Signalling pathways involved in multisite phosphorylation of the transcription factor ATF-2

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Abstract The multisite phosphorylation of the transcription factor ATF-2 was investigated using transformed embryonic fibroblasts from wild-type mice and mice deficient in c-Jun N-terminal kinases (JNK)1 and 2, and in the presence and absence of inhibitors of p38 mitogen-activated protein kinase (p38 MAPK) and the classical MAP kinase cascade. In wild-type cells, p38 MAPK and extracellular signal-regulated protein kinase (ERK)1/2 were not rate limiting for the phosphorylation of Thr69, Thr71 or Ser90. In JNK-deficient cells, p38 MAPK substituted for JNK partially in the phosphorylation of Thr69 and p38 MAPK or ERK1/2 in the phosphorylation of Thr71. JNK was the only MAP kinase that phosphorylated Ser90 under the conditions examined.

Keywords: c-Jun N-terminal kinase; p38; Extracellular signal-regulated protein kinase; Tumour necrosis factor; Epidermal growth factor

1. Introduction

Activating transcription factor-2 (ATF-2) [1] is a ubiquitously expressed member of the ATF/cyclic AMP-response element (CRE)-binding protein family of basic region-leucine zipper (bZIP) transcription factors. Upon activation, ATF-2 forms homodimers or heterodimers with other transcription factors of the ATF or activating protein-1 (AP-1) families [2–5], which then target gene promoters via the CRE or AP-1 binding sites.

In unstimulated cells, ATF-2 is maintained in an inhibited form by an intramolecular interaction between its activation and bZIP domains [6]. A transcriptional response can be induced either by interaction with regulatory proteins such as the retinoblastoma (Rb) tumour suppressor protein [7] and the early adenovirus 1A (E1A) protein [8–12], or by the phosphorylation of residues within its N-terminal transcriptional activation domain.

The phosphorylation of ATF-2 in vitro by a p54 MAP kinase (subsequently identified as an isoform of c-Jun N-terminal kinase (JNK)) and p42 MAP kinase (usually called extracellular-signal regulated protein kinase 2 (ERK2)) was first reported to increase its DNA-binding activity 12 years ago [13]. Subsequently, JNK was found to become activated in response to ultraviolet (UV) radiation, pro-inflammatory cytokines and genotoxic agents, while UV-radiation was shown to induce the phosphorylation of ATF-2 [14–16], suggesting that JNK might mediate the phosphorylation and activation of ATF-2 in vivo. Phosphopeptide mapping and mutational analysis revealed that the UV-induced phosphorylation of ATF-2 in cells occurred at Thr69, Thr71 and Ser90. The phosphorylation of Thr69 and Thr71 was found to be necessary for the transcriptional activation of ATF-2 in response to UV and genotoxic agents, because activation did not occur if either or both of these two sites were mutated to Ala [14–16]. In contrast, the mutation of Ser90 to Ala had little effect on transcriptional activity [15,16]. The phosphorylation of Thr69 and Thr71 was also required for E1A- and Rb-stimulated gene expression [14,15] and for the intrinsic histone acetyltransferase (HAT) activity of ATF-2 [17], but Ser90 phosphorylation was not required for the response to E1A [15] or HAT activity [17].

The importance of JNK in mediating the phosphorylation of ATF-2 was subsequently established in a number of ways, but most notably by the generation of mice that do not express JNK1 or JNK2, in which the tumour necrosis factor (TNF)α-induced phosphorylation of ATF-2 at Thr71 was greatly reduced [18]. However, another group reported that, in response to different stimuli, the phosphorylation of ATF-2 at Thr69 and Thr71 was reduced, but not abolished in JNK-deficient cells, indicating that other protein kinases are capable of phosphorylating these sites, if JNK isoforms are not expressed [19].

In this paper, we have generated a phospho-specific antibody that recognises ATF-2 phosphorylated at Ser90 and used this and other commercially available phospho-specific antibodies that recognise ATF-2 phosphorylated at Thr69 and Thr71 to analyse the phosphorylation of all three sites in response to a variety of agonists in transformed embryonic

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Abbreviations: AP-1, activating protein 1; ATF-2, activating transcription factor-2; CRE, cyclic AMP-response element; E1A, early adenovirus protein 1A; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; GST, glutathione *S*-transferase; HAT, histone acetyl transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP-K2, MAPK-activated protein kinase 2; PMA, phorbol-12-myristate 13-acetate; Rb, retinoblastoma protein; TNF, tumour necrosis factor; bZIP, basic region-leucine zipper

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fibroblasts generated from both control mice and mice that do not express either JNK1 or JNK2.

2. Materials and methods

2.1. Materials

The sources of all reagents, other than those detailed below, are given elsewhere [20].

