c-Jun N-terminal protein kinase (JNK), a member of the mitogen-activated protein (MAP) kinase family, regulates gene expression in response to various extracellular stimuli. JNK is activated by JNK-activating kinase (JNKK1 and JNKK2), a subfamily of the dual specificity MAP kinase kinase (MEK) family, through phosphorylation on threonine (Thr) 183 and tyrosine (Tyr) 185 residues. The physiological functions of the JNK pathway, however, are not completely understood. A major obstacle is the lack of specific and activated kinase components that can stimulate the JNK pathway in the absence of any stimulus. Here we show that fusion of JNK1 to its upstream activator JNKK2 resulted in its constitutive activation. In HeLa cells, the JNKK2-JNK1 fusion protein showed significant JNK activity, which was comparable with that of JNK1 activated by many stimuli and activators, including EGF, TNF-α, anisomycin, UV irradiation, MEKK1, and small GTP binding proteins Rac1 and Cdc42Hs. Immunoblotting analysis indicated that JNK1 was phosphorylated by JNKK2 in the fusion protein on both Thr183 and Tyr185 residues. Like JNKK2, the JNKK2-JNK1 fusion protein was highly specific for the JNK pathway and did not activate either p38 or ERK2. Transient transfection assays demonstrated that the JNKK2-JNK1 fusion protein was sufficient to stimulate c-Jun transcriptional activity in the absence of any stimulus. Immunofluorescence analysis revealed that the JNKK2-JNK1 fusion protein was predominantly located in the nucleus of transfected HeLa cells. These results indicate that the JNKK2-JNK1 fusion protein is a constitutively active Jun kinase, which will facilitate the investigation of the physiological roles of the JNK pathway.

JNK1 (also known as stress-activated protein kinase, SAPK), a subfamily of the MAP kinase family, regulates gene expression in response to various extracellular stimuli (1). Activation of JNK requires its phosphorylation on both Thr183 and Tyr185 residues (2–4). The MAP kinase kinases that phosphorylate and activate JNK are JNK-activating kinases (JNKK1 and JNKK2, also known as SEK1/MKK4/SAPKK and MKK7, respectively) (5–14), which are members of the dual specificity MAP kinase kinase (MEK) family. The MAP kinase kinase kinases that activate JNKK include MEKKs (6, 15–20), MLK (21), TAK1 (22), and ASK1 (23). This MEKK/JNKK/JNK module appears to be critical in mediating the effects of extracellular stimuli on the activities of several transcription factors, such as c-Jun, ATF-2, and Elk, which are phosphorylated by JNK and control expression of many genes involved in cell growth, differentiation, programmed cell death, and transformation (24).

The JNK pathway can be activated by a variety of extracellular stimuli such as growth factors, cytokines, tumor promoters, protein synthesis inhibitors, ultraviolet (UV) irradiation, and oncoproteins (1, 24). Genetic ablation of JNK1 (25), JNK2 (26, 27), JNK3 (28), and SEK1/MKK4/JNKK1 (29, 30) indicates that the JNK pathway is likely required for embryonic development, immune response, and cell survival and death. However, its function in many physiological processes is still poorly understood.

Specific chemical inhibitors and constitutively active kinase mutants have successfully been used in exploring the physiological functions of the ERK and p38 MAP kinase pathways (31–38). Such inhibitors and constitutively active mutants, however, have yet to be reported for the JNK pathway. A great deal of effort has been made to generate constitutively active kinase mutants for the JNK pathway but with no success. At the level of MAP kinase kinase kinase, a truncated form of MEKK1, MEKKΔ, has very high activity and can preferentially activate the JNK pathway when expressed at a low level (16). However, it still stimulates the ERK and p38 pathways in transfected cells, even though to a lesser extent (39). In addition, MEKKΔ also activates the IkB kinase complex, which plays a critical role in NF-κB activation (40–43). It has been reported that MEKK4 is a more specific activator for the JNK pathway (20), but its human homologue MTK1 also activates p38 (18). It is known that activation of MAP kinase kinases depends on phosphorylation of two conserved Ser/Thr residues between the subkinase domains VII and VIII in the activation loop (44). Replacement of these Ser/Thr residues with acidic amino acids, such as glutamic acid (Glu) or aspartic acid (Asp), has resulted in several constitutively active MAP kinase kinase mutants, including MEK1(ED) (31, 32) and MKK6b(EE) (34). Because these Ser/Thr residues are also conserved in JNKK1 and JNKK2, it was thought that the same strategy might be used to yield a constitutively active JNKK. Surprisingly, the corresponding JNKK mutants, JNKK1(2E) and JNKK2(3E), are catalytically inactive.5 This suggests that the activation of
JNK may be more complicated than previously thought.

