Constitutive JNK Activation in NIH 3T3 Fibroblasts Induces a Partially Transformed Phenotype*

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The c-Jun N-terminal kinases (JNKs) (also known as stress-activated protein kinases or SAPKs), members of the mitogen-activated protein kinase (MAPK) family, regulate gene expression in response to a variety of physiological and unphysiological stimuli. Gene knockout experiments and the use of dominant interfering mutants have pointed to a role for JNKs in the processes of cell differentiation and survival as well as oncogenic transformation. Direct analysis of the transforming potential of JNKs has been hampered so far by the lack of constitutively active forms of these kinases. Recently, such mutants have become available by fusion of the MAPK with its direct upstream activator kinase. We have generated a constitutively active SAPKβ-MKK7 hybrid protein and, using this constitutively active kinase, we are able to demonstrate the transforming potential of activated JNK, which is weaker than that of classical oncogenes such as Ras or Raf. The inducible expression of SAPKβ-MKK7 caused morphological transformation of NIH 3T3 fibroblasts. Additionally, these cells formed small foci of transformed cells and grew anchorage-independent in soft agar. Furthermore, similar to oncogenic Ras and Raf, the expression of activated SAPKβ resulted in the disassembly of F-actin stress fibers. Our data suggest that constitutive JNK activation elicits major aspects of cellular transformation but is unable to induce the complete set of changes which are required to establish the fully transformed phenotype.

Mitogen-activated protein (MAP) kinase (MAPK) kinases (MAPKs) are essential signaling molecules that translate extracellular stimuli into nuclear responses through the phosphorylation of transcription factors. Three major subfamilies of MAPKs have been isolated to date: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase or SAPK, and the p38 MAP kinase (1, 2). MAP kinase pathways are evolutionarily conserved and respond differentially to multiple physiological and non-physiological stimuli (3). Signals for ERK activation, including growth and differentiation factors (hormones as well as tumor promoters) (4), are relayed from the cell surface to the nucleus through a conserved signal transduction pathway, which includes the small G-protein Ras and a downstream kinase cascade consisting of Raf, MEK (MAPK/ERK kinase), and ERK (5). Two components of this pathway, Ras and Raf, have initially been identified as oncogenes, stressing the role of this pathway in deregulated growth (6). Moreover, the Raf → MEK → ERK cascade is essential for transformation by various classes of oncogenes (7–9).

In contrast to ERKs, SAPK/JNKs and p38 are poorly activated by mitogens but strongly stimulated in response to stress inducers like UV-light, ionizing radiation, osmotic or redox stress, heat shock, and inflammatory cytokines (e.g. tumor necrosis factor α and interleukin-1) (10). They have been implicated in embryonic development, immune response, DNA repair, cell proliferation, cell survival, and apoptosis (11, 12).

More recently, a role for JNK/SAPK in oncogenic transformation and tumor development has been postulated (13–15). Moreover, overexpression of the JNK substrate c-Jun transforms chicken embryo fibroblasts (16), and c-Jun is required for the transformation of mammalian or rodent fibroblasts by different oncogenes (17–19). Although these data suggest that SAPK/JNK may be a universal mediator of cellular transformation, an evaluation of the contribution of SAPK/JNK signaling to this process has been prevented by the lack of constitutively active mutants of this kinase. Until now, attempts to generate structure-based, site-directed mutants of MAP kinases with constitutive activity have failed. Only recently did the direct enzyme-substrate fusion yield a constitutively active form of the MAPK ERK (20). In an analogous approach, we fused the MAP kinase SAPKβ in-frame with its direct upstream activator MKK7 to generate a constitutively active SAPKβ-MKK7 (Ref. 21 and this paper). Employing this tool, we were able to show that SAPKβ-MKK7 behaves as a moderately transforming oncogene. Inducible SAPKβ-MKK7 expression in NIH 3T3 fibroblasts resulted in a morphologically transformed phenotype. In these cells the Filamentous)-actin cytoskeleton was disassembled, and cells expressing SAPKβ-MKK7 were able to grow anchorage-independent in soft agar and form small foci of transformed cells in monolayers.

MATERIALS AND METHODS

Cell Lines—Mouse NIH 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (heat inactivated at 56 °C for 45 min), 2 mM t-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C in humidified air with 5% CO₂. Generation of the tetracycline-regulated NIH 3T3 tet-off cell line (NIH 3T3...
has been described (22). In a second step, SAPKβ-MKK7 or SAPKβ-MKK7 KD, cloned into the tet-controlled vector pREV-TRE (CLON-TECH), which carries a hygromycin resistance gene, were transfected into the NIH 3T3 tet-off cell line. Cells were thereafter cultured as detailed above in the presence of 2 μg/ml tetracycline and 200 μg/ml hygromycin B.