2.2. Cloning and expression of ATF-2

Full length ATF-2 was cloned and inserted into a pGEX vector. Glutathione-*S*-transferase (GST)-ATF-2 was then expressed in *Escherichia coli* BL21, purified by affinity chromatography on glutathione–Sepharose and used for antibody production and as a substrate for different MAP kinase family members.

2.3. Protein kinases and their assay

JNK1α1, p38α mitogen-activated protein kinase (MAPK), p38β MAPK and ERK2 were expressed, purified, activated and assayed as described previously [21].

2.4. Antibodies

An antibody capable of immunoprecipitating ATF-2 (anti-ATF-2) was raised in a sheep against GST-ATF-2 and the antisera affinity purified on CH-Sepharose to which GST-ATF-2 had been coupled covalently, then passed through GST-Sepharose to remove the anti-GST antibodies. The phosphopeptide FNELApSPFENE (where pS is phosphoserine), corresponding to residues 85–95 of human ATF-2, was coupled separately to bovine serum albumin and keyhole limpet haemocyanin before being mixed and injected into a sheep. The antisera were affinity purified on CH-Sepharose to which the phosphorylated peptide had been coupled covalently. These anti-pSer90 antibodies were used for immunoblotting in the presence of the unphosphorylated form of the peptide antigen (10 μg/ml) to neutralise any antibodies that recognised unphosphorylated ATF-2. Antibodies that recognise ATF-2 phosphorylated at Thr71 (anti-pThr71), or both Thr69 and Thr71 (anti-pThr69/pThr71) were purchased from Cell Signalling Technologies (Hitchin, UK). The sources of other antibodies are given elsewhere [20].

2.5. Cell culture and stimulation

Wild-type and JNK−/− fibroblasts were cultured, lysed, exposed to pharmacological inhibitors and stimulated with agonists as described elsewhere [20]. Further details are given in the figure legends.

2.6. Immunoprecipitation and immunoblotting of ATF-2

ATF-2 was immunoprecipitated from 2 mg cell lysate protein with anti-ATF-2 and then immunoblotted with the phosphospecific antibodies that recognise ATF-2 at particular sites. The exact procedure is as detailed previously for anti-c-Jun and the phospho-specific antibodies that recognise this protein [20].

3. Results

3.1. Characterisation of anti-ATF-2 antibodies

The antibodies required to analyse the in vivo phosphorylation state of ATF-2 were first characterised in vitro. The specificity of the antibody that recognises ATF-2 phosphorylated at Ser90 was verified by the demonstration that recognition was prevented by preincubation with the phosphopeptide immunogen, but not by incubation with phosphopeptides corresponding to the sequences surrounding the other N-terminal phosphorylation sites (Fig. 1). The specificity of this antibody was also verified by the demonstration that when preincubated with the unphosphorylated form of the peptide immunogen, it only recognised ATF-2 after phosphorylation by JNK. In contrast, the antibody raised against the ATF-2 protein recognised phosphorylated and unphosphorylated ATF-2 equally well (Fig. 1).

The specificities of the commercially available antibodies that recognise ATF-2 when phosphorylated at both Thr69 and Thr71, or only phosphorylated at Thr71, were verified in analogous experiments to those described above for the anti-pSer90 antibody (Fig. 1). The antibody recognising ATF-2 phosphorylated at both Thr69 and Thr71 was not neutralised by either of the two singly phosphorylated peptides alone