To generate constitutively active kinase components for the JNK pathway, we used the approach of enzyme-substrate fusion. The rate of an enzymatic reaction is greatly influenced by the proximity between the enzyme and its substrate. We reasoned that JNK might become constitutively activated if it is physically linked to its upstream activator JNKK. Unlike JNKK1 that activates both JNK and p38 (5, 6), JNKK2 only stimulates JNK activity (8–14, 49). In addition, JNKK2 has considerable basal enzymatic activity when overexpressed in cells (6). Thus, we fused JNK1 to JNKK2 via a short peptide linker and created the JNKK2-JNK1 fusion protein. The JNKK2-JNK1 fusion protein showed profound JNK activity and was able to stimulate c-Jun transcriptional activity in the absence of any stimulus. The constitutively active JNKK2-JNK1 fusion protein will provide a powerful tool for investigating the physiological functions of the JNK pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa and human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml of streptomycin.

cDNA Constructs—To construct the JNKK2-JNK1 fusion construct, pSRa3HA-JNK2 was first digested with NotI and BgII to release the C-terminal part of JNK2 (796–1206). A PCR-generated NotI-NotI fragment encoding the C-terminal part of JNK2 with a (Gly-Glu)₅ linker, linker at its 3’-end and an NotI-BgII fragment encoding JNK1 were inserted into the NotI-BgII-digested pSRa3HA-JNK2. A Chameleon™ mutagenesis kit ( Stratagene) was used to replace lysine (Lys) 149 with methionine (Met) to create the hemagglutinin (HA)-JNKK2(K149M)-JNK1 fusion protein. To construct pSRa3HA-JNKK2-JNK1 (APY) construct, the JNK1 part in the JNKK2-JNK1 fusion construct was replaced by an NotI-BgII fragment encoding JNK1 (APY) (9). The constructs were confirmed by DNA dyeoxonucleotide sequencing. Expression vectors of HA-tagged JNKK2, Flag (M2)-tagged JNK1, MEKKA, Rac1 V12, Cdc42Hs V12, HA-MEK1(3NED), HA-ERK2, HA-JNK6b(E), M2-p38, GAL4-e-Jun, and GAL4-e-Jun (AA6373), have been described previously (6, 9, 37, 38). The reporter gene 5× GAL4-Luc, in which the GAL4 DNA-binding domain was fused to the luciferase gene, has also been described previously (6).

Purification of Recombinant Proteins—GST-c-Jun-(1–79) and GST-AFP2, GST-JNK1, and GST-p38 were purified on glutathione-agarose, as described (6, 9). Histidine-tagged ERK2 was purified on a nickel-chelate column, according to the manufacturer’s procedure (Amersham Pharmacia Biotech Inc.).

Transfections and Immunoprecipitation—HeLa or 293 cells were transiently transfected with various expression vectors using Lipofectamine Plus (Life Technologies, Inc., NY), according to the manufacturer’s procedure. After 40 h, the cells were treated with different stimuli or left untreated, as indicated in the figure legends. The cells were harvested and the lysates were prepared, as described previously (6). HA-tagged or M2-tagged protein kinases were immunoprecipitated with specific antibodies for 3 h at 4 °C.