DNA Constructs

Generation of pcDNA3-Myc-SAPKβ-MKK7

A full-length SAPKβ cDNA (GenBank™ accession number L27128; provided by Dr. J. Woodgett, Ontario Cancer Institute, Toronto, Canada) was cloned into the XhoI/XbaI sites of the mammalian transformation vector pcDNA3neo (Invitrogen) following PCR amplification with the following forward and reverse primers: 5′-CCG TCT CAG AGC AAA AGC AAG GTA GAT AAC CAG TTC TTC AC-3′ and 5′-TGC TCT AGA CTC GGC ATG TGC GTG CCT ACG GAT CCA ACC CAC GGG CGG TCC CGC CGA-3′. Behind the stop codon SAPKβ, a PCR-generated fragment of the coding sequence of mouse MKK7 (GenBank™ accession number AF00298126; provided by Dr. J. M. Penninger, Ontario Cancer Institute, Toronto, Canada) was cloned in-frame into the XbaI sites using the following primers: 5′-TGG CTC AGA GCT GTT AAC GAC CAG ATG CAG GAG ATG AAG-3′ and 5′-TGG CTC TCT AGA CTA GCT CAA GAA GGG GAG ATG ATG TGG CTG ACT CAG GAC-3′. Between the two cDNAs, an alternating combination of 10 amino acids of glutamic acid and glycine was inserted in-frame into the Nhel/HpaI sites. This linker was synthesized by invitro annealing of the following forward and reverse primers: 5′-CTA AGC GCT AGG GAG GCC GAA GGA GAT CAC TAT GTC CGG CTG ACT GAG GAC-3′ and 5′-CCA TCG TAT ATG TTA AGC CCC TCT CTC CTT CCG TCA CCT CGT CTA AG-3′. At the 5′-end of the Nhel/HpaI sequence encoded the Myc tag epitope was inserted by BamHI/XhoI. pcDNA3-Myc-SAPKβ-MKK7 KD, a kinase-inactive mutant form of this hybrid protein, has been described previously (21).

Cloning of pREV-TRE-SAPKβ-MKK7 and SAPKβ-MKK7 KD

The cDNA was released from pcDNA3 by a SphiI/BamHI digest and subcloned into pREV-TRE. pcDNA3-FLAG-H1P1 was kindly provided by Dr. R.J. Davis (University of Massachusetts, Worcester, MA). pcDNA3-HA-JBD (JIP-1; amino acids 126–281; GenBank™ accession number AF003115; provided by Dr. R.J. Davis, University of Massachusetts, Worcester, MA) was released with EcoRI/NotI from pcACT2, blunt ended, and subcloned into the EcoRV site of pcDNA3, which contains an HA (influenza hemagglutinin) tag. pcS3-MKK7 S3A was kindly provided by Dr. M. Kracht (Max Planck Institute of Immunology, Hänover, Germany), and Dr. D. Bohmann (EMBL, Heidelberg Germany) contributed the pMT35-c-Jun construct. The EH neo-v-Raf plasmid has been described (22). In a second step, SAPKβ-MKK7/KD, a sequence encoding the Myc tag epitope was inserted by Dr. R.J. Davis (University of Massachusetts, Worcester, MA). pcDNA3-FLAG-H1P1 (clone no. sc-571); phospho-c-Jun; KM-1 (mouse monoclonal; Santa Cruz Biotechnology catalog no. sc-571); phospho-c-Jun; KM-1 (mouse monoclonal; Santa Cruz Biotechnology catalog no. sc-822); ERK1 (rabbit polyclonal; Santa Cruz Biotechnology catalog no. sc-94). SAPKβ-MKK7/KD cell extracts were commercially available from New England Biolabs.

Immunostaining/Indirect Immunofluorescence—NIH 3T3 fibroblasts were seeded on glass cover slides. 36 h post-transfection the cells were washed three times with PBS and fixed with 3.7% paraformaldehyde at 4 °C for 20 min. After washing three times in PBS, cells were permeabilized in PBS supplemented with 0.2% (v/v) Triton X-100 for 3–4 min at room temperature. For immunodetection the PBS-washed cells were incubated for 1 h in the presence of 10 μl of anti-Myc (9E10) antibody diluted in 40 μl of PBS followed by three PBS washing steps. Cells were then incubated for 1 h with a mix of Cy3-labeled goat anti-mouse antibody (1:200 dilution; Jackson ImmunoResearch Laboratories) and fluorescein isothiocyanate-conjugated phalloidin (1:15 dilution; Molecular Probes). After washing with PBS, cells were mounted with Mowiol and viewed with a fluorescence microscope (Leitz DMRB).

