Induction of Apoptosis by DPC4, a Transcriptional Factor Regulated by Transforming Growth Factor-β through Stress-activated Protein Kinase/c-Jun N-terminal Kinase (SAPK/JNK) Signaling Pathway*

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Many of the actions of serine/threonine kinase receptors for the transforming growth factor-β (TGFβ) are mediated by DPC4, a human MAD-related protein identified as a tumor suppressor gene in pancreatic carcinoma. Overexpression of DPC4 is sufficient to induce the activation of gene expression and cell cycle arrest, characteristic of the TGFβ response. The stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) is also one of the downstream targets required for TGFβ-mediated signaling. Here we report that expression of the dominant-interfering mutant of various components of the SAPK/JNK cascade specifically blocked both TGFβ and DPC4-induced gene expression. These dominant-interfering mutants also inhibited TGFβ-stimulated DPC4 transcriptional activity. Moreover, we find that overexpression of DPC4 causes transfected cells to undergo the morphological changes typical of apoptosis. These findings define a mechanism whereby TGFβ signals mediated by DPC4 and SAPK/JNK cascade are integrated in the nucleus to activate gene expression and identify a new cellular function for DPC4.

The transforming growth factor-β (TGFβ) is a multifunctional factor that regulates a variety of cellular processes including extracellular matrix formation, cell proliferation, differentiation, and apoptosis (1–5). Two types of single transmembrane serine/threonine kinase receptors, the type I and type II, have been found to mediate the cellular effects of the TGFβ family ligands (4, 6, 7). The signaling pathways downstream of the TGFβ receptor complex that lead to the pleiotropic effects of TGFβ are still poorly understood. However, the recent identification of DPC4 and the related protein Smad3 (mothers against decapentaplegic) have provided initial insight into the mechanism of the TGFβ receptor signal transduction (8–10). Smad3 but not DPC4 associates with the TGFβ receptors and is directly phosphorylated by the ligand-bound receptor complex (8). Coexpression of Smad3 along with DPC4 resulted in the synergistic activation of TGFβ-like responses suggesting that Smad3 act in partnership with DPC4 to initiate TGFβ signaling (8). Since DPC4 does not associate with nor become phosphorylated by the TGFβ receptors, these data raise the possibility that DPC4 may fulfill some function that is not regulated by receptor-dependent phosphorylation. Because TGFβ receptor can also trigger stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activation (11, 12), we investigated whether the TGFβ-regulated function of DPC4 involved the activation of SAPK/JNK signaling pathway.

EXPERIMENTAL PROCEDURES

Plasmids—The p3TP-Lux reporter construct (a gift from Dr. Joan Massagué) contains three consecutive 12-O-tetradecanoylphorbol-13-acetate response elements, the plasminogen activator inhibitor promoter, and a luciferase reporter gene. pRK5-Flag-DPC4 and pRK5-Flag-Smad3 were kindly provided by Dr. Rick Derynck. Expression plasmids for the dominant-negative mutant of c-Jun (pCMV5 MEKK1(K432A)), and the dominant-interfering pcDNA3-Flag-MKK4(Ala) mutant have been described previously (11). Gal4-DPC4 was a gift from Dr. Joan Massagué. G15E1b-luc and Gal4-VP16 were kindly provided by Dr. Roger Davis and Michael R. Green, respectively. The pEGFP vector encoding for the green fluorescence protein (GFP) was purchased from CLONTECH. GST-Jun(1-179) was expressed in Escherichia coli as described (13).

Cell Culture, Transfection, and Gene Expression Analysis—MDCK cells were transfected with expression vectors by the LipofectAMINE™ method (Life Technologies, Inc.). Cells were subsequently incubated in the presence or absence of human TGFβ1 (2 ng/ml) for 12 h. The luciferase activities were normalized on the basis of β-galactosidase expression from pCMV5.LacZ-Control vector and protein content.

