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TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway

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Transforming growth factor-β (TGF-β) exerts its effects on cell proliferation, differentiation and migration in part through its modulation of extracellular matrix components, such as fibronectin and plasminogen activator inhibitor-1 (PAI-1). Although the SMAD family of proteins recently has been shown to be a key participant in TGF-β signaling, other signaling pathways have also been shown to be activated by TGF-β. We report here that c-Jun N-terminal kinase (JNK), a member of the MAP kinase family, is activated in response to TGF-β in the human fibrosarcoma HT1080-derived cell line BAHgpt. Stable expression of dominant-negative forms of JNK1 and MKK4, an upstream activator of JNK, results in loss of TGF-β-stimulated fibronectin mRNA and protein induction, while having little effect on TGF-β-induced levels of PAI-1. The human fibronectin promoter contains three CRE elements, one of which has been shown to bind a c-Jun–ATF-2 heterodimer. Utilizing a GAL4 fusion trans-reporting system, we demonstrate a decrease in transactivating potential of GAL4–c-Jun and GAL4–ATF-2 in dominant-negative JNK1- and MKK4-expressing cells. Finally, we show that TGF-β-induced fibronectin synthesis is independent of Smad4. These results demonstrate that TGF-β-mediated fibronectin induction requires activation of JNK which in turn modulates the activity of c-Jun and ATF-2 in a Smad4-independent manner.

Keywords: c-Jun/c-Jun N-terminal kinase (JNK)/ fibronectin/Smad4/transforming growth factor-β (TGF-β)

Introduction

Coordinated regulation of production and turnover of extracellular matrix (ECM) components is essential for normal tissue homeostasis. The composition of the ECM can influence cell growth, state of differentiation and specific gene induction. Fibronectin (FN), a major component of the ECM, plays an important role during development and wound healing by promoting cell adhesion, migration and cytoskeletal organization (Hynes, 1985; Kornblihtt *et al.*, 1996). Overproduction of matrix components including FN is the main pathological finding in tissue fibrosis (Border and Noble, 1994). Decreased FN production, however, is often observed following oncogenic transformation, leading to decreased adhesion and increased metastatic potential (Kornblihtt *et al.*, 1996). Transforming growth factor-β (TGF-β), which is released by degranulating platelets at a wound site, has been shown to potently induce FN expression, at both the mRNA and protein levels (Ignotz and Massagué, 1986; Dean *et al.*, 1988; Wrana *et al.*, 1991). TGF-β promotes net matrix deposition by increasing the expression of specific ECM components such as FN and collagen, up-regulating the expression of inhibitors of ECM proteases, such as plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of matrix metalloproteinases (TIMPs), while simultaneously down-regulating proteases which degrade matrix components, such as interstitial collagenase (Massagué, 1990; Roberts and Sporn, 1990).

Further insight into TGF-β signaling has been gained following the recent identification and cloning of the SMAD family of proteins, consisting of the pathway-restricted SMADs, the common-mediator SMADs and the inhibitory SMADs (Heldin *et al.*, 1997; Whitman, 1997; Massagué, 1998). TGF-β signaling is initiated following ligand binding to the TGF-β type II receptor, TβR-II. This allows the recruitment of the TGF-β type I receptor, TβR-I, into a heteromeric complex, resulting in trans-phosphorylation of TβR-I by TβR-II (Heldin *et al.*, 1997; Massagué, 1998). Following phosphorylation of Smad2 or Smad3 by the activated TβR-I, a heteromeric complex is formed with Smad4, resulting in translocation of the complex to the nucleus (Abdollah *et al*., 1997; Liu *et al*., 1997; Souchelnytskyi *et al*., 1997) where the complex can directly (Dennler *et al.*, 1998; Song *et al*., 1998) or indirectly, by interactions with other transcription factors (Chen *et al*., 1996; Labbe *et al.*, 1998), regulate gene transcription.

Other signaling pathways have also been shown to be mediators of TGF-β signaling, including the mitogen-activated protein kinase (MAPK) family. This family consists of the extracellular signal-regulated kinase (ERK) pathway (Marais and Marshall, 1996) and two stress-activated pathways, the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway (Kyriakis and Avruch, 1996; Woodgett *et al.*, 1996). Signaling initiated by the MAPK pathway proceeds through sequential activation of an MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), ultimately leading to activation of an MAPK.

Targets for the activated MAPKs are commonly transcription factors; ERK activation leads to phosphorylation and activation of CREB (Xing *et al.*, 1996) and members of the ternary complex (TCF) family such as Elk-1 (Treisman, 1996). p38 activation has been shown to lead to activation of ATF-2 (Jiang *et al.*, 1996) and CREB (Iordanov *et al.*, 1997). JNK is the only known kinase which phosphorylates c-Jun (Hibi *et al*., 1993; Derijard *et al.*, 1994; Kyriakis *et al*., 1994); however, JNK can

also phosphorylate ATF-2 (Gupta *et al.*, 1995) and Elk-1 (Whitmarsh *et al.*, 1995). c-Jun, a member of the AP-1 family of transcription factors, can bind to and activate transcription from AP-1 or TRE sites (Angel and Karin, 1991), while CREB and ATF-2 bind to CRE sites (Karin and Smeal, 1992). Heterodimers of c-Jun and ATF-2 have also been shown to bind to CRE sites (Jvashkiv *et al.*, 1990; Hai and Curran, 1991). The observation that many TGF-β-regulated gene promoters contain AP-1- or CRE-binding sites, as well as the cloning of the MAPK kinase kinase homolog TAK1 which is activated by TGF-β (Yamaguchi *et al.*, 1995), has prompted researchers to investigate the activation of the MAPK kinase pathways by TGF-β. ERK activity was shown to be stimulated by TGF-β in epithelial cells (Hartsough and Mulder, 1995), breast cancer cells (Frey and Mulder, 1997) and NIH 3T3 cells (Mucsi *et al.*, 1996). JNK activation was observed following TGF-β stimulation in HepG2, MDCK, CHO (Atfi *et al.*, 1997b) and breast cancer cells (Frey and Mulder, 1997), while p38 activity was shown to be stimulated transiently by TGF-β in human neutrophils (Hannigan *et al.*, 1998).

Although TGF-β-stimulated activation of ERK, JNK and p38 activity has been demonstrated previously in different cell types, the contributions of these pathways to the TGF-β-mediated regulation of endogenous genes has not yet been determined. We report here that the JNK pathway is stimulated rapidly by TGF-β in BAHgpt cells, an HT1080-derived human fibrosarcoma cell line, and that this activity is essential for TGF-β-mediated FN induction. In addition, we demonstrate that Smad4 is not required for TGF-β-mediated FN induction. Our data provide the first report of an endogenous gene response regulated by TGF-β in a Smad4-independent fashion.

Results

TGF-β activates c-Jun N-terminal kinase (JNK)

Many promoters of TGF-β-regulated genes contain either AP-1 sites, such as the PAI-1 (Keeton *et al.*, 1991; Descheemaeker *et al.*, 1992), TIMP-1 (Campbell *et al.*, 1991), TGF-β1 (Kim *et al.*, 1990), c-Jun (Angel *et al.*, 1988), α2(I) collagen (Chung *et al.*, 1996) and apoJ/clusterin promoters (Jin and Howe, 1998), or CRE sites, such as the FN (Dean *et al.*, 1987; Bowlus *et al.*, 1991), c-Jun (van Dam *et al.*, 1993) and TGF-β2 (Kim *et al.*, 1992) promoters. It seems plausible, therefore, that TGF-β-mediated gene induction would involve activation of JNK, since JNK has been shown to modulate promoters containing both AP-1 and CRE sites through its phosphorylation and activation of c-Jun and ATF-2. To investigate this hypothesis, we first determined whether JNK was activated following TGF-β treatment in BAHgpt cells. We have shown previously that BAHgpt cells, which are derived from HT1080 human fibrosarcoma cells, express wild-type receptors for TGF-β, are transcriptionally responsive to TGF-β utilizing a TGF-β-responsive reporter construct 3TPLux, and induce endogenous PAI-1 and FN synthesis following TGF-β treatment (Hocevar and Howe, 1996). Additionally, these cells have been used to study the TGF-β-mediated suppression of interferon (IFN)-γ-induced class II MHC gene expression (Lee *et al.*, 1997) and recently have been used to identify proteins which participate in TGF-β-stimulated PAI-1 promoter induction (Hua *et al.*, 1998).