Soft Agar Assay—6-cm tissue culture plates were covered with 5 ml of 0.5% soft agar (SeaPlaque). Cells were suspended in 2 ml of 0.3% soft agar (SeaPlaque) and added to each plate. Cells were plated in triplicate at a density of 106 cells/plate. Cells were refed twice weekly. 15 days post-transfection the cells were seeded on 9-cm tissue culture plates. Cells were stained with Giemsa to visualize the foci of transformed cells. Data shown were obtained from triplicate plates for each data point.

Results

Generation of a Constitutively Active SAPKβ Mutant—Until now, structure-based, site-directed mutagenesis failed to produce constitutively active forms of MAP kinases. As an alternative approach, the direct enzyme-substrate fusion was first successfully applied to generate a constitutively active ERK2 (20). After the addition of 200 μg/ml protein A-garose (Roche Molecular Biochemicals), the suspension was mixed again for 2 h at 4 °C. Immune complexes were washed three times in ICKA lysis buffer and once with a 50 μl of HEPES, 1 mM dihydrothreitol buffer and once with a 50 μl of HEPES, 1 mM dihydrothreitol buffer. For the kinase reaction, 30 μl of a mix containing kinase buffer (50 μl HEPES, pH 7.5, 10 mM magnesium chloride, 1 mM dihydrothreitol, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 2.5 mM EGTA, 20 μM cold ATP, and 10 μCi of [γ-32P]ATP supplemented with bacterially expressed and purified glutathione S-transferase GST-c-Jun N-terminal protein (amino acids 1–135) (24) was added. The reaction was carried out for 30 min at 37 °C under constant agitation and terminated by adding 10 μl of 5× Laemmli buffer (for 1× concentration, see above) and boiling the suspension for 5 min at 100 °C. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylation of the substrate protein was visualized by autoradiography. The amount of immunoprecipitated protein was controlled by immunoblotting.

Immunoblotting—Cells were washed three times with PBS and fixed with 3.7% paraformaldehyde at 4 °C for 20 min. After washing three times in PBS, cells were permeabilized in PBS supplemented with 0.2% (v/v) Triton X-100 for 3–4 min at room temperature. For immunodetection the PBS-washed cells were incubated for 1 h in the presence of 10 μl of anti-Myc (9E10) antibody diluted in 40 μl of PBS followed by three PBS washing steps. Cells were then incubated for 1 h with a mix of Cy3-labeled goat anti-mouse antibody (1:200 dilution; Jackson Immunoresearch Laboratories) and fluorescein isothiocyanate-conjugated phalloidin (1:15 dilution; Molecular Probes). After washing with PBS, cells were mounted with Mowiol and viewed with a fluorescence microscope (Leitz DMRB).

Soft Agar Assay—6-cm tissue culture plates were covered with 5 ml of 0.5% soft agar (SeaPlaque). Cells were suspended in 2 ml of 0.3% soft agar and added to each plate. Cells were plated in triplicate at a density of 106, 105, and 104 cells/plate and incubated for 18 days. Dishes were examined for formation of tetracycline-induced foci (1:15 dilution; Molecular Probes). After washing with PBS, cells were mounted with Mowiol and viewed with a fluorescence microscope (Leitz DMRB).
of the fusion protein (21). Western blot analysis of transiently transfected NIH 3T3 cells showed the expression of the expected 90-kDa SAPKβ-MKK7 fusion protein and also its kinase dead form, SAPKβ-MKK7 KD, in which the critical lysine residues in the ATP-binding sites of SAPKβ (K55A and K56A) and MKK7 (K76E) had been replaced by nonphosphorylatable amino acids (Fig. 1B, lanes 2 and 3).