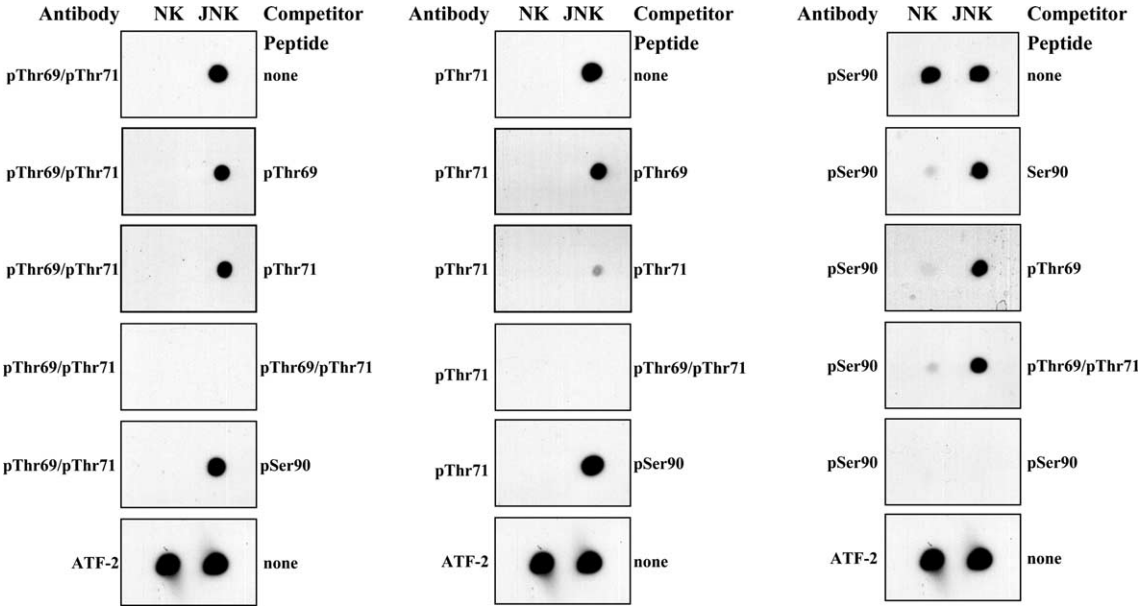

Fig. 1. Characterisation of phospho-specific antibodies that recognise ATF-2 phosphorylated at Thr69, Thr71 or Ser90. Bacterially expressed ATF-2 was left unphosphorylated (no kinase, NK) or maximally phosphorylated with JNK and 100 ng aliquots spotted onto a nitrocellulose membrane. Immunoblotting was then carried out using the phospho-specific antibodies indicated or with an antibody that recognises phosphorylated and unphosphorylated ATF-2 equally well (ATF-2), in the presence or absence (none) of the competitor peptides shown. The competitor peptides were: FNELASPFENE (Ser90); FNELApSPFENE (pSer90); VADQpTPTPTRFLK (pThr69); VADQTPpTPTRFLK (pThr71); and VADQpTPpTPTRFLK (pThr69/pThr71). The prefix "p" denotes the phosphorylated residue.

(Fig. 1) or in combination (data not shown), but only neutralised by the doubly phosphorylated peptide (Fig. 1). Therefore, this antibody recognises ATF-2 only when phosphorylated at both Thr69 and Thr71. The antibody that recognises ATF-2 only when phosphorylated at Thr71 was unaffected by the peptide phosphorylated at Thr69, but neutralised by the peptide phosphorylated at Thr71 or the peptide phosphorylated at both Thr69 and Thr71 (Fig. 1). Therefore, this antibody recognises ATF-2 phosphorylated at Thr71, whether or not Thr69 is phosphorylated.

3.2. Site-specific phosphorylation of ATF-2 in vitro by MAP kinase family members

MAP kinase family members normally phosphorylate serine and threonine residues that are followed by proline, and Thr69, Thr71 and Ser90 all lie in such motifs. In order to assess which sites could be phosphorylated by different MAP kinase family members in vitro, we incubated ATF-2 with MgATP in the presence of relatively high concentrations of these protein kinases and examined the phosphorylation of each site using the phospho-specific antibodies characterised above. These experiments showed that JNK1α1 phosphorylated all three sites, while ERK2 was only able to phosphorylate Thr71 (Fig. 2). As reported previously [22], p38α and p38β MAP kinases phosphorylated Thr69 and Thr71, but not Ser90.

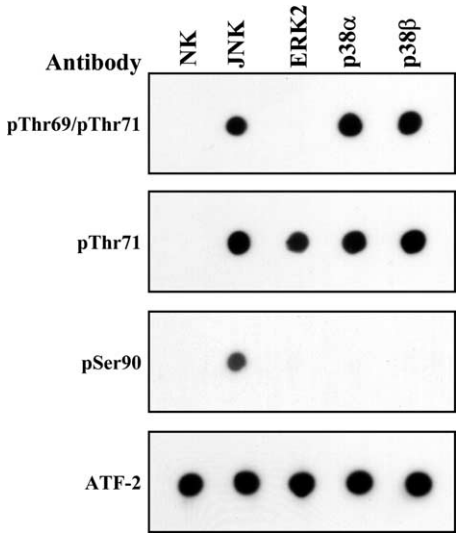

Fig. 2. Site-specific phosphorylation of ATF-2 in vitro by different MAP kinases. Bacterially expressed ATF-2 was left unphosphorylated (no kinase, NK) or phosphorylated for 30 min at 30 °C with 1 U/ml of JNK1α1, ERK2, p38α MAPK or p38β MAPK. Aliquots (100 ng) were spotted onto a nitrocellulose membrane and immunoblotted with the phospho-specific antibodies indicated or with an antibody that recognizes phosphorylated and unphosphorylated ATF-2 equally well (ATF-2). The prefix "p" denotes the phosphorylated residue.