The JNK family has been shown to consist of at least 10 isoforms, which arise from alternative splicing of the JNK1, JNK2 and JNK3 genes (Gupta *et al.*, 1996). Since it is not known which isoforms are activated following stimulation with TGF-β, and since JNK has been identified as the only known kinase which can phosphorylate c-Jun (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994), we performed JNK kinase assays on total cell lysates prepared from BAHgpt cells, rather than on immunoprecipitates of individual JNK isoforms. Cell lysates prepared from BAHgpt cells stimulated with TGF-β for varying times were incubated either with glutathione *S*-transferase (GST)–c-Jun, a substrate specific for JNK (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994), or GST–ATF-2, which can be phosphorylated by both JNK and p38 (Gupta *et al.*, 1995; Jiang *et al.*, 1996), to demonstrate JNK kinase activation in an *in vitro* kinase assay. As shown in Figure 1A, TGF-β stimulates JNK kinase activity rapidly and potently, resulting in phosphorylation of Ser73 on c-Jun within 5 min, which is detected by Western blot analysis utilizing a phospho-specific antibody. TGF-β-stimulated JNK phosphorylation of c-Jun appears to exhibit a biphasic response; the initial peak of activity declines after 1 h of stimulation followed by a second peak of activity which persists until 8 h. In contrast, the basal phosphorylation level of GST–ATF-2 is relatively high and remains elevated throughout the time course, which may reflect not only JNK activity but also p38 activity (Figure 1A). It should be noted that these assays are performed on cells in serum-containing media, indicating that TGF-β is capable of stimulating JNK activity in exponentially growing cells.

JNK kinases are activated by the upstream MAPK kinases MKK4 (Derijard *et al.*, 1995; Lin *et al.*, 1995) and MKK7 (Moriguchi *et al.*, 1997; Tournier *et al.*, 1997), which in turn can be activated by the upstream signaling component MEKK1 (Minden *et al.*, 1994). To confirm that the JNK pathway is involved in TGF-β signaling, we assessed whether transfection of a constitutively active form of MEKK1 (Minden *et al.*, 1994) could activate reporter constructs which are responsive to TGF-β. The 3TPLux reporter construct is comprised of three TRE elements from the human collagenase gene linked to the –740/–636 region of the PAI-1 promoter and has been shown to be induced selectively by TGF-β (Wrana *et al.*, 1992; Carcamo *et al.*, 1995). This reporter construct is utilized widely to determine the responsiveness of mammalian cells to TGF-β (Wrana *et al.*, 1992; Carcamo *et al.*, 1995; Zhou *et al.*, 1998). As shown in Figure 1B, transient transfection of activated MEKK1 into BAHgpt cells can stimulate basal luciferase activity of 3TPLux to almost the same extent as TGF-β treatment itself in the absence of added MEKK1. Addition of activated MEKK1 also elevates basal luciferase activity of both AP-1-containing and NF-κB-containing luciferase constructs; however, as in the case of the 3TPLux promoter, TGF-β can augment this induction further. Additionally, we also investigated the effect of transfected activated MEKK1 on the same reporter constructs in another TGF-β-responsive cell line, the mink lung epithelial MvLu cell line (Figure 1C). In contrast to the BAHgpt cells, MvLu cells