**SAPKβ-MKK7 Fusion Protein Is Constitutively Active and Does Not Stimulate Endogenous SAPK/JNKs—MKK7 activates SAPK/JNK by phosphorylating a threonine-proline-tyrosine (TPY) motif in the central part of the protein.** The presence of phosphorylation at these sites is indicative of the activated form of the kinase. To analyze whether SAPKβ-MKK7 is constitutively active in mammalian cells, the construct was transiently transfected into NIH 3T3 fibroblasts. Before the preparation of lysates, the cells were cultured for 24 h in the presence of 0.05% serum to shut down serum growth factor signaling. As shown in Fig. 2, the inactive SAPKβ-MKK7 KD fusion protein (lane 2) was present as an unphosphorylated protein in cells, whereas SAPKβ-MKK7 was constitutively phosphorylated (lane 1). Furthermore, the expression of the constitutively active SAPKβ-MKK7 fusion protein did not affect the activation status of the endogenous SAPK/JNKs. Comparable basal levels of both p54 and p46 SAPK phosphorylation were detectable in the cell lysates of SAPKβ-MKK7 and SAPKβ-MKK7 KD or in serum-starved NIH 3T3 cells (Fig. 2, lanes 1, 2, and 3). An increase in phosphorylation could only be observed when cells were treated with the known JNK activators arsenite (lane 4) or anisomycin (lane 5) but not after the overexpression of SAPKβ-MKK7 (lane 1). To confirm the identity of the p46/p54 SAPK/JNK proteins, their migratory and immunoblot behavior was compared with SAPK/JNKs present in commercially available control lysates and found to be identical (data not included in Fig. 2). An analysis of MAPK signaling has revealed a high degree of cross-talk between these related signaling pathways. Using the same approach as above, we therefore tested these lysates for a potential activation of p38 or ERK1/2. Consistent with a previous report (25), we failed to detect any effect of SAPKβ-MKK7 on the activity of these MAPKs (data not shown).

**SAPKβ-MKK7 Possesses c-Jun Kinase Activity in Vivo**—To test whether the constitutively active SAPKβ-MKK7 fusion protein is able to phosphorylate the SAPK/JNK substrate c-Jun in vivo, SAPKβ-MKK7 and c-Jun were co-expressed in NIH 3T3 cells. An immunoblot was performed using an antibody that recognizes unphosphorylated c-Jun as well as the slower migrating phosphorylated c-Jun as well as the slower migrating phosphorylated c-Jun (Fig. 3). Whereas the kinase dead SAPKβ-MKK7 fusion protein caused no activation of c-Jun in vivo (Fig. 3, lane 1), the expression of constitutively active SAPKβ-MKK7 lead to significant phosphorylation of c-Jun as indicated by the electrophoretic mobility shift (Fig. 3, lane 2) (see also Ref. 21). c-Jun transactivation activities are regulated by phosphorylation of serines 63 and 73 through SAPK/JNKs. SAPKβ-MKK7 appears...
to be able to phosphorylate both serine residues because a third intermediate c-Jun band could be observed most likely representing the singly phosphorylated form of c-Jun. The activation of c-Jun by SAPKβ-MKK7 could be completely inhibited when the SAPK/JNK binding domain of JIP-1 is coexpressed (Fig. 3, lane 3). JIP-1 has been described as a scaffold protein that interacts with different components of its SAPK/JNK signaling pathway, and overexpression of the SAPK/JNK binding domain blocked signaling by JNK/SAPK, presumably by the cytoplasmic retention of the kinase (26). These results are in support of our in vitro data where SAPKβ-MKK7 phosphorylated recombinant GST-c-Jun (amino acids 1–135) in an immune complex kinase assay (21).

**Subcellular Localization of SAPKβ-MKK7**—Upon stimulation, SAPK/JNK kinases translocate from the cytoplasm to the nucleus where they phosphorylate a variety of transcription factors including members of the Jun family ATF2 (activating transcription factor 2) and Elk1 but also p53, c-Myc, and NFAT4 (nuclear factor of activated T cells 4) (27–31). In stimulated cells, a small fraction of activated SAPK/JNK also remains in the cytoplasm to potentially interact with or phosphorylate proteins that are present in this compartment, for instance p38 (24) or MAP-2 (microtubules-associated protein 2) (32). To analyze the distribution of SAPKβ-MKK7, NIH 3T3 cells transiently transfected with SAPKβ-MKK7 or the kinase dead mutant SAPKβ-MKK7 KD were stained with an antibody directed against the Myc tag epitope (9E10) present in the fusion protein. SAPKβ-MKK7 was found predominantly in the nucleus (Fig. 4). Also, in cells expressing lower amounts of the protein the majority of SAPKβ-MKK7 was always detected in the nucleus, whereas its inactive mutant form (Fig. 4) was in all cases totally excluded from the nucleus. These findings are in good agreement with the data of Zheng et al. (25).

**Tetracycline-regulated SAPKβ-MKK7 Expression Results in c-Jun Phosphorylation in Vitro and in Vivo**—To further elucidate the physiological function of SAPK/JNK kinase in mammalian cells, we generated an inducible NIH 3T3 tet-off cell line expressing SAPKβ-MKK7. For this purpose SAPKβ-MKK7 or its inactive form, SAPKβ-MKK7 KD, were cloned into the tet-off-inducible expression vector pEV-TRE and transfected into the previously established NIH 3T3 fibroblast cell line NIH 5.15 (22). Following selection, we identified several clones in which the expression of SAPKβ-MKK7 or SAPKβ-MKK7 KD was induced upon the removal of tetracycline. In comparison to transiently transfected cells, all inducible NIH3T3 tet-off clones expressed SAPKβ-MKK7 at lower amounts. Over time the extent of SAPKβ-MKK7 expression gradually declined (data not shown), suggesting that cells cannot tolerate higher amounts of constitutively active SAPKβ-MKK7 for longer time periods.