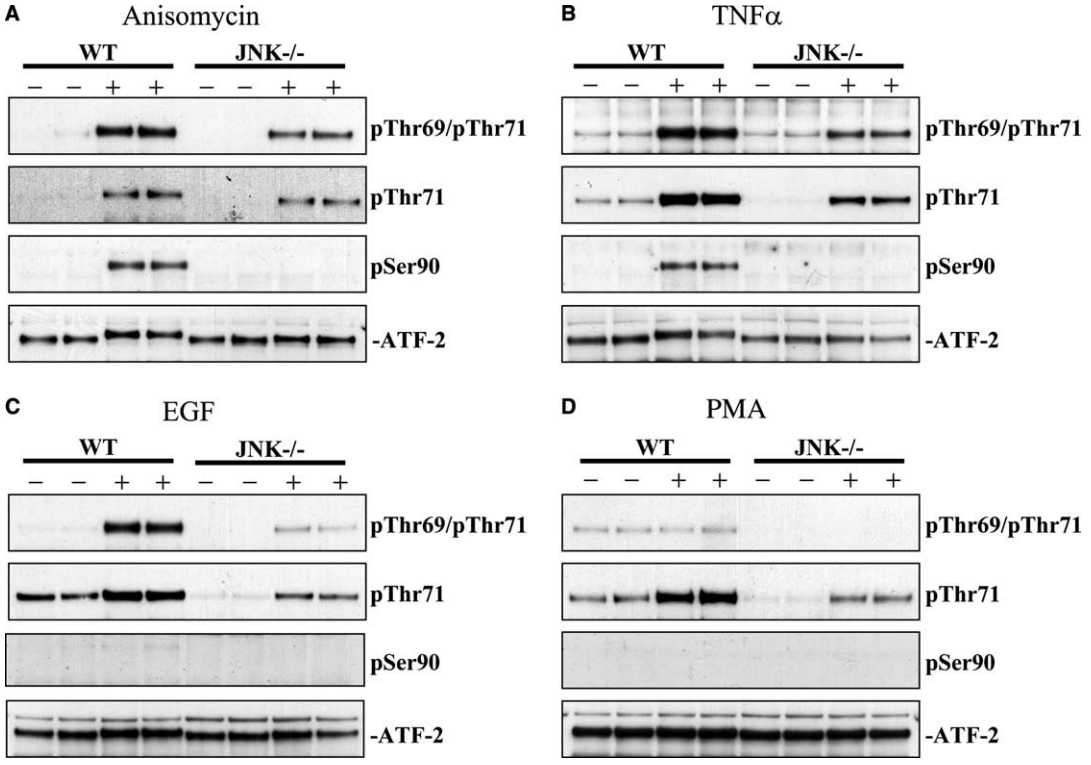

Fig. 3. Phosphorylation of three sites in ATF-2 in embryonic fibroblasts from wild-type (WT) and JNK−/− mice. Cells were stimulated (A) for 30 min with anisomycin (10 μg/ml), (B) for 15 min with TNFα (10 ng/ml), (C) for 15 min with EGF (100 ng/ml) and (D) for 30 min with PMA (100 ng/ml), and lysed. ATF-2 was immunoprecipitated from the lysates, denatured in SDS, subjected to SDS–PAGE and transferred to nitrocellulose membrane. The membranes were immunoblotted with antibodies that recognise ATF-2 phosphorylated at Thr69/Thr71, Thr71 and Ser90, as well as with an antibody that recognises the phosphorylated and unphosphorylated forms of ATF-2 equally well. Similar results were obtained in another independent experiment. The prefix "p" denotes the phosphorylated residue.

3.3. The phosphorylation of ATF-2 in wild-type and JNK−/− embryonic fibroblasts in response to anisomycin, TNFα, epidermal growth factor and phorbol-12-myristate 13-acetate

Transformed fibroblasts from wild-type and JNK−/− cells (that do not express JNK1 or JNK2) express similar levels of ATF-2 (Fig. 3) and were used to analyse the phosphorylation of ATF-2 in response to a variety of stimuli. The JNK3 isoform is not expressed in these cells. The protein synthesis inhibitor anisomycin (Fig. 3A) and TNFα (Fig. 3B) induced the phosphorylation of ATF-2 at Thr69, Thr71 and Ser90 in wild-type fibroblasts, but no phosphorylation of Ser90 occurred in the JNK−/− fibroblasts. In contrast, the phosphorylation of ATF-2 at Thr69 and Thr71 in the JNK−/− fibroblasts was reduced, but not abolished. Epidermal growth factor (EGF) also induced the phosphorylation of ATF-2 at Thr69 and Thr71 in wild-type fibroblasts, but no EGF-induced phosphorylation of Ser90 was detected in either these or JNK−/− fibroblasts (Fig. 3C). As for TNFα-induced phosphorylation, the EGF-induced phosphorylation of Thr69 and Thr71 was reduced, but not abolished in the JNK−/− fibroblasts (Fig. 3C). The tumour-promoter phorbol-12-myristate 13-acetate (PMA) induced phosphorylation of Thr71 in wild-type fibroblasts, which was reduced but not abolished in JNK−/− fibroblasts, but this agonist did not increase Thr69 or Ser phosphorylation in wild-type or JNK−/− fibroblasts (Fig. 3D).