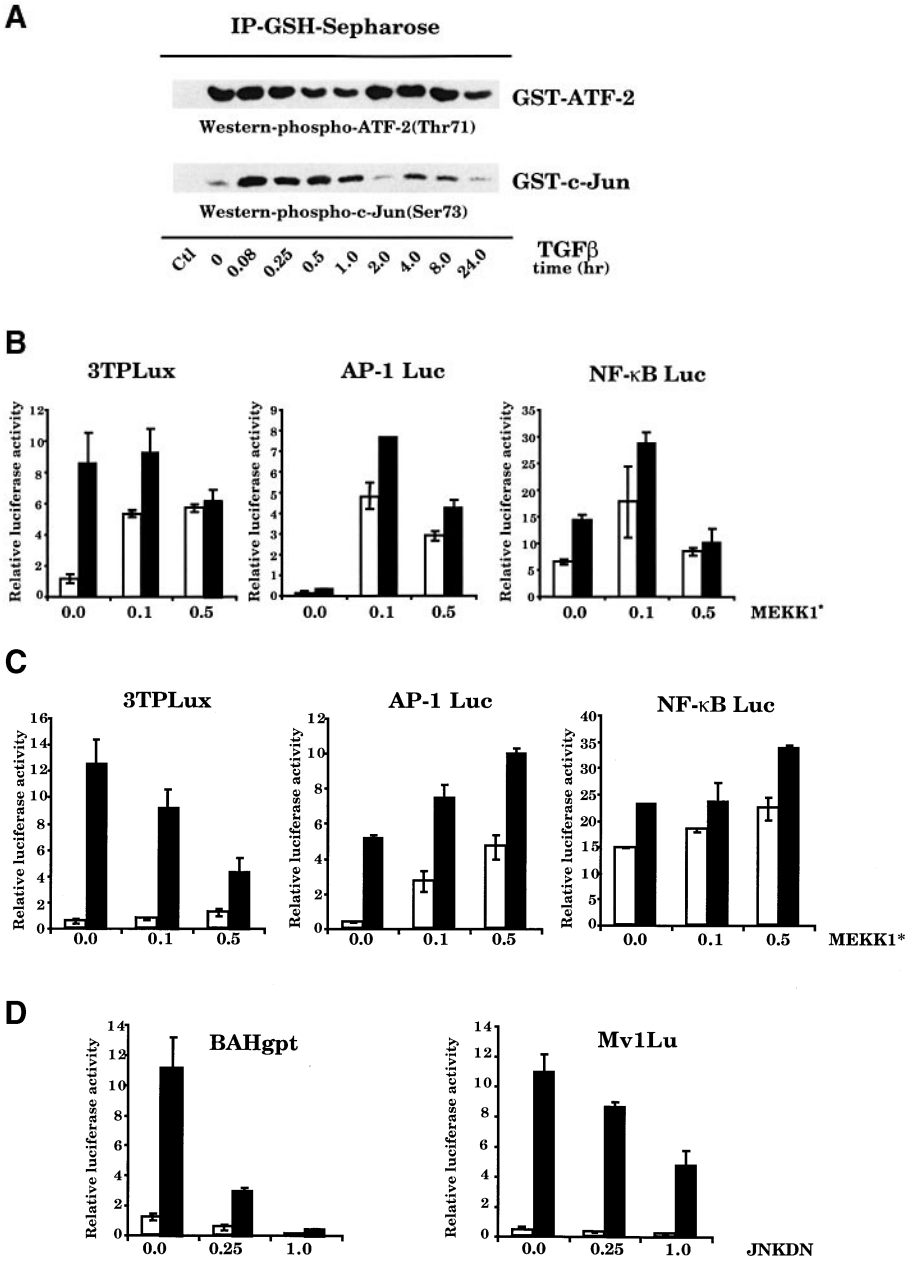

Fig. 1. Activation of JNK kinase by TGF-β. (**A**) BAHgpt cells were treated with 5 ng/ml of TGF-β for the times indicated. Lysates were prepared and kinase assays were performed utilizing 2 μg of GST–c-Jun or GST–ATF-2 as substrates as described in Materials and methods. Reaction products were analyzed on 10% SDS–PAGE gels, transferred to Immobilon membrane and subjected to Western analysis utilizing either anti-phospho-ATF-2 (Thr71) or anti-phospho-c-Jun (Ser73). The control (Ctl) reaction contains only the relevant GST construct without the addition of cellular lysate to demonstrate that the phospho-specific antibodies recognize only phosphorylated reaction products. (**B** and **C**) BAHgpt cells (B) or MvLu cells (C) were transiently co-transfected with 1 μg of 3TPLux, AP-1 Luc, or NF-κB Luc and 0.2 μg of SV40-RL, with or without the indicated additions of constitutively active MEKK1 (μg) (designated MEKK1*). The blank vector pcDNA3.1 was included where needed to maintain the same amount of total transfected DNA. After 18 h, cells were incubated in the absence (open bars) or presence of TGF-β (5 ng/ml) (closed bars) for an additional 24 h. Cells were lysed and luciferase activity determined using the Promega Dual Luciferase Reporter Assay according to the manufacturer's instructions. Luciferase activity is expressed as the ratio of specific luciferase activity divided by the luciferase activity of the internal standard. Shown is the mean ± SD of duplicates from a representative experiment. (**D**) BAHgpt and MvLu cells were transiently co-transfected with 1 μg of 3TPLux and 0.2 μg of SV40-RL, with or without the addition of the indicated amounts of JNKDN plasmid (μg). pcDNA3.1 was included where needed to maintain a constant amount of transfected DNA. After 18 h, cells were incubated in the absence (open bars) or presence of TGF-β (5 ng/ml) (closed bars) for an additional 24 h. Cells were lysed and luciferase activity determined as described above. Shown is the mean ± SD of duplicates from a representative experiment.

do not display the large increase in basal luciferase activity of the 3TPLux and NF-κB Luc reporters with increased activated MEKK1 addition; however, similar to the results we obtained in BAHgpt cells, basal luciferase activity of the AP-1 Luc reporter is stimulated potently by increasing activated MEKK1 (Figure 1C).

To confirm that JNK kinase activity is indeed involved in TGF-β signaling, we assessed the effects of transient

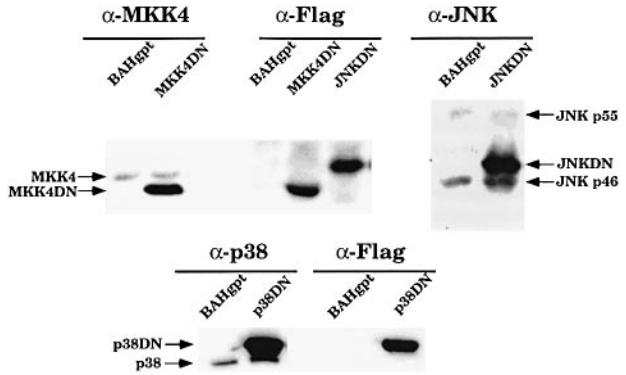

Fig. 2. Stable expression of JNKDN, MKK4DN and p38DN in BAHgpt cells. BAHgpt cells were transfected with JNK1(APF), MKK4(A) or p38(AGF) plus pSV_2neo followed by selection in 500 μg/ml of G418. Individual neo-resistant clones were pooled and maintained for further analysis. To demonstrate the expression of Flag-tagged DN constructs and endogenous JNK, MKK4 or p38, 100 μg of total cellular protein was resolved on 10% SDS–PAGE gels, transferred to Immobilon membrane and subjected to Western analysis using either a monoclonal antibody to the Flag epitope (Anti-Flag M2, Kodak/IBI) or polyclonal antibodies to JNK, MKK4 or p38 (Santa Cruz). The position of cellular JNK, MKK4 and p38, as well as the Flag-tagged JNKDN, MKK4DN and p38DN, is as indicated.

transfection of a dominant-negative form of JNK1 kinase (Derijard *et al*., 1994) on TGF-β-stimulated induction of the 3TPLux reporter construct. As shown in Figure 1D, addition of increasing amounts of the dominant-negative JNK1 construct (JNKDN) is capable of blocking TGF-β-stimulated induction of 3TPLux in both BAHgpt and MvLu cell lines. Although the transfection of the JNKDN construct is more efficient in blocking the TGF-β-stimulated response in BAHgpt cells, transfection of 1.0 μg of the JNKDN construct still decreases TGF-β-stimulated induction of the 3TPLux reporter in MvLu cells by 50%. The differences we observe between these two cell lines may reflect the high basal JNK activity which we observe in exponentially growing MvLu cells (data not shown), in contrast to BAHgpt cells (Figure 1A). The direct activation of JNK kinase activity by TGF-β, the ability of activated MEKK1 to stimulate TGF-β-responsive reporter constructs and the ability of a dominant-negative form of JNK1 to block TGF-β-stimulated reporter induction all indicate that JNK kinases play a role in TGF-β-stimulated gene induction.

Activation of JNK kinase is required for TGF-β-mediated induction of fibronectin, but not PAI-1 synthesis

To study further the involvement of the JNK family in TGF-β-mediated signaling, we generated pools of cells which stably express dominant-negative forms of both JNK1 and MKK4, one of the upstream activators of JNK (Derijard *et al.*, 1994; Whitmarsh *et al.*, 1995). As a control for the specificity of the responses we observe for the JNK pathway, we also generated pools of cells which stably express a dominant-negative form of the p38 kinase, p38(AGF) (Raingeaud *et al.*, 1995). We chose to utilize the BAHgpt cell line for these studies, since we demonstrated that they are more responsive to the effects of both the stimulation and inhibition of the JNK pathway, as compared with MvLu cells (Figure 1). As shown in Figure 2, expression of the dominant-negative forms of

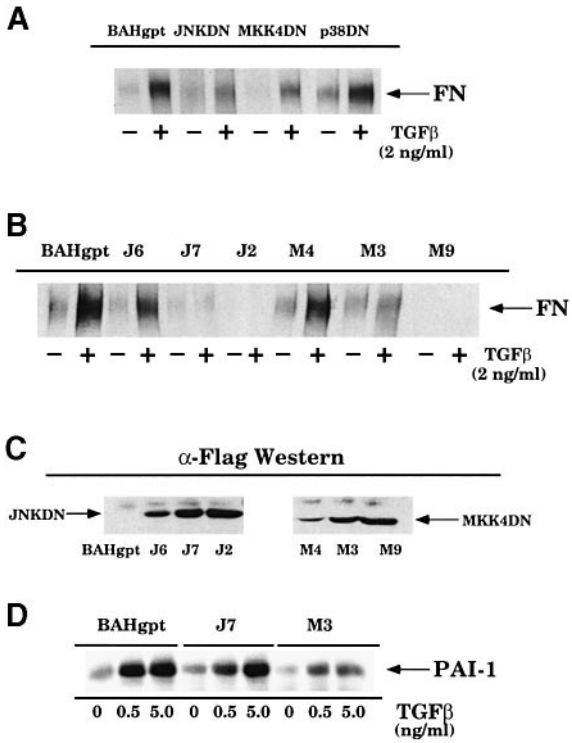

Fig. 3. TGF-β-mediated induction of fibronectin requires activation of JNK kinase. (**A**) FN induction following TGF-β stimulation was assayed in BAHgpt, JNKDN, MKK4DN and p38DN pools of cells by immunoprecipitation of ^{35}S-labeled FN as described in Materials and methods. Immunocomplexes were resolved on 6% SDS–PAGE gels, subjected to fluorography and visualized by autoradiography. The position of FN is indicated by the arrow. (**B**) Individual clonal cell lines were isolated from the JNKDN- and MKK4DN-expressing pools, designated by either J or M and clone number, referring to a JNKDN- or MKK4DN-derived clone, respectively. These individual cell lines as well as parental BAHgpt cells were assayed for induction of FN synthesis as described above. (**C**) Lysates were prepared from BAHgpt, and individual clonal cell lines designated as described in Materials and methods. Expression of Flag-tagged JNKDN and MKK4DN was analyzed from 100 μg of total cell lysate, resolved on 10% SDS–PAGE gels, transferred to Immobilon membrane and subjected to Western analysis utilizing a monoclonal antibody to the Flag epitope (Anti-Flag M2, Kodak/IBI). The position of Flag-tagged JNKDN and MKK4DN is indicated by the arrow. (**D**) For analysis of PAI-1 synthesis, BAHgpt and the individual clonal cell lines J7 and M3 were treated with the indicated doses of TGF-β and assayed for PAI-1 deposition in the ECM as described in Materials and methods. PAI-1 is indicated by the arrow.

