

Constitutively Active Forms of c-Jun NH₂-terminal Kinase Are Expressed in Primary Glial Tumors¹

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ABSTRACT

The c-Jun NH₂-terminal kinases (JNKs) have a role both in promoting apoptosis and tumorigenesis. The JNKs are encoded by three separate genes (*JNK1*, 2, and 3), which are spliced alternatively to create 10 JNK isoforms that are either M_r 55,000 or 46,000 in size. However, the functional significance and distinct role for each splice variant remains unclear. We have noted previously that 86% of primary human glial tumors show activation of almost exclusively the M_r 55,000 isoforms of JNK. To further study which isoforms are involved, we constructed glutathione *S*-transferase fusion proteins for all 10 JNK isoforms and examined kinase activity with or without the activating upstream kinase. Surprisingly, five JNK isoforms demonstrate autophosphorylation activity, and in addition, all four JNK2 isoforms (either M_r 55,000 or 46,000) show a high basal level of substrate kinase activity in the absence of the upstream kinase, especially a M_r 55,000 JNK2 isoform. Examination revealed autophosphorylation activity at the T-P-Y motif, which is critical for JNK activation, because a mutant lacking the dual phosphorylation sites did not show autophosphorylation or basal kinase activity. Using green fluorescence protein-JNK expression vectors, transient transfection into U87MG cells demonstrates that although the JNK1 isoforms localize predominantly to the cytoplasm, the JNK2 isoforms localize to the nucleus and are phosphorylated, confirming the constitutive activation seen *in vitro*. We then examined which JNK isoforms are active in glial tumors by performing two-dimensional electrophoresis. This revealed that the M_r 55,000 isoforms of JNK2 are the principal active JNK isoforms present in tumors. Collectively, these results suggest that these constitutively active JNK isoforms play a significant role in glial tumors. Aside from epidermal growth factor receptor vIII, this is the only other kinase that has been shown to be basally active in glioma. The presence of constitutively active JNK isoforms may have implications for the design of inhibitors of the JNK pathway.

INTRODUCTION

JNK,⁴ a member of the MAP kinase family, regulates gene expression through the phosphorylation of various transcription factors. It has an important role in regulating various cellular functions, including proliferation, apoptosis, and development (1–3). There are three separate genes (*JNK1*, *JNK2*, and *JNK3*), and these genes are spliced alternatively in the center (α versus β) or COOH terminus (1 versus 2) to create 10 JNK isoforms (JNK1 α 1, JNK1 α 2, JNK1 β 1, JNK1 β 2, JNK2 α 1, JNK2 α 2, JNK2 β 1, JNK2 β 2, JNK3 α 1, and JNK3 α 2; Ref. 4). The alternate splicing at the COOH terminus leads to proteins that

are either M_r 46,000 (JNK1 α 1, JNK1 β 1, JNK2 α 1, JNK2 β 1, and JNK3 α 1) or 55,000 (JNK1 α 2, JNK1 β 2, JNK2 α 2, JNK2 β 2, and JNK3 α 2) in size. Like all MAP kinase pathways, the JNK proteins require phosphorylation via an upstream kinase cascade to become active. The major MAPKKs have been shown to be MKK4 and MKK7, although other kinases can also phosphorylate the JNKs (1).

The role of the JNK pathway in mediating cellular responses to extracellular stimuli and the proapoptotic function of activated JNK have been studied extensively (1–3). For several cell lineages, various cytokines, cellular stresses, or trophic withdrawal can induce JNK activation with subsequent apoptosis. Indeed, inhibition of the JNK kinase cascade has been proposed as a therapeutic treatment for neuronal apoptosis caused by Alzheimer's disease or trauma. There are also several lines of evidence that suggest the JNK pathway can play a significant role in cellular transformation and tumor cell growth. JNK activation is required for cellular transformation by certain oncogenes, and inhibition of the JNK pathway can reverse a transformed phenotype (5–7). It has also been shown that the JNK pathway is constitutively activated in various tumor cell lines (8–10). Thus, it has been proposed that JNK inhibition can also be used as an antitumor agent.