Protein Kinase Assays and Immunoblotting—The activity of the immunocomplex was assayed at 30 °C for 30 min in 30 μl of kinase buffer (6) in the presence of 10 μM ATP/10 μCi [γ-³²P]ATP (10 Ci/mmol) with appropriate substrates, as indicated in the figure legends. The reactions were terminated with 4× Laemmli sample buffers. The proteins were resolved by 13% SDS-polyacrylamide gel electrophoresis, followed by autoradiography. The phosphorylated proteins were quantitated by a PhosphoImager (Molecular Dynamics Inc.). For immunoblotting analysis, proteins were resolved by SDS-polyacrylamide gel electrophoresis on 13% gels, blotted onto Immobilon P membranes (Millipore), and subjected to immunoblotting analysis using anti-HA monoclonal antibody (Santa Cruz Biotechnology), anti-M2 monoclonal antibody (Sigma), or anti-phospho-JNK (on both Thr¹⁸³ and Tyr¹⁸⁵) polyclonal antibody (New England Biolabs Inc.), as indicated in the figure legends. The antibody-antigen complexes were visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) according to the manufacturer’s procedure.

Immunofluorescence Analysis—Immunofluorescence analysis was performed as described previously (45). Briefly, HeLa cells were plated onto coverslips and transfected with various expression vectors. After 36 h, the cells were fixed with ice-cold methanol for 7 min and washed with phosphate-buffered saline (PBS) three times. The coverslips were incubated with anti-HA antibody (1:50) in PBS at 37 °C for 30 min and then washed with PBS. Immune complexes were detected with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (1:400, Jackson ImmunoResearch, Inc.) in PBS at room temperature for 1 h. The nuclei were stained with H33258 (1:500) in PBS for 2 min. The coverslips were mounted in Vectashield (Vector Laboratories, Inc.). Fluorescence microscopy was performed with a Zeiss Axioplan microscope.

RESULTS

Generation of JNKK2-JNK1 Fusion Proteins—Because the approach of site-directed mutagenesis failed to produce a constitutively active JNKK,² we explored the possibility of generating a constitutively active JNK through enzyme-substrate fusion. JNK1 was fused in frame with its specific activator JNKK2 (Fig. 1A). A decapeptide linker (Gly-Glu)₅, was inserted between the coding sequences of JNKK2 and JNK1 to facilitate the folding (46). Cell-free translation of in vitro generated JNKK2-JNK1 transcripts produced a single polypeptide with an apparent molecular mass of 86 kDa, as expected (Fig. 1B). Using the same strategy, we also constructed JNKK2(KM)-JNK1, in which the lysine (Lys) 149 in the ATP binding domain of the JNKK2 moiety was replaced by methionine (Met), and JNKK2-JNK1 (APY), in which the Tyr¹⁸⁵ residue in the JNK1 moiety was replaced by a nonphosphorylatable alanine (Ala). The JNKK2-JNK1 Fusion Protein Has Significant Jun Kinase Activity—We tested whether the JNKK2-JNK1 fusion protein has Jun kinase activity and, if so, whether it is a more active Jun kinase than JNK1 stimulated by cotransfection with JNKK2.

HeLa cells were transiently transfected with expression vectors encoding HA-JNKK2-JNK1, HA-JNKK2(K149M)-JNK1, HA-JNKK2-JNK1 (APY), M2-JNK1 with or without HA-JNK2, or empty expression vector. After 40 h, the cells were harvested and the transfected kinases were isolated by immunoprecipitation. The kinase activity was measured by immunocomplex kinase assays with GST-e-Jun-(1–79) as a substrate (6). As reported previously (9), coexpression of JNKK2 activated JNK1 moderately (Fig. 2, lanes 2–4). In contrast, the JNKK2-JNK1 fusion protein itself was 30-fold more active than the nonstimulated JNK1 (Fig. 2, lanes 5–7). This activity was not a result of differences in the expression between HA-JNKK2-JNK1 and M2-JNK1/HA-JNKK2, as demonstrated by immunoblotting analysis (Fig. 2). Under the same conditions, the JNKK2(K149M)-JNK1 fusion protein had no detectable Jun kinase activity (Fig. 2, lane 8) nor did the JNKK2-JNK1 (APY) fusion protein (data not shown). These results indicate that the JNKK2-JNK1 fusion protein has profound Jun kinase activity, which likely results from activation of JNK1 by the JNKK2 fusion protein.