JNK1 (JNKDN), MKK4 (MKK4DN) and p38 (p38DN) is much greater than endogenous levels of kinase present in the cells. The use of pools of cells which stably express the dominant-negative construct allows us to evaluate TGF-β-responsiveness in a population where the majority of the cells express the construct, as compared with transient transfection where only a small minority of the cells express the construct of interest.

To define the role of JNK kinases in endogenous TGF-β-stimulated responses, the effects of the JNKDN, MKK4DN and p38DN constructs were first examined on TGF-β-mediated induction of FN protein synthesis. When BAHgpt cells were stimulated with TGF-β, a marked elevation of FN production was observed (Figure 3A). In sharp contrast, expression of the JNKDN and MKK4DN constructs dramatically decreased TGF-β-stimulated FN induction, with little effect on the basal level of expression

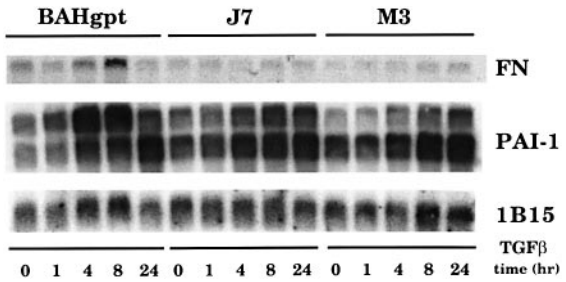

Fig. 4. Northern analysis of TGF-β-mediated fibronectin and PAI-1 mRNA induction in BAHgpt, J7 and M3 cell lines. BAHgpt and the individual clonal cell lines J7 and M3 were treated with 5 ng/ml TGF-β for the indicated times. Poly(A)$^+$ RNA was prepared from the cells as described in Materials and methods, and 5 μg was resolved on a 1.2% agarose/formaldehyde gel, transferred to nitrocellulose and subjected to Northern analysis using a probe to human FN. The filter was stripped sequentially and reprobed with a probe to human PAI-1, which detects both transcripts for PAI-1, and cyclophilin (1B15), as described in Materials and methods.

of FN (Figure 3A). Expression of the p38DN construct, however, does not block TGF-β-mediated FN induction, suggesting that the JNK pathway is required specifically for TGF-β-stimulated FN induction (Figure 3A). We next isolated individual clonal cell lines from the JNKDN- or MKK4DN-expressing pools to determine the effects of different levels of dominant-negative expression on TGF-β-mediated FN induction. As shown in Figure 3B and C, clones which express an increasing amount of the JNKDN or MKK4DN construct display a decrease in TGF-β-stimulated FN induction, which correlates well with the level of dominant-negative expression. For example, J6 and M4, which express the lowest levels of JNKDN and MKK4DN, respectively, show the least inhibition of the TGF-β response.

To assess the effects of the JNKDN and MKK4DN constructs on another component of the ECM, TGF-β-mediated induction of PAI-1 expression was evaluated. Although TGF-β induces a strong dose-dependent response in the BAHgpt cells, this response is attenuated in both the J7 and M3 cells, two clonal cell lines which express an intermediate amount of dominant-negative construct (Figure 3D). Expression of the p38DN construct, however, did not have any effect on TGF-β-stimulated PAI-1 induction (data not shown).

To characterize further the effects of the JNKDN and MKK4DN constructs on FN and PAI-1 induction, Northern blot analysis of the different cell lines was used to examine mRNA levels. For these analyses, we chose the same two individual clonal cell lines, J7 and M3, which stably express a moderate level of JNKDN and MKK4DN construct, respectively. As shown in Figure 4, FN message was induced by TGF-β in BAHgpt cells beginning at 4 h, reaching a peak at 8 h. In the J7 and M3 cell lines, however, TGF-β is unable to up-regulate FN mRNA levels, although a basal message is present (Figure 4). In contrast, PAI-1 mRNA levels in BAHgpt cells are induced by 1 h post-TGF-β treatment, reaching a peak at 4 h which is maintained up to 8 h (Figure 4). TGF-β is also capable of inducing both PAI-1 transcripts in J7 and M3 cells, although to a lesser extent than in the parental cells. Together, these results indicate that the JNK pathway is required for TGF-β-mediated induction of FN protein and mRNA levels, while JNK may play a modulatory role in TGF-β-mediated induction of PAI-1.

Stable expression of dominant-negative forms of the JNK pathway alters TGF-β-stimulated transcriptional responses

To analyze the mechanism of the effects of JNKDN and MKK4DN constructs on TGF-β-mediated gene induction, J7 and M3 cell lines were examined for responsiveness to different TGF-β-stimulated transcriptional reporter constructs. As shown, treatment with TGF-β is capable of inducing activation of both the 3TPLux and AP-1 Luc reporter constructs in BAHgpt cells; however, this response is abrogated in both the J7 and M3 cells (Figure 5A). In contrast, although TGF-β-stimulated induction of NF-κB Luc is blocked in the JNKDN-expressing J7 cell line, TGF-β-stimulated induction of NF-κB Luc is not affected by expression of the MKK4DN construct in the M3 cell line (Figure 5A).

To define the effects of blockade of the JNK signaling pathway on specific transcription factors, the ability of the BAHgpt, J7 and M3 cell lines to transactivate plasmids containing the activating domains of c-Jun, ATF-2 and Elk-1 fused to the GAL4 DNA-binding domain was examined. In this system, luciferase induction only occurs if the transactivating domain of the construct becomes phosphorylated as a result of activation of a specific signaling pathway. In BAHgpt cells, TGF-β stimulates transactivation of all three reporters; however, TGF-β-stimulated transactivation is blocked efficiently in both the J7 and M3 cell lines (Figure 5B). Additionally, expression of the JNKDN construct in the J7 cell line is capable of blocking basal transactivation, while expression of the MKK4DN construct in the M3 cell line is less efficient. Similarly, the JNKDN- and MKK4DN-expressing pools from which these cells were derived display the same attenuation of these TGF-β-stimulated reporter constructs (data not shown). Together, these results suggest that TGF-β-mediated induction of the 3TPLux, AP-1 Luc and NF-κB Luc reporter constructs requires cellular JNK activity, and suggest that activation of these promoters may occur through the TGF-β-stimulated transactivation of the transcription factors c-Jun, ATF-2 and Elk-1.