The phosphorylation of ATF-2 induced by anisomycin, TNFα and EGF, but not by PMA, was accompanied by a decrease in the mobility of this protein. Taken together, these observations suggest that the decrease in mobility of ATF-2 results from the combined phosphorylation of Thr69 and Thr71.

3.4. Effects of protein kinase inhibitors on the anisomycin and PMA-induced phosphorylation of ATF-2

In fibroblasts, anisomycin is a potent activator of JNK and p38 MAPK, but a much weaker activator of ERK1/ERK2. Conversely, PMA is a potent activator of ERK1/ERK2 and weak activator of JNK and p38 MAPK. We therefore, studied the site-specific phosphorylation of ATF-2 in the presence and absence of SB 203580 (a potent and relatively specific inhibitor of p38 MAPK) [23] and/or PD 184352 (a potent and specific inhibitor of MAPK kinase 1, the enzyme that activates ERK1 and ERK2) [24]. These experiments revealed that, in wild-type cells, SB 203580 did not affect the anisomycin-induced phosphorylation of Thr69 and Thr71 (Fig. 4A), while PD 184352 did not affect the PMA-induced phosphorylation of Thr71 (Fig. 4B). In contrast, SB 203580 abolished the anisomycin-induced phosphorylation of Thr69 and Thr71 (Fig. 4C), while PD 184352 abolished the PMA-induced phosphorylation (Fig. 4D) of Thr71 in the JNK−/− cells. The phosphorylation of MAPK-activated protein kinase-2 (MAPKAP-K2), an established downstream substrate of p38α, was prevented by SB