Fig. 1. Generation of the JNKK2-JNK1 fusion protein. A, a schematic illustration of the JNKK2-JNK1 fusion protein. The coding region of JNK1 was fused in frame to the 3’-end of the stop codon-less JNKK2 through a peptide linker (Gly-Glu)₅. B, ³⁵S-labeled in vitro translated JNKK2-JNK1 fusion protein.

Generation of JNKK2-JNK1 Fusion Proteins
Because the JNKK2-JNK1 functions as an activated Jun kinase, we examined whether JNK1 is phosphorylated by JNKK2 in the fusion protein. HeLa cells were transiently transfected with expression vectors encoding HA-JNKK2-JNK1, HA-JNKK2(KM)-JNK1, or empty vector. The cells were treated with anisomycin (which is a strong stimulus of JNK) for 15 min or left untreated. The cells were harvested and the extracts were fractionated by SDS-gel electrophoresis, followed by immunoblotting with the anti-JNK antibody (Pharmingen) or the specific anti-phospho-JNK antibody (New England Biolabs, Inc.), which only recognizes the dual-phosphorylated JNK (on both Thr183 and Tyr185).

As expected, the anti-phospho-JNK antibody only recognized anisomycin-stimulated, but not nonstimulated, endogenous JNK1 and JNKK2 (Fig. 3, left panel, lanes 1 and 2). The anti-phospho-JNK antibody also recognized the transfected HA-JNKK2-JNK1 fusion protein (Fig. 3, left panel, lane 3). Under the same conditions, it failed to detect the inactive HA-JNKK2(KM)-JNK1 mutant (Fig. 3, left panel, lane 4). This was not a result of differences in protein expression, as demonstrated by immunoblotting analysis with anti-JNK antibody (Fig. 3, right panel). Thus, activation of JNK1 in the fusion protein may result from its phosphorylation by JNKK2 on Thr183 and Tyr185 residues.

**The JNKK2-JNK1 Fusion Protein Functions As a Constitutively Active Jun Kinase—**To determine whether the JNKK2-JNK1 fusion protein is a constitutively active Jun kinase, we compared its activity with that of JNK1 stimulated by various activators and extracellular stimuli. HeLa cells were transiently transfected with expression vectors encoding HA-JNKK2-JNK1, HA-JNK1 with or without the active forms of MEKK1, Rac1, Cdc42, or empty expression vector. After 40 h, the cells were treated with EGF, TNF-α, anisomycin, UV, or left untreated, as indicated. The activity of HA-JNKK2-JNK1 was equivalent or comparable with that of JNKK2-JNK1 functions as an activated Jun kinase.

**The JNKK2-JNK1 Fusion Protein Is Highly Specific for the JNK Pathway—**JNKK2 is a highly specific JNK activator that does not activate p38 or ERK2 (8–14, 49). To ensure that the JNKK2-JNK1 fusion protein still maintained this high specificity, we determined its effect on the activity of JNK1, p38, and ERK2.

In HeLa cells, coexpression of HA-JNKK2-JNK1, but not
HA–JNKK2(149M)–JNK1, stimulated M2–JNK1 activity significantly (Fig. 5, lanes 3 and 4). Under the same conditions, the HA–JNKK2–JNK1 fusion protein failed to stimulate M2–p38 or HA–ERK2 activity (Fig. 5, lanes 6 and 9). However, M2–p38 and HA–ERK2 were activated by their specific upstream kinases, MKK6b(EE) and MEK1(ΔNED), respectively (Fig. 5, lane 7 and 10). These results demonstrate that like JNKK2, the JNKK2–JNK1 fusion protein is a highly specific activator for the JNK pathway.

