein as an activator or a repressor of gene transcription.

Since bZIP DBDs are highly conserved, we performed a genome-wide alignment of this domain in order to identify other bZIP factors containing serine at a position analogous to serine-43 in BATF [36,37]. A portion of this alignment is presented in Figure 6(A). Indeed, this position is highly conserved as a cysteine or a serine residue in 55% and 35% respectively of bZIP proteins (Figure 6B). Additional analysis of the data revealed that DBDs within the C/EBP, PAR (TEF), OASIS and ATF6 protein subfamilies contain a serine at this conserved position (Figure 6A and results not shown). Interestingly, BATF and a highly related protein, JDP-1 [38], are the only AP-1 factors containing a serine at this position. All other AP-1 factors contain a cysteine.

The functional role of the cysteine residue found in the DBDs of 55% of all bZIP proteins has been investigated. The oxidation state of this cysteine, which can be controlled by the activity of the Ref-1 protein [25], regulates DNA binding. Dimers in which both partner protein cysteines are oxidized are unable to bind DNA. This has proven to be a regulatory paradigm, since

Figure 6 Analysis of amino acid conservation in bZIP DBDs

(A) Alignment of selected bZIP DBDs, highlighting the position analogous to serine-43 of BATF. CREB, cAMP response element binding protein. (B) Genome-wide alignment (65 human bZIP factors) [36] was used to establish the distribution of amino acids at positions analogous to serine-43 of BATF. The category labelled 'Other' includes three proteins with alanine, glycine and glutamine respectively.

all cysteine-containing bZIP factors examined to date appear to be regulated by this mechanism. Here we present evidence for another regulatory paradigm that could affect the 40% of all bZIP factors containing a potential site of phosphorylation (serine or tyrosine) at an analogous position within their DBDs. In contrast with the redox regulation of DNA binding, our experiments have demonstrated that the effect of phosphorylating one DBD of a heterodimer is functionally dominant. Under reducing conditions, AP-1 DNA binding is observed for the c-Fos:c-Jun heterodimer, but not for the BATF(S43D):c-Jun heterodimer (Figure 4D).

The studies described in the present paper have provided insight into how phosphorylation at serine-43 affects the most rudimentary functions of BATF. However, several outstanding questions remain under investigation. Clearly, identifying the kinase(s) responsible for phosphorylating BATF is critical if we are to place BATF into cellular signal transduction pathways. Moreover, delineating situations where the phosphorylation of BATF is regulated will provide additional information on the role of BATF in specific cellular processes. Perhaps the most intriguing experiments on the horizon involve the *in vivo* documentation of this phosphorylation event for other bZIP factors possessing different dimerization properties, DNA binding preferences and transactivation potentials, and the dramatic consequences that this modification could have on their function. Our demonstration that the phosphorylation of serine-43 of BATF occurs *in vivo*, and that this modification prevents DNA binding, has established yet another intriguing mechanism through which the activities of bZIP transcription factor complexes can be regulated in cells.

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