TGF-β-stimulated induction of fibronectin synthesis does not require the ERK or p38 pathways

The promoter of the FN gene contains three CRE sites, all of which previously have been demonstrated to be responsive to cAMP induction (Bowlus *et al.*, 1991). To assess whether TGF-β-mediated induction of FN occurs through CREB activation, which is stimulated by both the ERK and p38 pathways (Cohen, 1997), we utilized pharmacological agents which have been shown to block these signaling pathways effectively. Pre-treatment of BAHgpt cells with PD98059, which blocks ERK activation (Alessi *et al.*, 1995), or SB203580, which inhibits p38 (Tong *et al.*, 1997), prior to addition of TGF-β had no effect on basal or TGF-β-stimulated levels of FN induction (Figure 6A and B). Similarly, pre-treatment of BAHgpt cells with PD98059 did not block TGF-β-stimulated induction of PAI-1 (Figure 6C). Although pre-treatment

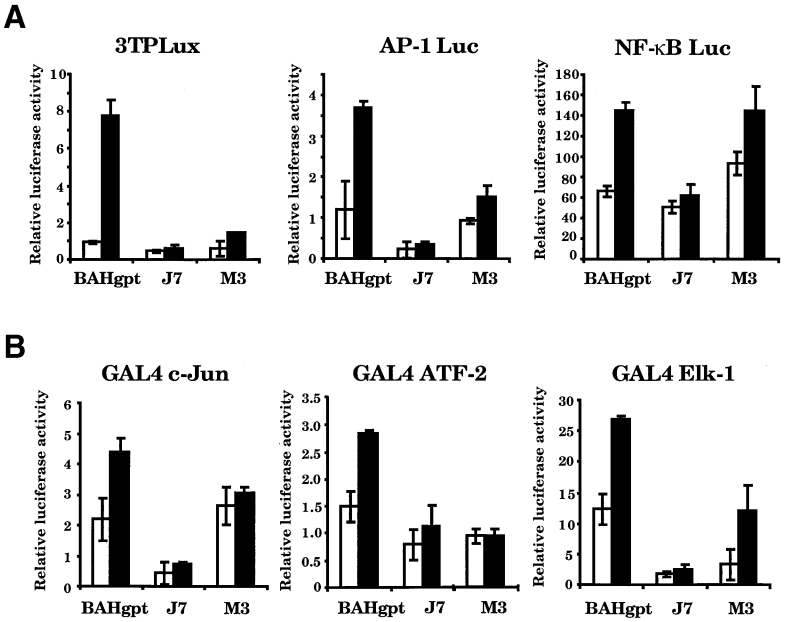

Fig. 5. Expression of JNKDN and MKK4DN alters TGF-β-stimulated transcriptional responses. (**A**) BAHgpt and the individual clonal cell lines J7 and M3 were co-transfected transiently with 1 μg of 3TPLux, AP-1 Luc or NF-κB Luc and 0.2 μg of SV40-RL as described in Materials and methods. After 18 h, cells were incubated in the absence (open bars) or presence of TGF-β (5 ng/ml) (closed bars) for an additional 24 h. Cells were lysed and luciferase activity determined using the Promega Dual Luciferase Reporter Assay according to the manufacturer's instructions. Luciferase activity is expressed as the ratio of specific luciferase activity divided by the luciferase activity of the internal standard. Shown is the mean ± SD of duplicates from a representative experiment. (**B**) BAHgpt and the individual clonal cell lines J7 and M3 were co-transfected with 1 μg of pFR-Luc, 0.2 μg of SV40-RL and 0.25 μg of either GAL4–cJun, GAL4–ATF-2 or GAL4–Elk-1 (PathDetect *Trans*-Reporting System, Stratagene). After 18 h, cells were incubated in the absence (open bars) or presence of TGF-β (5 ng/ml) (closed bars) for an additional 24 h. Cells were lysed and luciferase activity determined as above. Shown is the mean ± SD of duplicates from a representative experiment.

of BAHgpt cells with SB203580 appeared to decrease the stimulation of PAI-1 synthesis by 0.5 ng/ml TGF-β, we did not observe any dimunition of TGF-β-stimulated PAI-1 synthesis in the p38DN-expressing cells (data not shown). These results indicate that TGF-β-mediated FN or PAI-1 induction does not require the ERK or p38 pathways. These results also confirm the inability of p38DN to block TGF-β-stimulated FN induction (Figure 3A).

Smad4 TGF-β-mediated induction of fibronectin is Smad4 independent

The SMAD family of proteins have been identified as components of the signaling pathways utilized by members of the TGF-β superfamily (Heldin *et al.*, 1997; Massagué, 1998). Recently, Smad3 and Smad4 have been shown to interact with the transcription factors c-Jun and c-Fos (Zhang *et al.*, 1998). To ascertain whether the SMAD proteins are required for TGF-β-mediated FN induction, we utilized several cell lines which do not express endogenous Smad4. Both the MDA-MB 468 cell line (Schutte *et al.*, 1996; de Winter *et al.*, 1997) and the BxPC3 cell line (Le Dai *et al*., 1998; Villanueva *et al*., 1998) previously have been shown to have a homozygous deletion of the Smad4 gene. Additionally, the cell line SW480.7 has been reported to lack expression of Smad4 (Zhang *et al*., 1996). TGF-β treatment of Smad4-deficient MDA-MB 468, BxPC3 and SW480.7 cell lines, however, is still able to stimulate FN induction (Figure 7A), whereas TGF-β-mediated induction of PAI-1 synthesis in MDA-MB 468 cells is abrogated (Figure 7B). These results demonstrate that TGF-β-mediated induction of FN synthesis is Smad4-independent.

TGF-β signaling mutants deficient in TGF-β-mediated fibronectin induction show reduced TGF-β-stimulated JNK kinase activity

We previously have isolated mutant cell lines derived from BAHgpt cells which are deficient in TGF-β-mediated signaling (Hocevar and Howe, 1996). During the characterization of these mutant cell lines, we identified two clones, M 7-5 (Hocevar and Howe, 1996) and M 903 (Lee *et al.*, 1997), which are deficient in TGF-β-mediated FN induction (Figure 8A). An additional TGF-β-signaling mutant cell line was isolated, M 5-3, which retains TGF-β-stimulated FN induction (Hocevar and Howe, 1996; Figure 8A). To determine whether the lack of FN induction could be due to a defect in JNK activation, kinase assays were performed on the mutants M 7-5, M 903 and M 5-3 following TGF-β treatment. Both of the mutant cell lines which fail to induce FN synthesis following TGF-β stimulation displayed diminished TGF-β-stimulated JNK activity towards the substrate GST–c-Jun compared with parental BAHgpt cells (Figure 8B). The mutant cell line M 5-3, which induces FN synthesis following TGF-β treatment, however, displays TGF-β-stimulated JNK activation (Figure 8B). Finally, the ability of the mutant cell lines to exhibit TGF-β-stimulated transactivation of the GAL4–c-Jun and GAL4–ATF-2 constructs was examined. TGF-β-mediated transactivation of both reporter constructs is attenuated in the signaling mutants which fail to induce FN synthesis following TGF-β treatment (Figure 8C), consistent with the decreased JNK kinase

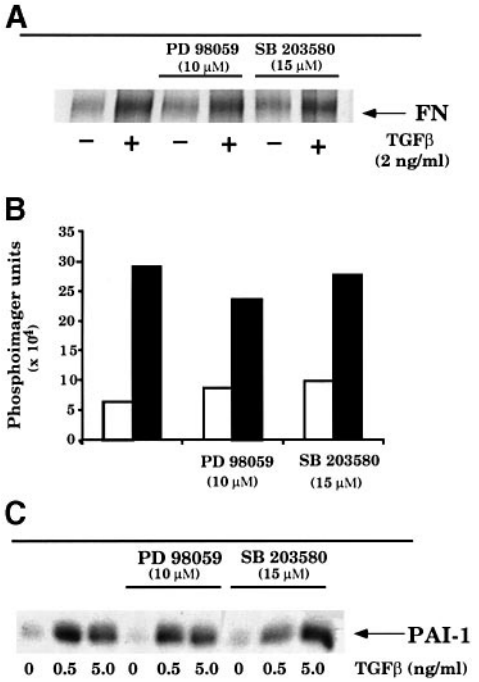

Fig. 6. Effect of MAP kinase inhibitors on TGF-β-mediated fibronectin and PAI-1 induction. (**A**) For the determination of the effects of MAP kinase inhibitors on TGF-β-stimulated FN synthesis, BAHgpt cells were pre-treated with 10 μM PD98059 or 15 μM SB203580 for 30 min prior to addition of 2 ng/ml of TGF-β for 18 h. Induction of FN was then assayed as described in Materials and methods. (**B**) FN synthesis depicted in (A) was quantitated by phosphoimager analysis and expressed as relative phosphoimager units. Closed bars represent the presence and open bars the absence of TGF-β. (**C**) For the determination of the effects of MAP kinase inhibitors on TGF-β-stimulated PAI-1 production, cells were pre-treated for 30 min with either 10 μM PD98059 or 15 μM SB203580 prior to addition of TGF-β where indicated. PAI-1 production was assayed as described in Materials and methods.