Although the JNKK2–JNK1 fusion protein was able to stimulate cotransfected M2–JNK1 (Fig. 5, lane 3), it only slightly stimulated endogenous JNK1 (data not shown). It is possible that the endogenous JNK may not be able to compete with the linked JNK1 to serve as a JNKK2 substrate because its amount is relatively smaller in comparison to the linked JNK1.

The JNKK2–JNK1 Fusion Protein Stimulates c-Jun Transcriptional Activity in the Absence of Any Stimuli—Previously, we demonstrated that JNKK2 was able to potentiate the stimulatory effect of MEKK1 on c-Jun transcriptional activity, though itself was unable to enhance c-Jun activity (9). Because the JNKK2–JNK1 fusion protein appears to be a constitutively active Jun kinase, we tested whether by itself it is able to stimulate c-Jun transcriptional activity.

Human embryonic kidney 293 cells were transiently transfected with the GAL4–c-Jun fusion protein in the presence or absence of expression vectors encoding MEKK1, HA–JNKK2–JNK1, HA–JNKK2 plus HA–JNK1, or empty expression vector. As expected, expression of MEKK1 was able to stimulate GAL4–c-Jun activity, as measured by the luciferase reporter gene driven by a GAL4 response promoter (Fig. 6, lane 4). Expression of HA–JNKK2–JNK1 also significantly stimulated GAL4–c-Jun activity in a dose-dependent manner (Fig. 6, lanes 5–8). In fact, the maximal stimulation of GAL4–c-Jun activity by the JNKK2–JNK1 fusion protein was even stronger than the effect of MEKK1 under these conditions (Fig. 6, compare lane 8 with lane 4). The stimulation was specific, because expression of HA–JNKK2–JNK1 did not stimulate the activity of GAL4–c-Jun (AA63/73), in which both Ser63 and Ser73 have been replaced with alanine residues (Fig. 6, lanes 9–12). On the other hand, coexpression of JNKK2 and JNK1 together only slightly stimulated GAL4–c-Jun activity (Fig. 6, lanes 13–16).

Expression of the JNKK2–JNK1 fusion protein also stimulated c-Jun transcriptional activity in HeLa cells (data not shown). These results demonstrate that the JNKK2–JNK1 fusion protein is sufficient to stimulate c-Jun transcriptional activity in the absence of any stimuli.

Subcellular Localization of the JNKK2–JNK1 Fusion Protein—It is known that upon stimulation, JNK translocates from the cytoplasm to the nucleus (47), where it stimulates the transcription activity of several transcription factors such as c-Jun. On the other hand, JNKK2 has been reported to be distributed in both the cytoplasm and the nucleus (48). The ability of the JNKK2–JNK1 fusion protein by itself to stimulate c-Jun transcriptional activity prompted us to examine its subcellular localization.

HeLa cells were transiently transfected with expression vectors encoding HA–JNK1, HA–JNKK2, or HA–JNKK2–JNK1. After 40 h, the cells were treated with UV irradiation, or left untreated. The subcellular localization of transfected kinases was detected by immunofluorescence analysis. In nonstimulated control cells, HA–JNK1 was predominantly localized in the cytoplasm (Fig. 7). Upon UV irradiation, the majority of HA–JNK1 translocated to the nucleus (Fig. 7). On the other hand, HA–JNKK2 was localized in both the cytoplasm and the nucleus (Fig. 7). However, the JNKK2–JNK1 fusion protein was predominantly present in the nucleus (Fig. 7). This result suggests that the JNKK2–JNK1 fusion protein is localized in the nucleus, where it is able to stimulate c-Jun transcriptional activity.