Protein Kinase and Apoptosis Assays—Assays for SAPK/JNK activity were carried out as described (11). We analyzed MDCK cells for apoptosis by phase-contrast microscopy 48 h after transfection. Transfected MDCK cells were fixed in PBS containing 0.5% glutaraldehyde for 15 min followed by washing in PBS with 5 mM MgCl2. Fixed cells were stained overnight with PBS containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-galactopyranoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.02% Nonidet P-40. The number of blue-staining cells was determined microscopically. For the GFP, cells were fixed in PBS containing 4% paraformaldehyde, stained with Hoechst (2 μg/ml), and mounted.

DNA fragmentation (TUNEL staining) was determined using the in situ cell death detection kit (Boehringer Mannheim). The TUNEL-stained cells were visualized by microscopy.

To assess the apoptotic DNA damage, cells were collected, rinsed with Ca2+- and Mg2+-free PBS, lysed in lysis buffer (10 mM EDTA, 50 mM Tris (pH 8.0), 0.5% sodium lauryl sarcosine, 0.5 mg of proteinase K/ml), and incubated at 50 °C for 1 h. RNase A (0.5 mg/ml) was added, and lysates were incubated for an additional hour. DNA was electrophoresed in a 1% agarose gel in 0.5 × TBE running buffer (4.5 mM Tris, 4.5 mM boric acid, 62.5 mM EDTA). RESULTS AND DISCUSSION

In comparison with the immediate and transient SAPK/JNK activity induced by other stimuli such as tumor necrosis factor-α (5 min) and anisomycin (13, 14), treatment of MDCK
cells with human TGFβ1 induces a delayed and persistent increase in SAPK/JNK activity that peaks at 8 h and reaches a maximal value of about 3–5-fold over the basal activity (11). Therefore, we used conditioned medium from cells treated with TGFβ under conditions in which SAPK/JNK activation was readily observed (8 h) to address the question of whether TGFβ-induced SAPK/JNK activation occurs via a paracrine mechanism. This possibility seems more unlikely, since conditioned medium stimulation did not significantly induce the activation of SAPK/JNK in any time up to 30 min, although the addition of aspartic acid to these cells led to the increase in SAPK/JNK activity under these experimental conditions (data not shown). In contrast with SAPK/JNK, TGFβ failed to activate p38 kinase under the same experimental conditions (data not shown). Thus, the increase in SAPK/JNK activity most likely resulted from phosphorylation and activation by an upstream kinase such as mitogen-activated protein kinase (MAPK) kinase 4 (MKK4, also termed Sek1 or JNKK), a dual specificity protein kinase, which is structurally related to MKK3, the kinase that phosphorylates and promotes activation of p38 MAP kinase (15–18). The activation of SAPK/JNK indicated by our observations agrees with recent studies showing that TGFβ activates a novel MAPK kinase kinase (MAPKKK), known as TAK1, that may be involved in signal transmission by members of the TGFβ superfamily (15, 19). Activated TAK1 phosphorylates and promotes activation of MKK4, the kinase that controls activation of SAPK/JNK (19). Whether TAK1 kinase is downstream of the TGFβ receptor in the biochemical route to SAPK/JNK warrants further investigation.

To investigate whether the SAPK/JNK signaling pathway participates in TGFβ signaling, we examined the effect of dominant-negative mutants of MEKK1, MKK4, and c-Jun expression on transcriptional activation by TGFβ. We made use of p3TP-Lux, a reporter construct that directs luciferase expression in response to TGFβ (6). Expression of dominant-negative mutants MEKK1(K432A), MKK4(Ala), and c-Jun(TAM67) suppressed the TGFβ-induced reporter gene activity; similar results were obtained with all of the SAPK/JNK signaling pathway components in HepG2 and CHO cells (11 and data not shown). Further evidence for the specificity of JNK signaling pathways in mediating these processes is provided by the inability of the dominant-interfering mutant of MKK3(Ala) to inhibit reporter gene activation by TGFβ (data not shown).