To test whether SAPKβ-MKK7 is able to phosphorylate c-Jun in vitro, we performed an immune complex kinase assay using GST-c-Jun (amino acids 1–135) as the substrate. Three independent NIH 3T3 tet-off clones were grown in the absence of tetracycline to induce the expression of SAPKβ-MKK7. In parallel, cells from the same clones were maintained in the presence of tetracycline to shut down protein synthesis. SAPKβ-MKK7 was immunoprecipitated using the anti-Myc antibody (9E10) and assayed for kinase activity (Fig. 5). Immunoprecipitates from all three clones were able to phospho-
FIG. 5. Activated SAPKβ-MKK7 expressed by inducible NIH 3T3 tet-off cell lines phosphorylates c-Jun in vitro. An autoradiogram of phosphorylated GST-c-Jun is shown. Three independently isolated clones expressing SAPKβ-MKK7 were grown for 48 h in the presence (+) or absence (−) of tetracycline (2 μg/ml) in full medium. Afterward, cells were lysed and proteins were immunoprecipitated (IP) with the anti-Myc antibody 9E10. The kinase activity was measured using an in vitro immune complex kinase assay with GST-c-Jun (amino acids 1–135) as the substrate. The expression of immunoprecipitated proteins was controlled by immunoblotting (IB) with the 9E10 antibody.

FIG. 6. Inducible expression of SAPKβ-MKK7 causes electrophoretic mobility shift of c-Jun in vivo. Cells from different clones were grown for 24 h in 10% serum with (+) or without (−) tetracycline prior to transfection with the c-Jun expression plasmid pMT35 (1.5 μg). 24 h before the preparation of lysates, cells were grown in medium with 0.3% serum. The activation status of c-Jun was analyzed with the anti-c-Jun antibody. The lower panel shows the expression of SAPKβ-MKK7 in the inducible NIH3T3 tet-off clones using an anti-Myc antibody (9E10).

Fig. 5 shows the expression of SAPKβ-MKK7 KD, which resulted in a dramatic reduction to a complete loss of actin stress fibers, whereas the inactive form, SAPKβ-MKK7, had no such effect (Fig. 8C). Approximately 12 days later, the formation of small cell clusters became clearly visible (Fig. 7C, −tet). On average, ~19.9 ± 4.6% of the cells seeded yielded colonies in the absence of tetracycline in comparison to 5.4 ± 0.6% when grown in the presence of tetracycline.

Additionally, we transiently transfected NIH 3T3 fibroblasts with an expression vector encoding SAPKβ-MKK7 or the empty vector (Fig. 7D). After 1 week selections with G418, cells were seeded in soft agar. Under these experimental conditions SAPKβ-MKK7-expressing cells also showed growth in soft agar (SAPKβ-MKK7 KD 13.3% ± 2.6) in contrast with the vector control (pcDNA3 6.1% ± 2.8) or the inactive fusion protein (SAPKβ-MKK7 KD 3.0% ± 1.3) (Fig. 7D).

Constitutively Active SAPKβ-MKK7 Causes Disassembly of the F-actin Cytoskeleton in NIH 3T3 Fibroblasts—Morphological changes seen in transformed cells are linked to changes in the cytoskeleton (33). Small GTPases of the Rho family are critical in this process. Interestingly, Rac and Cdc42, which regulate the formation of filopodia and lamellipodia and are also important for cell migration, have been identified as upstream activators of SAPK/JNK kinases (34). Therefore, we performed analysis to see whether active SAPKβ-MKK7 could lead to alterations in the cytoskeleton. Cells of the inducible NIH 3T3 tet-off cell line (clone 3) were grown for 48 h in the presence or absence of tetracycline. Afterward, the F-actin of the cytoskeleton was stained with phalloidin (Fig. 8A). Cells depicted in Fig. 8 expressed SAPKβ-MKK7 at relatively high amounts. In the presence of tetracycline, which blocks SAPKβ-MKK7 expression, cells showed an intact F-actin cytoskeleton (Fig. 8A, −tet). In contrast to this, the expression of SAPKβ-MKK7 caused a partial or in some cases even a complete loss of stress fibers (Fig. 8A, −tet). To control for SAPKβ-MKK7, expression cells were stained in parallel with the anti-Myc antibody (9E10) (Fig. 8A, ±tet). Similar effects of activated JNK were also seen in cells after selection for growth in soft agar (data not shown). Additionally, the transient expression of SAPKβ-MKK7 resulted in a dramatic reduction to a complete loss of actin stress fibers, whereas the inactive form, SAPKβ-MKK7 KD, had no such effect (Fig. 8B).