DISCUSSION

In this report, we show that fusion of JNK1 to its upstream kinase JNKK2 generated a specific and constitutively active Jun kinase. This conclusion is based on several lines of evidence. First, the JNKK2–JNK1 fusion protein had significant Jun kinase activity, which was equivalent or comparable with that of JNK1 stimulated by various activators, such as active forms of MEKK1, Rac, and Cdc42Hs, and extracellular stimuli like EGF, TNF-α, anisomycin, and UV. Second, the JNK1 moiety in the JNKK2–JNK1 fusion protein was apparently phosphorylated on both Thr183 and Tyr185 residues, which are essential for JNK1 activation. Third, the JNKK2–JNK1 fusion protein only activated JNK, but not p38 or ERK2. Fourth, the JNKK2–JNK1 fusion protein was predominantly localized in the nucleus and was able to stimulate c-Jun transcriptional activity in the absence of any stimulus. To our knowledge, this is the first time that a specific and constitutive activator of the JNK pathway has been characterized.

Constitutively active mutants of MAP kinase kinases have played a critical role in elucidating the physiological functions of the MAP kinase pathways (31–38). Site-directed mutagene-
Generation of a Constitutively Active JNK

**Fig. 7. Subcellular localization of the JNKK2-JNK1 fusion protein.** HeLa cells were transfected with expression vectors encoding HA-JNK1, HA-JNKK2, or HA-JNKK2-JNK1 (1 µg each). After 40 h, the cells were treated with UV (80 J/m²) and incubated for 1 h, or left untreated, as indicated. The transfected kinases were detected by immunofluorescence analysis using anti-HA monoclonal antibody as a primary antibody and fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody as a second antibody. The cell nucleus was stained with H33258.

The formation of signaling complexes composed of multiple protein kinases of the same signaling pathway has proved to be an important mechanism employed by eukaryotic cells to ensure the specificity and efficiency of signal transduction. The association is usually carried out through specific scaffold/adaptor proteins, such as Ste5 in the yeast *Saccharomyces cerevisiae* (51–53), and MP1 (54) and JIP-1 (55) in mammalian cells. Theoretically, physical association of protein kinases in a sequential signaling pathway should enhance the efficiency of the enzymatic reaction, resulting in activation of the downstream kinase. Indeed, Cobb and co-workers (46) have recently demonstrated that fusion of ERK2 to its upstream kinase MEK1 (the ERK2-MEK1 fusion protein) results in a constitutively active ERK, even though ERK2 and MEK1 themselves have very low activity. The JNKK2-JNK1 fusion protein that we have reported here appears to be a constitutively active JNK (Figs. 2–4). It is likely that fusion of the upstream protein kinase with its downstream protein kinase will, in general, lead to constitutive activation of the downstream kinase.

In contrast to the ERK2-MEK1 fusion protein that only activates the linked ERK2 (46), the JNKK2-JNK1 fusion protein can also activate cotransfected M2-JNK1 (Fig. 5). However, it only slightly activated endogenous JNK1 (data not shown). One possibility is that the JNKK2-JNK1 fusion protein might have flexibility that allows its JNKK2 moiety to interact with and activate nonlinked JNK1. Although the majority of the JNKK2-JNK1 fusion protein is localized in the nucleus and the transfected JNK1 is in the cytoplasm (Fig. 7), it is conceivable that the JNKK2-JNK1 fusion protein may be present in the cytoplasm before it finally resides in the nucleus. In that case, coexpressed M2-JNK1 may be able to compete with the linked JNK1 to serve as a JNKK2 substrate. On the other hand, endogenous JNK1 may have the disadvantage of competing with the linked JNK1, because its amount is relatively smaller. This could explain why the activation of endogenous JNK1 by the JNKK2-JNK1 was much weaker. Another possibility is that the topology of the JNKK2-JNK1 fusion protein may enable it to interact with and activate nonlinked M2-JNK1. Note that the ERK2-MEK1 fusion protein is constructed as NH₂-ERK2-MEK1-COOH (46). This kind of topology may somehow prevent the MEK1 moiety from interacting with and activating nonlinked ERK. On the other hand, the JNKK2-JNK1 fusion protein is constructed as NH₂-JNK2-JNK1-COOH (Fig. 1A), which may allow the JNKK2 moiety to interact with and activate cotransfected JNK1.