DPC4 and the related protein Smad3 are also regulated by the TGFβ signal transduction pathway and may function to transmit TGFβ signals from the cytoplasm to the nucleus (4, 8, 9). Consistent with previous studies, overexpression of wild-type DPC4, but not Smad3, into MDCK cells strongly increased the basal level of p3TP-Lux expression and potentiated the ability of TGFβ to induce reporter gene activity (8, 9). Coexpression of Smad3 along with DPC4 resulted in the synergetic activation of gene reporter expression, and this effect was enhanced by TGFβ addition (Fig. 1A). The ability of the dominant-interfering mutant of c-Jun (TAM67) to inhibit TGFβ-induced transcriptional activation allows us to address the functional role of SAPK/JNK pathways in Smad3 and DPC4-mediated signaling events. TAM67 acts as a dominant-interfering mutant because of a deletion in the N-terminal transactivation domain of c-Jun that includes the binding site for SAPK/JNK (20, 21). Expression of TAM67 is sufficient to block the superinduction of the gene reporter activity by TGFβ in cells coexpressing wild-type Smad3 and DPC4. Immunoblotting with anti-Flag antibody confirms that expression of TAM67 did not alter expression of Flag-Smad3 and Flag-DPC4 proteins (data not shown). Similar results were obtained with the dominant-negative mutant MEKK1(K432A) (Fig. 1A), which is consistent with the hypothesis that SAPK/JNK function is required for transcriptional activation by Smad3 and DPC4 proteins.

To determine the potential mechanism underlying the dominant-negative activity of TAM67, we investigated whether this mutant may alter the regulation of DPC4 transcriptional activity by TGFβ. When fused to the DNA binding domain of the yeast transactivator Gal4 (147), the C-terminal domain of DPC4 activated transcription from a promoter containing Gal4-binding sites (22), thus raising the interesting possibility that DPC4 may act as a transcriptional activator to initiate TGFβ responses. In support of this hypothesis, we show that a Gal4 fusion protein containing the full-length DPC4 failed to stimulate transcription in this assay, but a strong increase of Gal4-DPC4 transcriptional activity was detected in MDCK cells expressing both Gal4-DPC4 and full-length Smad3 in the presence of TGFβ (Fig. 1B). Similar to its effect on p3TP-Lux promoter activity, expression of TAM67 inhibits both basal and TGFβ-induced Gal4-DPC4 transcriptional activity (Fig. 1B). As a control (Fig. 1B), transfection of TAM67 produces little or no
effect on a Gal4 fusion containing the potent acidic activating region of the herpes simplex virus VP16 protein (23). From these results, it is becoming evident that TAM67 acts specifically as a dominant-negative inhibitor in TGFβ signaling by blocking the function of endogenous DPC4 and Smad3 proteins and that inhibition of DPC4 transcriptional activity occurs at a level downstream of the SAPK/JNK signaling pathway.

Our analysis of the regulation of DPC4 transcriptional activity by c-Jun was consistent with the possibility that c-Jun and DPC4 could interact and produce trans-activation of the 3TP-Lux reporter. One attractive possibility is that the dominant-interfering mutant of c-Jun (TAM67) binds and sequesters DPC4, thus removing it from its natural target. However, we were unable to detect any interactions between DPC4 and TAM67 using different methods, including immunoprecipitation followed by Western blotting and in vitro binding assays with GST-DPC4 produced in bacteria. Because association could be transient or unstable, these negative results do not completely rule out direct interactions of DPC4 and c-Jun.