To further confirm the transforming potential of constitutively active SAPKβ-MKK7, we analyzed SAPKβ-MKK7 expressing cells for their potential to grow in soft agar. In a first attempt the inducible NIH 3T3 tet-off cell line expressing SAPKβ-MKK7 was used. 1000 cells (clone 3) were plated in soft agar in the presence or absence of tetracycline (Fig. 7C, ±tet). Approximately 12 days later, the formation of small cell clusters became clearly visible (Fig. 7C, −tet). On average, ~19.9 ± 4.6% of the cells seeded yielded colonies in the absence of tetracycline in comparison to 5.4 ± 0.6% when grown in the presence of tetracycline.

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Fig. 7. SAPKβ-MKK7 demonstrate properties of growth transformation. A, constitutively active SAPKβ-MKK7 induces morphological transformation of NIH 3T3 tet-off cells. Cells from clone 3 were cultivated for 4 days in 10% serum in the presence (+) or absence (−) of tetracycline. B, SAPKβ-MKK7 induces foci formation in the NIH 3T3 tet-off cell line. Parallel dishes from the experiment described in A were grown for 24 days in the presence (+) or absence (−) of tetracycline (2 μg/ml) in 10% serum. Plates were fixed with methanol and stained with Giemsa. For the focus formation assay, the same number of cells was plated in triplicate onto 90-mm dishes. The number of foci on each dish was counted. 115.0 ± 21.6 foci were formed by SAPKβ-MKK7-expressing cells (−tet) in comparison to 77.5 ± 13.2 foci in the presence of tetracycline (+tet). NIH 3T3 cells stably expressing oncogenic v-Raf were used as a positive control (shown only as a section). C, SAPKβ-MKK7 expression induces anchorage-independent growth. 1000 cells from the inducible NIH 3T3 tet-off cell line expressing SAPKβ-MKK7 (clone 3) were seeded into 0.3% soft agar over a 0.6% agar bottom layer. Colonies were photographed on day 18. In the absence of tetracycline, cells expressed SAPKβ-MKK7 and formed little colonies (−tet 19.9% ± 4.6% vs tet 5.4% ± 0.6%, n = 3). D, SAPKβ-MKK7 transfected into NIH 3T3 cells results in anchorage-independent growth. 2 × 10^5 cells were transiently transfected with 1.5 μg of the following plasmids: pcDNA3neo encoding SAPKβ-MKK7 (13.3% ± 2.6%), SAPKβ-MKK7 KD (3.0% ± 1.3%), or the empty expression vector (6.1% ± 2.8%). One week after selection with G418 (450 μg/ml), cells were pooled and 5000 cells per dish were seeded in soft agar (n = 3). Stably transfected NIH 3T3 cells expressing oncogenic v-Raf were used as a positive control. All pictures were taken after 18 days in soft agar

shown). The majority of cells expressing MKK7 SE3 had their F-actin cytoskeletons preserved, and only a few high expressing cells were seen that had a completely remodeled cytoskeleton. In contrast, when MKK7 (SE3) was coexpressed with SAPKβ the disassembly of the F-actin stress fibers was pronounced (data not shown). Identical results were obtained when cotransfecting NIH 3T3 cells with the upstream activators of SAPK/JNKs, MLK3 or Cot (35–37), together with SAPKβ (data not shown). Following expression of a kinase dead version of MKK7 SA3, all transfected cells demonstrated an intact cytoskeleton (Fig. 8B).

Analysis of the subcellular location of SAPKβ-MKK7 had shown that the kinase was predominantly located in the nucleus with only a fraction of the protein remaining in the cytosol. We thus tried to determine whether the cytosolic fraction of SAPKβ-MKK7 was responsible for the observed effects on the cytoskeleton. As seen in the bottom panel of Fig. 8B, the coexpression of SAPKβ-MKK7 together with the scaffold protein JIP-1 prevented the translocation of the constitutive active fusion protein into the nucleus. Inspection of these cells, which excluded SAPKβ-MKK7 from the nucleus, revealed no detectable remodeling of the cytoskeleton. This finding could suggest that perhaps the nuclear fraction of SAPKβ-MKK7 is required for the observed effect. However, we cannot exclude the possibility that JIP-1 sequesters SAPKβ-MKK7 away from its cytoskeletal target(s) and therefore prevents disassembly of the actin stress fibers. Apart from modulating cellular localization, the effects of JIP-1 may also be the direct result of its inhibition of SAPKβ-MKK7 activity (62).²