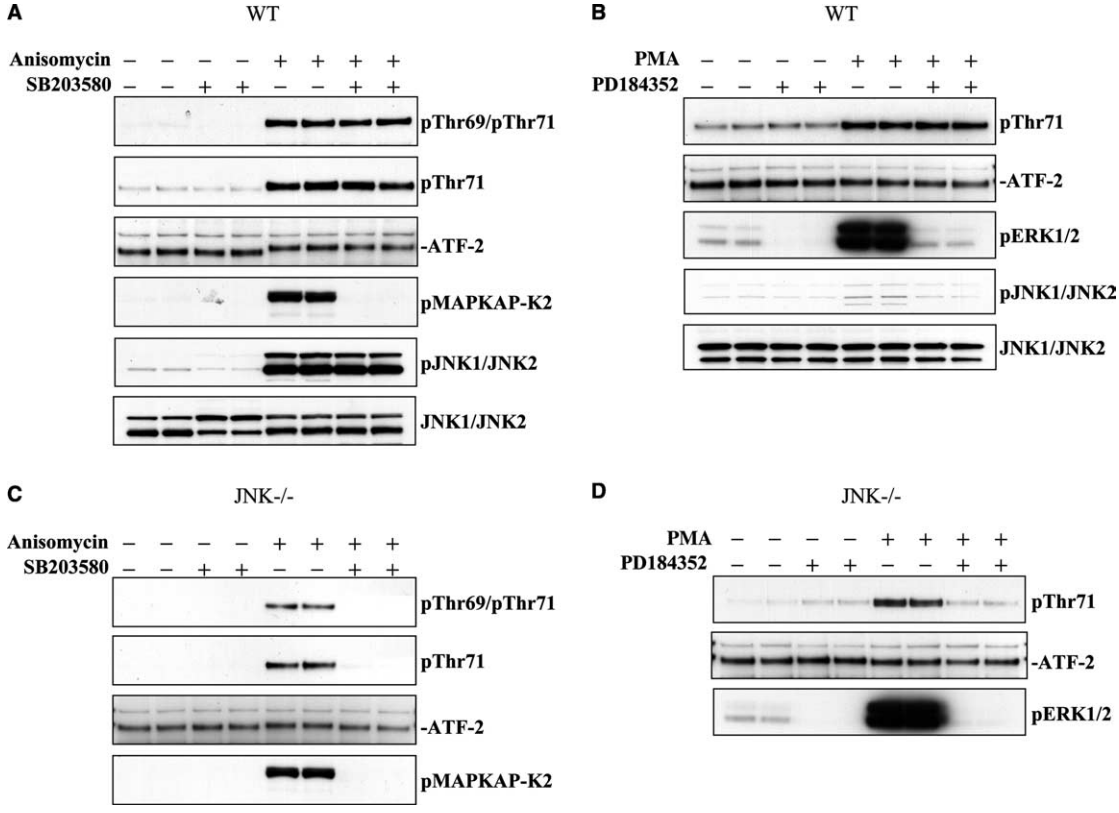

Fig. 4. Effect of protein kinase inhibitors on the anisomycin- and PMA-induced phosphorylation of ATF-2 at Thr69 and Thr71 in wild-type and JNK−/− fibroblasts. Wild-type (WT) (A, B) and JNK−/− (C, D) cells were incubated for 1 h with (+) or without (−) 10 μM SB 203580 or 2μM PD 184352, stimulated for 30 min with or without anisomycin (10 μg/ml) or PMA (100 ng/ml) and lysed. Immunoblotting was then performed with antibodies that recognise ATF-2 phosphorylated at Thr69/Thr71 or Thr71 alone, and with an antibody that recognises the phosphorylated and unphosphorylated forms of ATF-2 equally well (see legend to Fig. 3). Further aliquots of the cell lysates were immunoblotted (without immunoprecipitation) using antibodies that recognise MAPKAP-K2 phosphorylated at Thr334 (A, C), phosphorylated ERK1 and ERK2 (B, D), phosphorylated JNK (A, B) and an antibody that recognises the phosphorylated and unphosphorylated forms of JNK equally well (A, B). Similar results were obtained in another independent experiment. The prefix "p" denotes the phosphorylated residue.

203580 and the phosphorylation of ERK1 and ERK2 by PD 184352, as expected (Fig. 4).

3.5. Effects of protein kinase inhibitors on the TNFα and EGF-induced phosphorylation of ATF-2

We also studied the site-specific phosphorylation of ATF-2 after stimulating the fibroblasts with TNFα and EGF, two agonists that activate both p38 MAPK and the classical MAPK cascade in fibroblasts. As found with anisomycin or PMA, SB 203580 and/or PD 184352 did not affect the TNFα-induced (Fig. 5A and B) or EGF-induced (Fig. 6A) phosphorylation of Thr69 and Thr71 in wild-type cells. In contrast, SB 203580 abolished the TNFα- (Fig. 5C) or EGF-induced (Fig. 6B) phosphorylation of Thr69, but the combined addition of SB 203580 and PD 184352 was needed to abolish the phosphorylation of Thr71 by these agonists.