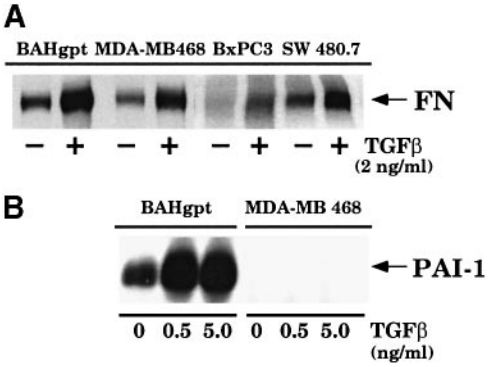

Fig. 7. TGF-β-mediated fibronectin induction is Smad4-independent. (**A**) BAHgpt, MDA-MB 468, BxPC3 and SW480.7 cells were assayed for TGF-β-stimulated FN induction by ^{35}S-labeling of cells followed by immunoprecipitation of labeled FN as described in Materials and methods and Figure 3A. (**B**) BAHgpt and MDA-MB 468 cells were treated with the indicated doses of TGF-β and assayed for PAI-1 induction as described in Materials and methods.

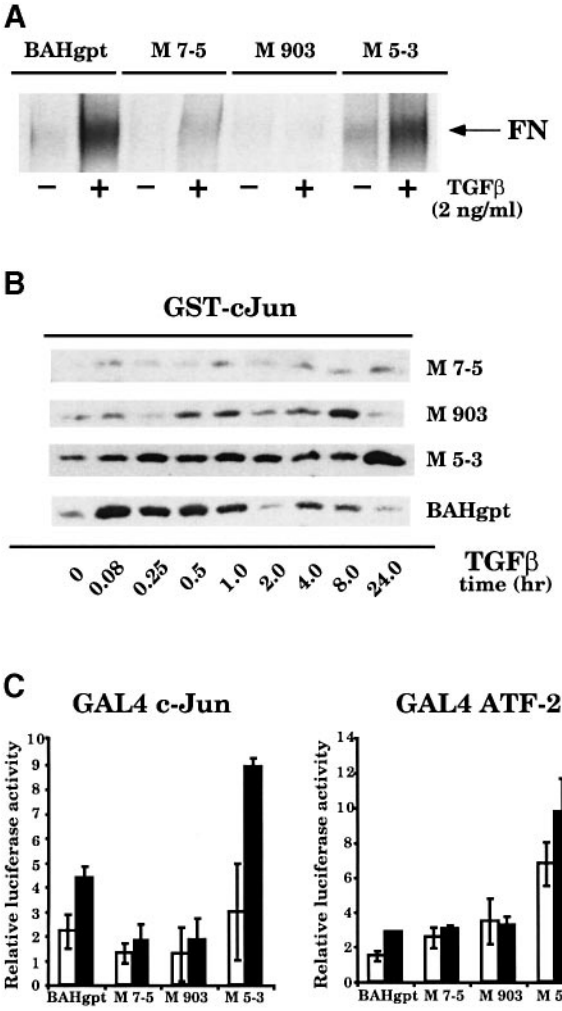

Fig. 8. Loss of TGF-β-mediated fibronectin induction is associated with decreased JNK activity in TGF-β-signaling mutants. (**A**) BAHgpt, M 7-5, M 903 and M 5-3 cells were assayed for TGF-β-mediated FN synthesis as described in Materials and methods. (**B**) BAHgpt, M 7-5, M 903 and M 5-3 cells were subjected to *in vitro* JNK kinase assays utilizing GST–c-Jun as substrate as described in Materials and methods. Phosphorylated GST–cJun at Ser73 was detected by Western analysis utilizing a phospho-specific antibody (New England Biolabs) as described in Materials and methods. (**C**) BAHgpt, M 7-5, M 903 and M 5-3 cells were assayed for TGF-β-stimulated GAL4–c-Jun and GAL4–ATF-2 transactivating potential using the PathDetect system by transient transfection assays as described in Materials and methods. Luciferase activity is expressed as the ratio of specific luciferase activity divided by the luciferase activity of the internal standard. Shown is the mean ± SD of duplicates from a representative experiment.

which was observed (Figure 8B). Additionally, the mutant cell line M 5-3 displays increased transactivation of the GAL4–c-Jun and GAL4–ATF-2 constructs as compared with the parental BAHgpt cells (Figure 8C), again consistent with the level of JNK activity which is observed in this cell line (Figure 8B). Taken together, these results support the hypothesis that JNK activity, which modulates the activity of c-Jun and ATF-2, is required for TGF-β-mediated induction of FN.

Discussion

FN, a widely distributed component of the ECM, promotes cell adhesion, migration and cytoskeletal organization, thereby influencing cellular proliferation and differentiation (Kornblihtt *et al.*, 1996). FN is essential for embryonic development as evidenced by the lethal phenotype of the FN knockout which exhibits defects in mesodermal migration, proliferation, adhesion and differentiation (George *et al.*, 1993). In the adult, FN provides

a matrix for fibroblast migration during wound healing (Greiling and Clark, 1997) and promotes the differentiation of fibroblasts to myofibroblasts (Serini *et al.*, 1998). Myofibroblasts are required for efficient and rapid wound closure (Martin, 1997); however, these cells are also responsible for the overproduction of ECM which is observed in many fibroproliferative disorders (Zhang *et al.*, 1994). TGF-β has often been implicated in a variety of fibrotic disorders, including fibrosis of the liver, lung and kidney (Border and Noble, 1994), as well as in atherosclerosis, where increased FN expression commonly is associated with increased TGF-β expression (Farhadian *et al.*, 1995; Rasmussen *et al.*, 1995).

In this study, we report that TGF-β-stimulated FN mRNA and protein induction depend on JNK kinase activity. In BAHgpt cells, we observed a biphasic stimulation of JNK activity, with an increase in JNK activity occurring as rapidly as 5 min and a second wave of activity persisiting through 8 h. This rapid activation of JNK was reported previously by Frey and Mulder (1997) and is similar to the rapid JNK activation seen after stimulation with tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) (Sluss *et al.*, 1994; Gupta *et al.*, 1996), both of which have been shown to induce FN (Coito *et al.*, 1995; Molossi *et al.*, 1995). In contrast, Atfi *et al.* (1997b) did not observe JNK activation until 2 h, with a maximum JNK activity occurring at 12 h, which may correspond to the second peak of activity which we observe in BAHgpt cells. These differences may reflect not only differences in cell types, but also differences in the way in which the kinase assays were performed. Our assays were designed to detect total TGF-β-stimulated JNK activity; however, the isoforms activated by TGF-β *in vivo* remain to be determined. Atfi *et al.* (1997a) suggested that activation of JNK was involved in the apoptotic response induced by Smad4, while Frey and Mulder (1997) show that the ability of TGF-β to mediate growth inhibition is correlated with JNK activation in breast cancer cells. In these studies, we demonstrate that JNK activity is required for TGF-β-stimulated induction of the reporter construct 3TPLux in both BAHgpt and MvLu cells. More importantly, however, we show a direct endogenous gene target for TGF-β-mediated JNK activation in BAHgpt cells, the FN gene. Taken together, these studies indicate that activation of the JNK pathway is required for responses which are mediated by TGF-β.