Another difference between the ERK2-MEK1 fusion protein and the JNKK2-JNK1 fusion protein is their subcellular localization. It is known that a fraction of ERK2 translocates into the nucleus upon stimulation (56–58). However, MEK1, which has a nuclear export sequence (NES), remains in the cytoplasm (59, 60). The ERK2-MEK1 fusion protein was found to remain predominantly in the cytoplasm. The NES of MEK1 appeared to be a dominant factor in determining the subcellular localization of the ERK2-MEK1 fusion protein because the ERK2-MEK1-AL fusion protein, in which the leucines in the NES were replaced by alanines, was localized in the nucleus (46). Like ERK2, JNK1 is localized in the cytoplasm in resting cells and translocates into the nucleus upon stimulation (55). In contrast to MEK1, JNK2 has no recognizable NES and distributes in both the cytoplasm and the nucleus (48). Interestingly, we found that the JNKK2-JNK1 fusion protein was predominantly localized in the nucleus (Fig. 7). It is possible that the overall structure of the JNKK2-JNK1 fusion protein may cause it to be localized in the nucleus. We cannot exclude the possibility, however, that this nuclear localization may be the result of overexpression. But it is not likely because overexpressed JNKK2 apparently distributed in both the cytoplasm and the nucleus (Fig. 7). It appears that the nuclear localization of the constitutively active JNKK2-JNK1 fusion protein allows it to stimulate the activity of the nuclear transcription factor c-Jun (Fig. 6).

The exact mechanism(s) underlying the activation of JNK1 by JNKK2 in the fusion protein has yet to be determined. The activation of JNK1 is not a result of the fusion per se because both functional JNK1 and JNKK2 are required. Fusion of the catalytic inactive JNKK2(K149M) mutant to JNK1 resulted in...
an inactive fusion protein (Fig. 2). Conversely, the JNK2-JNK1 (APY) fusion protein, in which Thr183 residue of the fused JNK1 was replaced by nonphosphorylatable alanine (Ala), was also inactive (data not shown). Furthermore, the activity of the JNK2-JNK1 fusion protein is not a result of the cleavage of the fusion protein and subsequent release of the JNK2 moiety. The JNK2-JNK1 fusion protein expressed as a single polypeptide with an apparent molecular mass of 8 kDa, as demonstrated by in vitro transcription/translation experiments (Fig. 1B) and immunoblotting analysis (Figs. 2 and 3A). Thus, it is clear that the activity of the JNK2-JNK1 fusion protein results from phosphorylation and activation of JNK1 by JNK2 in the fusion protein. JNK1 is present at an effectively infinite concentration in the fusion protein. Therefore it may be constantly phosphorylated and activated by JNK2 even though JNK2 only has basal activity. Indeed, JNK1 was found to be constantly phosphorylated by JNK2 in the fusion protein on both Thr183 and Tyr185 residues (Fig. 3). This may lead to constitutive activation of the JNK2-JNK1 fusion protein in vivo. We believe that in vivo activation of JNK1 is a result of the balance between phosphorylation by its upstream kinase(s) like JNK2 and dephosphorylation by its phosphatase(s). Fusion of JNK1 to JNK2 may change the balance toward activation because JNKK2 is always ready to act on JNK1 in the fusion protein, whereas the JNK1 phosphatase(s) may not always be located nearby. Mechanisms aside, the JNK2-JNK1 fusion protein provides us, for the first time, an opportunity to activate the JNK pathway in the absence of any extracellular stimulus. Future studies should determine whether the JNK2-JNK1 fusion protein is able to stimulate the JNK pathway in vivo, and if so, what are the physiological functions of the JNK pathway.

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