Another important biological activity mediated by the SAPK/JNK signaling pathway is the induction of programmed cell death (24–26). One mechanism by which JNK activation may contribute to cell death is by phosphorylating and enhancing the activity of c-Jun, which in turn regulates the activity of cell-killing genes. Consistent with this observation, death of serum-deprived PC12 cells does not require new gene transcription, although blockade of c-Jun activity by microinjection of anti-c-Jun antibodies or by ectopic expression of dominant-negative TAM67 inhibits their apoptotic death (24). Since the activity of DPC4 has been shown to be under the control of c-Jun, we were interested in determining whether DPC4 and Smad3 were able to trigger cell death. To test this hypothesis, we initially used a GFP cotransfection assay to determine the effects of DPC4 and Smad3 overexpression on cell viability. The MDCK cells expressing DPC4, but not Smad3, displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (Fig. 2). DNA staining with Hoechst 33258 (DNA dye bisbenzimide) showed that many of the DPC4-expressing cells had apoptotic nuclei, a feature not seen when control vector or Smad3 expression plasmids were used (Fig. 2A). DNA fragmentation analysis with the TUNEL method confirms that the death of MDCK cells induced by overexpression of DPC4 occurred by an apoptotic mechanism (Fig. 2B).

To further quantify the effects of ectopic expression of DPC4 and Smad3 on cell viability, in a second independent approach, cotransfections were performed with vector expressing β-galactosidase instead of GFP. In this assay, MDCK cells which die by apoptosis round up and detach from the dishes, making the relative number of blue cells a good indicator of
TGFβ, DPC4, and SAPK/JNK Cascade

Fig. 3. Smad3 synergizes with DPC4 to trigger cell death. A, MDCK cells were transiently transfected with 1 μg of a β-galactosidase expression vector (pCMV5-LacZ) in the presence or absence of 1 μg of the indicated expression constructs encoding DPC4 or Smad3, respectively. Data (mean ± S.D.) shown are the percentage of apoptotic cells among the total number of cells counted. B, MDCK cells were transiently transfected with 2 μg of either control plasmid or expression vectors for Smad3, DPC4, or Smad3 plus DPC4. Forty-eight h after transfection, the cells were fixed, and apoptosis in attached MDCK cells was examined using the TUNEL assay. The number of TUNEL-positive cells per plate was determined by counting five different fields. C, overexpression of Smad proteins induces chromatin cleavage. MDCK cells were transiently transfected with 2 μg of either control plasmid or expression vectors for Smad3, DPC4, or Smad3 plus DPC4. Transfection mixture was normalized to 6 μg of total plasmid DNA. Laddered electrophoretic patterns of oligonucleosomal DNA fragments were resolved 48 h post-transfection by electrophoresis.

DPC4 and Smad3 function (27, 28). By 48 h after transfection, over 57% of blue cells arising from cotransfection of DPC4 and the β-galactosidase showed morphological changes consistent with apoptosis, whereas only 10–20% of the blue cells that had been transfected with β-galactosidase plasmid or in combination with Smad3 exhibited such a phenotype (Fig. 3A). However, coexpression of Smad3 along with DPC4 resulted in a dramatic decrease in blue cell number, suggesting that Smad3 may act in partnership with DPC4 to trigger cell death (Fig. 3A). A similar conclusion could be drawn when the experiments were performed using the TUNEL detection method (Fig. 3B). In addition to morphological changes, the effects of DPC4 and Smad3 on apoptosis were associated with a marked increase in nuclear DNA fragmentation, because apoptosis is frequently accompanied by the cleavage of DNA at internucleosomal sites, which results in a distinct laddering pattern when analyzed by agarose gel electrophoresis (Fig. 3B). Collectively, these data provide the first demonstration that DPC4 and Smad3 together may function in a cooperative way to induce cell death.

Signaling by TGFβ family members is now relatively well understood at the level of receptor activation, and the diverse biological responses are becoming increasingly well characterized in terms of gene targets that are regulated by these signaling pathways (1–5, 11, 29). The recent identifications of the Smad protein family and SAPK/JNK signaling pathways provided an important clue as to how members of the TGFβ receptor superfamily signal downstream responses (4, 8, 9, 11, 12, 30, 31). The findings outlined in the present study provide molecular evidence that the activation of SAPK/JNK signaling pathways is required for the function of Smad proteins. The molecular framework that we proposed gives rise to a number of areas for future study, including the identification of downstream targets of DPC4 and Smad3 and to determine how these targets are regulated to initiate TGFβ responses and apoptotic cell death.

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