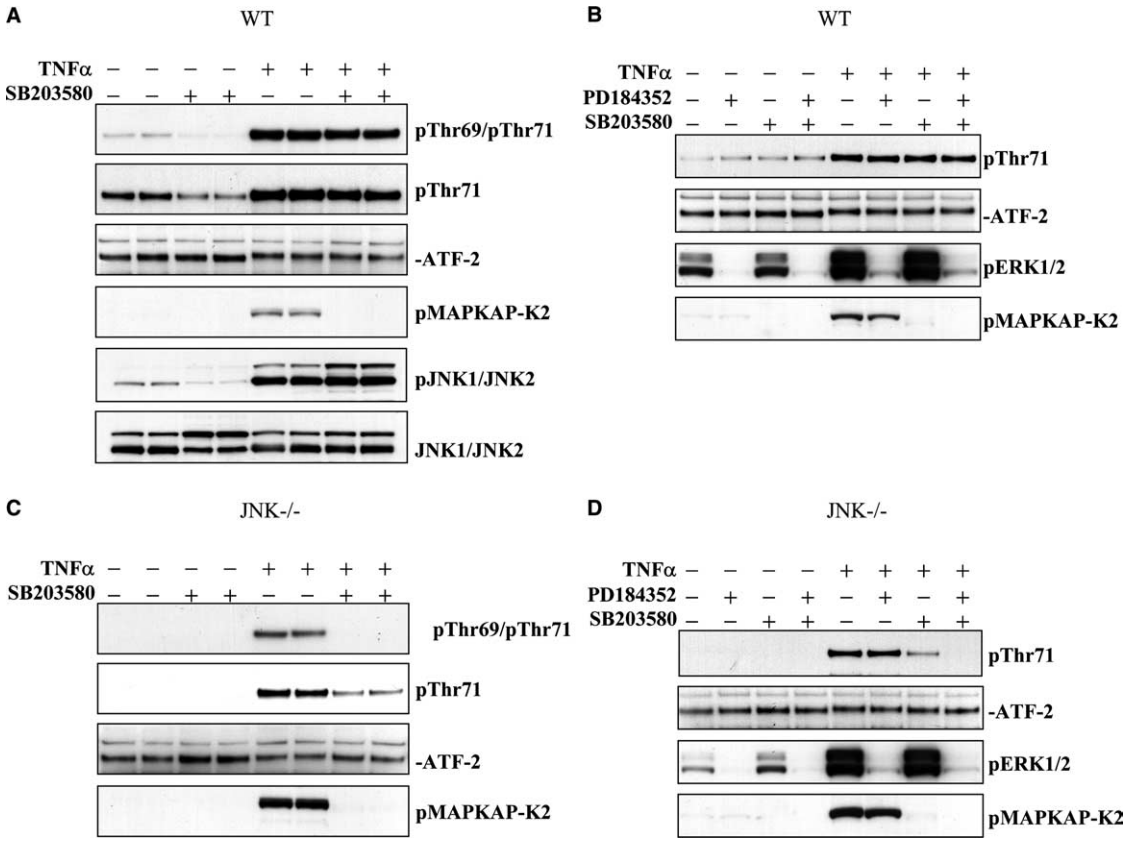

Fig. 5. Effect of SB 203580 and PD 184352 on the TNFα-induced phosphorylation of ATF-2 at Thr69 and Thr71 in wild-type and JNK−/− fibroblasts. Wild-type (WT) (A, B) and JNK−/− (C, D) cells were incubated for 1 h with (+) or without (−) 10 μM SB 203580 and/or PD 184352, then stimulated for 15 min with TNFα (10 ng/ml) and lysed. ATF-2 was immunoblotted with antibodies that recognise ATF-2 phosphorylated at Thr69/Thr71 or Thr71 alone, as well as with an antibody that recognises the phosphorylated and unphosphorylated forms of ATF-2 equally well, as described in the legend to Fig. 3. Further aliquots of the cell lysates were immunoblotted with antibodies that recognise the active form of MAPKAP-K2 phosphorylated at Thr334 (A–D), the active phosphorylated forms of JNK (A), and an antibody that recognises the phosphorylated forms of ERK1 and ERK2 (B, D). Similar results were obtained in a separate independent experiment. The prefix "p" denotes the phosphorylated residue.

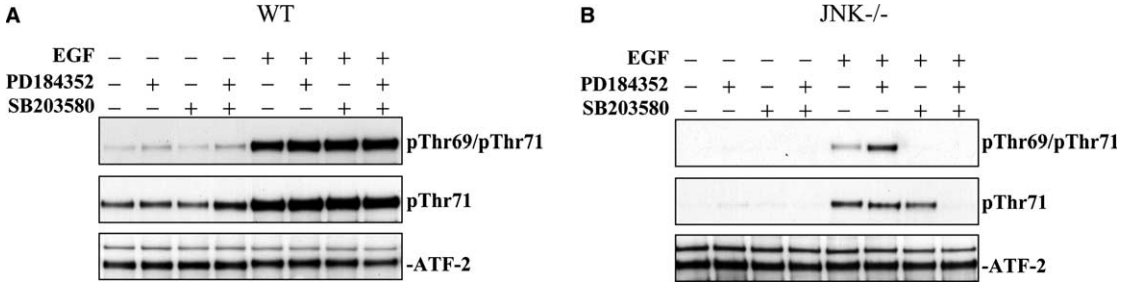

Fig. 6. Effect of PD 184352 and SB 203580 on the EGF-induced phosphorylation of ATF-2 at Thr69/Thr71 and Thr71 in wild-type and JNK−/− MEFs. Wild-type (WT) (A) and JNK-deficient (JNK−/−) (B) cells were incubated for 1 h with (+) or without (−) 2 μM PD 184352 and/or 10 μM SB 203580 and then stimulated for 15 min with EGF (100 ng/ml). ATF-2 was immunoblotted with antibodies that recognise ATF-2 phosphorylated at Thr69/Thr71 or Thr71 alone, as well as with an antibody that recognises the phosphorylated and unphosphorylated forms of ATF-2 equally well, as described in the legend to Fig. 3. Similar results were obtained in a separate independent experiment. The prefix "p" denotes the phosphorylated residue.