We demonstrate that TGF-β stimulation causes transcriptional activation of the reporter constructs 3TPLux, AP-1 Luc and NF-κB Luc. Although addition of activated MEKK1, an upstream activator of the JNK pathway, can mimic some of this induction, TGF-β can augment the response further. This suggests that additional components besides those activated by the JNK pathway are involved in TGF-β-mediated induction of these reporter constructs. In the case of the 3TPLux reporter, Smad3 and Smad4, which are activated in response to TGF-β, have been shown to bind directly to the PAI-1 promoter region present in the construct (Yingling *et al.*, 1997; Dennler *et al.*, 1998). However, Yingling *et al.* (1997) demonstrated that disruption of the AP-1 sites in the 3TPLux promoter results in loss of TGF-β-stimulated activity. Additionally, co-transfection of TAM67, a dominant-negative form of c-Jun which lacks both the site for JNK binding and activating phosphorylation sites, was shown to block TGF-β-stimulated induction of 3TPLux (Atfi *et al.*, 1997b). As shown here, stable expression of dominant-negative JNK1 and MKK4, as well as transient expression of JNKDN, also blocks TGF-β-mediated induction of 3TPLux. These data suggest that activation of c-Jun at the TRE site of 3TPLux is required for 3TPLux induction, while the Smad3–Smad4 complex may be recruited to the site following TGF-β stimulation to augment the response further. TGF-β has been shown to induce the expression of c-Fos (Pertovaara *et al.*, 1989), which may be involved in transactivation of the AP-1 Luc construct. By contrast, although it has been demonstrated previously that TGF-β can activate NF-κB (Li *et al.*, 1998), the mechanism by which this occurs is unknown. Our results suggest that TGF-β-mediated NF-κB activation may occur as a consequence of the activation of the JNK pathway; however, the exact mechanism by which this occurs remains to be determined. Taken together, these results indicate that TGF-β-stimulated activation of JNK proceeds through MKK4 to JNK at AP-1-mediated sites; however, TGF-β may also stimulate another activator of JNK, such as MKK7, to effect NK-κB-mediated induction since MKK4DN cannot block the TGF-β-stimulated response.

Heterodimers of ATF-2 and c-Jun can bind to CRE sites in DNA, many of which are found in inflammatory response genes, such as the urokinase (De Cesare *et al.*, 1995), IFN-β (Du *et al.*, 1993), E-selectin (Read *et al.*, 1997), TGF-β2 (Kim *et al.*, 1992), FN (Dean *et al.*, 1987; Bowlus *et al.*, 1991) and c-Jun (van Dam *et al.*, 1993) promoters. Previous studies have shown that a c-Jun–ATF-2 heterodimer bound to the positive domain II (PDII) in the E-selectin promoter was important for transcriptional regulation by TNF-α (Read *et al.*, 1997). Regulation of this domain by TNF-α was dependent on JNK and p38 activation (Read *et al.*, 1997). In our studies, we show that JNK activation is required for TGF-β-stimulated FN induction. The FN promoter contains three CRE sites (Bowlus *et al.*, 1991), one of which, CRE-170, has been shown to be occupied by c-Jun–ATF-2 (van Dam *et al.*, 1993). This CRE site at –170 is important for FN induction by cAMP (Bowlus *et al.*, 1991), serum (Dean *et al.*, 1990), E1A and the HPV16-E6 protein (Shino *et al.*, 1997). We postulate that TGF-β-stimulated transcriptional activation also proceeds through this element, which is supported by the demonstration that expression of dominant-negative JNK and MKK4 results in decreased TGF-β-stimulated transactivating ability of GAL4–c-Jun and GAL4–ATF2. In agreement with this, Dean *et al.* (1990) indicated that TGF-β was able to stimulate a construct containing the FN –170 CRE element; however, this level of induction was much less than the total induction of FN synthesis induced by TGF-β. This difference may be due to increased FN message stability, which is also regulated by TGF-β (Dean *et al.*, 1989; Wrana *et al.*, 1991). Whether activation of the JNK pathway can lead to increased FN message stability or whether JNK activates other transcription factors at other sites in the FN promoter currently is under investigation.

One of the most potent inducers of PAI-1 is TGF-β, which also has been shown to stimulate the transcription and message stability of PAI-1 (Westerhausen *et al.*, 1991). Early studies of the regulatory regions of the PAI-1

promoter responsible for TGF-β-mediated induction demonstrated the presence of several AP-1 sites (Keeton *et al.*, 1991; Descheemaeker *et al.*, 1992). Proteins bound to these sites could be competed with oligonucleotides containing consensus AP-1 sites, suggesting that members of the AP-1 family were important in TGF-β-mediated PAI-1 induction (Keeton *et al.*, 1991; Descheemaeker *et al.*, 1992). Recently, Dennler *et al.* (1998) have demonstrated that a Smad3–Smad4 heterodimer binds to three CAGA sequence elements in the PAI-1 promoter. One of the AP-1 sites identified previously is located within 10 bp of the –730 CAGA repeat, raising the possibility that transcription factors bound to the AP-1 site may physically interact with the Smad3–Smad4 heterodimer bound to the CAGA repeat. In support of this, it has been reported recently that a Smad3–Smad4 heterodimer could interact with both c-Jun and c-Fos bound to the AP-1 site from the collagenase promoter (Zhang *et al.*, 1998). Our studies demonstrate a requirement for Smad4 in the induction of PAI-1 by TGF-β, which is suggested by the lack of TGF-β-mediated PAI-1 induction in the Smad4-deficient MDA-MB 468 cells. In support of this, Dennler *et al.* (1998) have demonstrated the lack of TGF-β-mediated transcriptional activation of a PAI-1 promoter construct in MDA-MB 468 cells which could be restored by transfection of Smad4. Our results indicate that the JNK pathway through activation of c-Jun may also modulate TGF-β-mediated PAI-1 induction, since both the JNKDN- and MKK4DN-expressing cells exhibit an attenuation of TGF-β-stimulated message and protein levels; however, JNK activation is not sufficient. This is in contrast to TGF-β-mediated regulation of FN, which requires activation of the JNK pathway but does not require Smad4.

Our studies have shown that TGF-β-mediated activation of the JNK pathway proceeds through MKK4 to modulate FN induction. The upstream MKK4 activator or MAPK kinase kinase, which is stimulated by TGF-β leading to JNK pathway activation, currently is unknown. Whether it is MEKK1, which we have shown to mediate induction of TGF-β-responsive reporter constructs, or whether it is TAK1, which previously has been shown to activate the JNK pathway (Shirakabe *et al.*, 1997; Wang *et al.*, 1997), remains to be determined.

In summary, we have demonstrated that the JNK pathway is activated by TGF-β in BAHgpt cells and is required for TGF-β-mediated induction of FN message and protein synthesis. This may proceed solely through regulation of FN promoter activity by activation of the transcription factors c-Jun and ATF-2, or may also involve increased FN message stabilization. Additionally, we demonstrate that FN induction does not require the participation of Smad4. These studies are the first to identify a TGF-β-mediated gene, the FN gene, which is a direct target of the JNK pathway, and also the first to identify a gene which is modulated by TGF-β in a Smad4-independent manner. It still remains a possibility that Smad2 or Smad3 can act independently on the FN promoter, or that there are other common mediator Smads besides Smad4 which are involved in TGF-β-mediated FN induction. The identification of other TGF-β-responsive genes which are targeted by the JNK pathway currently is under investigation.