**DISCUSSION**

In this report we fully characterized a constitutively active version of SAPKβ and demonstrated that the expression of this mutant caused many aspects of morphological transformation in NIH 3T3 cells usually associated with oncogenes. The fusion of SAPKβ to its specific upstream activator, MKK7, resulted in a constitutively active JNK/SAPK (21) (Figs. 1 and 2). SAPKβ-MKK7 phosphorylated the c-Jun protein in vitro and also induced an electrophoretic mobility shift of c-Jun when expressed in NIH 3T3 cells, which is indicative of an in vivo phosphorylation (Figs. 3 and 5). The inducible expression of SAPKβ-MKK7 in the NIH 3T3 fibroblasts caused morphological transformation and allowed cells to grow in soft agar (Fig. 7). Additionally, the presence of activated JNK resulted in the disassembly of actin stress fibers in NIH 3T3 cells (Fig. 8). Thus our experiments revealed a transforming potential for constitutively active SAPKβ-MKK7 that is, however, much weaker than that of dominant oncogenes such as ras or raf.

Oncogenic transformation is characterized by enhanced growth rates but also by morphological changes resulting from alterations in the actin cytoskeleton and adhesive interactions (38). Small GTPases of the Ras superfamily play key roles in these processes and also have been shown to activate SAPK/JNK; members of the Ras subfamily regulate cell proliferation and differentiation, whereas members of the Rho subfamily (RhoA, RhoB, RhoC, Rac1, Rac2, and Cdc42) were first identified as regulators of the actin cytoskeleton but also affect gene expression and proliferation (39). RhoA controls the assembly of actin stress fibers and focal adhesion complexes (40, 41), and Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles, whereas Cdc42 stimulates the formation of filopodia (34, 42, 43). It has been reported that the transient expression of activated Ras, Rac, and RhoA in cells results in the formation of stress fibers (44–46), whereas chronic stimulation, as seen in Ras-transformed cells, leads to the inhibition of stress fiber formation (47). The potential mechanism responsible for the absence of stress fibers in cells transformed by Ras and Raf has been dissected recently (63). Sustained ERK activation down-regulates p160 Rho-associated coiled-coil-containing protein kinase (ROCK) and Lin11, Isl-1, and Mec-3 domain (LIM) kinase, two Rho effectors required for actin stress fiber formation (39). In

² U. E. E. Rennefahrt, J. Troppmair, and U. R. Rapp, unpublished data.
addition, oncogenic Raf blocks upstream activators of Rac and thus impairs Rac-mediated adhesion of cells to the extracellular matrix (63). Interference with Rac activation may involve the transcriptional down-regulation of Tiam1, an activator of Rac (48).

MAP kinases function as effectors of the Ras and the Rho subfamily of small GTPases. An essential role for the classical mitogenic cascade Ras → Raf → MEK → ERK in oncogenesis has been established. MEK/ERK signaling is required for transformation by most oncogenes (36, 49–51), and the expression of constitutively active mutants of ERK or MEK is sufficient for the establishment of the transformed phenotype (52).

Recently published data suggest a functional role for SAPK/JNK signaling in oncogenic transformation based on studies with dominant negative mutants that block transformation by oncogenes like ras (53), met (13), and ber-abl (14, 15). Additional evidence for a functional role of SAPK/JNK in this process has been obtained by the use of a dominant negative mutant of c-Jun that efficiently blocked transformation by various classes of oncogenes (14, 19, 54, 55). A role for SAPK/JNK in cellular transformation is further substantiated by the demonstration of the albeit limited oncogenic potential of kinases, whose expression results in the activation of SAPK/JNKs such as MLK3. The overexpression of MLK3 in NIH 3T3 fibroblasts results in a low level activation of MEK/ERK, which was required to achieve the fully transformed phenotype because the treatment of

FIG. 8. Expression of constitutively active SAPKβ-MKK7 results in the disassembly of F-actin. A, cells of the NIH 3T3 tet-off line expressing SAPKβ-MKK7 (clone 3) were grown for 48 h with (+) or without (−) tetracycline (2 µg/ml). F-actin was visualized with fluorescein isothiocyanate (FITC)-conjugated phalloidin. The same cells were also stained with the anti-Myc antibody (9E10) and a Cy3-labeled secondary anti-mouse antibody to detect the expression of SAPKβ-MKK7.