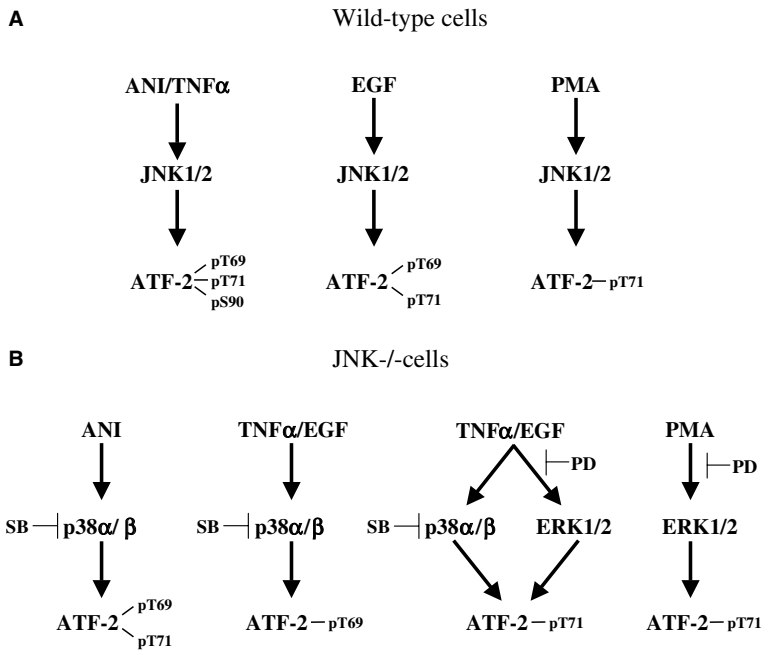

Fig. 7. Summary of the results obtained in this study. (A) Wild-type cells: p38α/β or ERK1/ERK2 are not rate limiting for the phosphorylation of ATF-2 at Thr69, Thr71 or Ser90. Anisomycin (ANI) or TNFα stimulates the phosphorylation of ATF-2 all three sites, EGF at Thr69 and Thr71 and TPA at Thr71 only. (B) JNK−/− cells: p38α/β are rate limiting for ANI, TNFα and EGF-induced phosphorylation of Thr69, and ANI–induced phosphorylation of Thr71. p38α/β or ERK1/ERK2 are both required for maximal phosphorylation of ATF-2 at Thr71 in response to TNFα or EGF. The TPA-induced phosphorylation of ATF-2 at Thr71 is mediated by ERK1/ERK2. Abbreviations: SB, SB 203580; PD, PD 184352.

4. Discussion

In this paper, we have analysed the multisite phosphorylation of ATF-2 in wild-type and JNK−/− cells and in the presence or absence of inhibitors of p38α/β MAPKs (SB 203580) and the classical MAPK cascade (PD 184352) and the results are summarised in Fig. 7. Although the phosphorylation of ATF-2 at Ser90 in response to UV radiation has been reported previously, the present study is the first to demonstrate that JNK isoforms are required for the phosphorylation of this site in anisomycin or TNFα-stimulated cells, since no phosphorylation of this residue was detected in JNK−/− cells (Fig. 3). However, it cannot yet be excluded that JNK does not phosphorylate Ser90 directly, and that phosphorylation in cells is catalysed by another proline-directed Ser90 kinase(s) whose synthesis and/or activation is dependent on JNK expression. In order to resolve this issue, a potent and selective small molecule inhibitor capable of suppressing JNK activity within minutes will be needed. However, compounds with the requisite potency and specificity have not yet been described (see [25]). Consistent with our finding that JNK, but not ERK2 (or p38α/β MAPK), phosphorylates Ser90 in vitro (Fig. 2), EGF or PMA, which are potent activators of the classical MAP kinase cascade, did not induce the phosphorylation of Ser90 in fibroblasts.

In contrast to the phosphorylation of Ser90, the phosphorylation of ATF-2 at both Thr69 and Thr71 was reduced in JNK−/− cells, but not abolished. The residual phosphorylation of Thr69 plus Thr71 in anisomycin-stimulated JNK−/− cells was prevented by SB 203580, while the residual phosphorylation of Thr71 in PMA-stimulated cells was suppressed by PD 184352. Incubation with SB 203580 was sufficient to prevent the phosphorylation of Thr69 plus Thr71 in TNFα – or EGF-stimulated cells, but the combined addition of SB 203580 and PD 184352 was required to abolish the phosphorylation of Thr71 by these agonists. These observations indicate that p38α/p38β MAPKs, but not ERK1/ERK2, can replace JNK isoforms in the phosphorylation of Thr69, and that both p38α/p38β MAPKs and ERK1/ERK2 can partially replace JNK isoforms in the phosphorylation of Thr71 if JNKs are not expressed (Fig. 7B). These findings are consistent with our observation that p38α/β MAPK can phosphorylate Thr69 and Thr71, while ERK2 can only phosphorylate Thr71 in vitro (Fig. 2).

The effects of inhibitors on the phosphorylation of Thr69 and Thr71 described in this paper are also consistent with those reported by another laboratory who stimulated transformed fibroblasts from JNK−/− cells with agonists that were mostly distinct from those used in the present study [19]. These investigators reported that the phosphorylation of Thr69 plus Thr71, or Thr71 alone, induced by UV-C radiation or the DNA alkylating agent methylmethane sulfonate, was abolished by SB 203580 in the JNK−/− cells, similar to our results in anisomycin-stimulated JNK−/− cells. They also found that SB 203580 or U0126 (another inhibitor of the classical MAPK cascade [26]) were both required to suppress the phosphorylation of Thr71 induced by insulin or EGF, similar to our results with TNFα or EGF.

In the transformed fibroblasts from wild-type mice, SB 203580 and/or PD 184352 had no effect on the phosphorylation of ATF-2 by any agonist tested, indicating that neither p38α /p38β MAPKs nor ERK1/ERK2 are rate limiting for the phosphorylation of this transcription factor under the conditions that we have studied (Fig. 7A). The lack of

effect of these inhibitors could either mean that JNK activity alone is sufficient for maximal phosphorylation of ATF-2 or that the binding of JNK to ATF-2 prevents phosphorylation by p38α/p38β and ERK1/ERK2. The development of a potent and specific pharmacological inhibitor of JNK would help to distinguish between these possibilities. However, in A14 fibroblasts, in which PMA does not activate JNK or p38 MAPK, the PMA-induced phosphorylation of ATF-2 at Thr71 was blocked by U0126, suggesting that ERK1/ERK2 may phosphorylate ATF-2 in at least one JNK-expressing cell [19].

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