Materials and methods

Cell culture, DNA constructs and TGF-β treatment

BAHgpt, M 7-5, M 903 and M 5-3 cell lines have been described previously (Hocevar and Howe, 1996; Lee *et al.*, 1997) and were cultured in DME/F12 media supplemented with 10% calf serum and 100 μg/ml hygromycin B (Gibco-BRL). MDA-MB 468 and MvLu (CCL64) cells, obtained from the American Type Culture Collection, along with BxPC3 and SW480.7 cells were cultured in DME/F12 media supplemented with 10% fetal bovine serum. In these studies, we utilized recombinant human TGF-β2 which was generously provided by Genzyme (Cambridge, MA). This TGF-β2 was found to be equivalent to TGF-β1 in all responses tested. The 3TPLux promoter construct has been described previously (Wrana *et al.*, 1992; Carcamo *et al.*, 1995). The AP-1 Luc (7× AP-1 sites), NF-κB Luc (5× NF-κB sites) and the GAL4-transactivating system (PathDetect) were all purchased from Stratagene. This PathDetect system consists of the reporter vector pFA-Luc (5× GAL4-binding element) and the GAL4 fusion vectors pFA-cJun (1–223), pFA-ATF-2 (1–96), pFA-Elk1 (307–427) and a constitutively active form of MEKK1, pFC-MEKK (380–672). The pcDNA3.1 blank vector was obtained from Invitrogen.

Establishment of stable dominant-negative cell lines

The dominant-negative constructs JNK1(APF), MKK4(A) and p38(AGF) have been described previously (Derijard *et al.*, 1994; Raingeaud *et al.*, 1995; Whitmarsh *et al.*, 1995). For transfection of JNK1(APF) and MKK4(A), BAHgpt cells were transfected with 10 μg of DNA per 100 mm dish with 10 μg/ml polybrene as previously described (Hocevar and Howe, 1996). The p38(AGF) construct (10 μg) was co-transfected with pSV_2neo (2 μg) to allow for selection of transfectants. Stable transfectants were selected and maintained in media containing 500 μg/ml geneticin (Gibco-BRL). Individual clones were isolated from pools of geneticin-resistant cells by limiting dilution or ring cloning.

Protein kinase assays and Western blot analysis

Cells were treated with 5 ng/ml TGF-β for various times followed by lysis in buffer D (20 mM Tris pH 7.5, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na_3VO_4 and EDTA-free protease inhibitor cocktail, complete; Boehringer Mannheim). Extracts were centrifuged at 14 000 *g* at 4°C for 15 min and 100 μg of total cellular protein were incubated with an equal volume of kinase buffer [25 mM HEPES pH 7.5, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 50 μM ATP, plus protease inhibitor] and 2 μg of either GST–c-Jun 1–79 (Stratagene) or GST–ATF-2 1–96 (Santa Cruz) followed by incubation at 30°C for 30 min. At this time, 25 μl of glutathione–agarose (Pharmacia) was added and samples were rocked for 30 min at 4°C. Pellets were washed three times in 1× phosphate-buffered saline (PBS) containing 1% Triton X-100 and resuspended in Laemmli sample buffer. Reaction products were resolved on 10% SDS–PAGE gels and transferred to Immobilon membrane (Millipore). Immunoblots were probed with antibodies specific to phosphorylated c-Jun at Ser73 or ATF-2 at Thr71 (New England Biolabs). Immunocomplexes were detected by enhanced chemiluminescence (Renaissance, NEN).

For detection of Flag-tagged JNKDN, MKK4DN and p38DN constructs, cells were lysed in buffer D as described above and 100 μg of total cellular protein was resolved on 10% SDS–PAGE gels, transferred to Immobilon membrane and subjected to Western analysis using either a monoclonal antibody to the Flag epitope (Anti-Flag M2, Kodak/IBI) or polyclonal antibodies to JNK (#sc 571, Santa Cruz), MKK4 (#sc 837, Santa Cruz) or p38 (#sc 728, Santa Cruz). Immunocomplexes were detected by enhanced chemiluminescence as described above.

Transient transfections and reporter gene measurements

For luciferase assays, MvLu cells were transfected with Fugene6 (Boehringer Mannheim) following the manufacturer's instructions. All other cell lines were transiently transfected by the use of cationic liposomes as previously described (Jin and Howe, 1998). Typically, 2×10^5 cells were plated in each well of a 6-well plate. The next day, cells were transfected with 1 μg/well of the specific luciferase construct (3TPLux, AP-1 Luc or NF-κB Luc) along with 0.2 μg/well SV40-RL (Promega) as an internal control for transfection efficiency. After 18 h, the medium was changed and TGF-β (5 ng/ml) was added for an additional 24 h. Cells were then lysed and luciferase activity determined using the Promega Dual Luciferase Reporter Assay as per the manufacturer's instructions. For the GAL4 fusion transactivator luciferase meas-

urements, cells were co-transfected with 1 μg of pFR-Luc, 0.2 μg of SV40-RL and 0.25 μg of either pFA-cJun, pFA-ATF-2 or pFA-Elk-1 (PathDetect *Trans*-Reporting System, Stratagene). After 18 h, cells were incubated in the absence or presence of TGF-β (5 ng/ml) for an additional 24 h. Cells were lysed and luciferase activity determined as above.

PAI-1 and fibronectin protein assays

FN production was determined as previously described (Hocevar and Howe, 1996). Briefly 4×10^5 of the indicated cells were plated in 60 mm plates in regular media and were untreated or treated with 2 ng/ml of TGF-β for 18 h. At this time, the medium was removed and replaced with methionine-free medium with or without TGF-β supplemented with 50 μCi/ml of [^{35}S]methionine. After 4 h, the medium was removed and subjected to immunoprecipitation using a polyclonal antibody to FN (Gibco-BRL) and captured with protein A–Sepharose beads (Sigma). Complexes were washed and resolved on 6% SDS–PAGE gels and subjected to fluorography followed by autoradiography. When phosphoimager analysis was performed, gels were not subjected to fluorography. Imaging was quantitated by Imagequant software. For experiments utilizing the inhibitors PD98059 (Alexis) and SB203580 (Calbiochem), BAHgpt cells were pre-treated with the indicated concentration of the inhibitors for 30 min before the addition of TGF-β. For examination of PAI-1 production, 2×10^5 of the indicated cells were treated with the indicated doses of TGF-β for 2 h in serum-free methionine-free media. At this time, 50 μCi/ml of [^{35}S]methionine was added for an additional 2 h. Extracellular matrices were prepared as previously described (Hocevar and Howe, 1996) and analyzed on 8% SDS–PAGE gels, subjected to fluorography and visualized by autoradiography. For experiments utilizing the inhibitors PD98059 and SB203580, BAHgpt cells were pre-treated with the indicated concentration of the inhibitors for 30 min before the addition of TGF-β.

Northern blot analysis

Poly(A)$^+$ RNA was isolated from BAHgpt, J7 and M3 cells after stimulation with 5 ng/ml TGF-β for the various times as previously described (Leof *et al.*, 1986). A 5 μg aliquot of poly(A)$^+$ RNA from each treatment was resolved in a 1.2% formaldehyde/agarose gel, transferred to nitrocellulose and subjected to Northern analysis as described (Jin and Howe, 1998). The same blot was stripped and reprobed for detection of FN, PAI-1 and cyclophilin (1B15) expression. The probes utilized were an *Eco*RI–*Ava*I fragment of clone pFH1 for human FN (Kornblihtt *et al.*, 1983), a *Bam*HI fragment of PAI-1 (Wun and Kretzmer, 1987) and a *Bam*HI–*Hin*dIII fragment of cyclophilin (Danielson *et al.*, 1988), which were all random primed with [α-^{32}P]dCTP using the Rediprime labeling kit (Amersham) according to the manufacturer's instructions.

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