B, NIH 3T3 was transiently transfected with the following constructs: Myc-SAPKβ-MKK7, Myc-SAPKβ-MKK7 KD, Myc-MKK7 S3A, and FLAG-JIP-1. For single plasmid transfections, 1.5 µg of DNA was used; for coexpression experiments, 0.75 µg of each DNA was transfected. Fluorescein isothiocyanate-conjugated phalloidin detects the formation of F-actin stress fibers. Detection of the expressed proteins was achieved by staining with the anti-Myc 9E10 antibody in combination with a Cy3-conjugated secondary anti-mouse antibody. The presence of the scaffold protein of JIP-1 in the transfected pool of NIH 3T3 cells was controlled with a Western blot using the anti-FLAG antibody.
MLK3-transformed NIH 3T3 cells with synthetic MEK inhibitors resulted in a partial reversion of the transformed phenotype (36). In the meantime, we have also begun to directly test the tumorigenic potential of SAPKβ-MKK7 in the nude mouse model. In agreement with the in vitro data presented in this paper, the injection of SAPKβ-MKK7-expressing fibroblasts resulted in the establishment of a well defined fibrosarcoma, although with a longer latency than in the case of v-raf transformed cells (17 days for v-raf versus 36 days for SAPKβ-MKK7).2 Tumor cells readily could be taken into culture, and, using a PCR approach, immunoblot analysis, and direct staining of cells with the Myc antibody 9E10, we could confirm the expression of SAPKβ-MKK7. Thus, the expression of activated JNK is sufficient to initiate tumor development in vivo. However, an analysis of tumors induced by the ERK-and JNK-activating kinase Cot demonstrated the lack of activation of SAPK/JNK.3 Thus, although the expression of activated SAPK/JNK induces certain aspects of morphological transformation in vitro, SAPK/JNK signaling may be redundant or deleterious in later stages of tumor growth in vivo.

A direct link between actin organization and SAPK/JNK has not been definitively established. The Drosophila JNK (DJNK) homologue Basket plays a role in the regulation of cell shape changes and actin reorganization in the process of dorsal closure during embryogenesis (56, 57). Recently, p150-Spir was found in a yeast two-hybrid screen as a new substrate of DJNK (21). It belongs to the Wiscott-Aldrich syndrome protein (WASP) homology domain (WH2) family of proteins involved in actin reorganization (58, 59). N-terminal sequences of Spir can interact with Rho family GTPases (60). p150-Spir, which co-localizes with F-actin in NIH 3T3 cells, acts as an initiator of the actin polymerization (21). Currently, it is not known whether SAPK/JNK could function as a positive or negative regulator of p150-Spir. Additionally, DJNK is known to directly phosphorylate and modify cytoskeletal components involved in dorsal closure such as Zipper (nonmuscle myosin), Coracle (Drosophila homologue of the vertebrate band 4.1 cytoskeletal protein), Inflated, or Myosinoid (integrins involved in cell adhesion). Alternatively, DJNK (or mammalian JNK/SAPK) could also modify the activity of transcription factors that are necessary for the process of dorsal closure (61).

The microinjection of activated Rac and Cdc42 into cells causes changes of the actin structures within minutes before de novo protein synthesis starts (2, 3). Contrary to this, our data obtained after artificial retention of activated SAPKβ in the cytoplasm by the overexpression of JIP-1 (Fig. 8) suggest that perhaps nuclear translocation, resulting most likely in the regulation of gene transcription, is required for the observed effects on the cytoskeleton. The differences may be explained in part by the fact that multiple effectors have been described for Rac/Cdc42 that may independently affect the process of cellular transformation, whereas in our studies we analyzed an isolated pathway. However, it is also possible that JIP-1 merely sequesters SAPKβ-MKK7 away from its cytoskeletal target(s). Furthermore, an inhibitory effect of JIP-1 on SAPK/JNK activity can not be excluded (62). Preliminary experiments using the inhibitors actinomycin D (0.1 μg/ml for 16 h) and cycloheximide (1 μg/ml for 16 h) support the notion that at least de novo protein synthesis is not required for SAPKβ-MKK7-induced disassembly of actin stress fibers (data not shown).

In summary, the data presented here provide direct evidence that constitutively active SAPKβ-MKK7 leads to a morphologically transformed phenotype when expressed in NIH 3T3 fibroblasts. Its transforming potential is weak in comparison to classical oncogenes like ras andraf. This may be due to the fact that SAPKβ-MKK7 expression only recapitulates some aspects of transformation such as the disassembly of stress fibers but is unable to induce the whole set of events required for establishing a transformed phenotype, e.g., enforced cell cycle progression. Despite these limited in vitro effects, our preliminary in vivo data suggest that the expression of activated JNK is able to trigger tumor formation in vivo.

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