Several studies have demonstrated biological differences among the three *JNK* genes (11–16), *e.g.*, the *JNK2* gene may play a more significant role in cancer because antisense RNA against *JNK2*, but not *JNK1*, inhibited transformation of lung carcinoma and glioblastoma cells (12, 16). *JNK2* knockout mice develop chemically induced papillomas at a much lower rate than wild-type or *JNK1* knockout mice (13). Although some isoforms have been shown to have distinct substrate affinities, which suggests a selective role in different biological contexts (4), no functional significance or distinct role for a specific splice variant has been demonstrated.

We have found previously that 86% of primary glial tumors show activation of the M_r 55,000 but not the M_r 46,000 form of JNK (17), suggesting that these isoforms play a more critical role in tumors. In an attempt to understand these differences, we initiated studies on the properties of individual isoforms. Here, we report that several JNK isoforms have autophosphorylation activity. Interestingly, all four splice forms of JNK2 show autophosphorylation activity and also enhanced basal kinase activity in the absence of the upstream kinase. Using gene product-specific antibodies combined with two-dimensional electrophoresis, we show that the M_r 55,000 form of JNK2 is expressed predominantly in primary glial tumors as compared with normal brain tissue. Moreover, the predominant phosphorylated form of JNK in tumors is composed of the M_r 55,000 form of JNK2. Collectively, these results suggest that these JNK2 isoforms play a more significant role than other JNK isoforms in the progression of primary glial tumors. Aside from EGFRvIII (18), JNK2 is the only other kinase that has been shown to be basally active in these tumors and suggests that tumors can up-regulate the JNK pathway simply through the up-regulation of specific isoforms. Moreover, the presence of constitutively active JNK isoforms should be taken into consideration in the design of therapeutics aimed at disabling this pathway.

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⁴ The abbreviations used are: JNK, c-Jun NH₂-terminal kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; GFP, green fluorescence protein; RT-PCR, reverse transcription-PCR; TBS, Tris-buffered saline; ATF, activating transcription factor; IEF, isoelectric focusing; ERK, extracellular signal-regulated kinase; MEKK, MKK, MAPKK, mitogen-activated protein kinase kinase.

MATERIALS AND METHODS

Culture Cells and Chemicals. U87MG, a human glioblastoma cell line, was obtained from the American Type Culture Collection and maintained as recommended. Anti-JNK1 antibody (C-17), anti-JNK2 antibody (D-2), and anti-GST antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-JNK3 antibody and recombinant GST-ATF2 protein were purchased from Upstate Biotechnology (Lake Placid, NY). Antiphospho-stress-activated protein kinase/JNK antibody, which recognizes the active form of JNK, was purchased from Cell Signaling Technology (Beverly, MA). Additional materials and chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) unless indicated.

Plasmids and Recombinant Protein Purifications. All JNK α isoforms cDNA were amplified by RT-PCR and cloned into pET42 (Novagen, Madison, WI). The JNK1 α 1, JNK1 α 2, JNK2 α 1, and JNK2 α 2 isoforms were cloned as a fusion protein with GFP into pEGFP-C1 (Clontech, Palo Alto, CA). Mutations of the T-P-Y motif (T183A and Y185F of JNK2 α 2) and cloning of the JNK β isoform were performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Sequencing confirmed that each construct matched that found in the GenBank Database. For the JNK2 isoform, the sequence from RT-PCR products was different from that of accession no. L31951 at the 152th nucleotide (G *versus* A resulting in Ser to Asn) but completely matched that of accession no. U09759. Moreover, we screened genomic DNA from 50 normal subjects, and all sequences matched with that of ours.

GST fusion proteins were purified as described previously (19). GST fusion proteins were expressed in *Escherichia coli* and purified using immobilized glutathione beads (Pierce, Rockford, IL) using manufacturer's protocols. The upstream MAPKKs, MKK4, and MKK7 and a synthetic constitutively active MAPKK kinase MEKK1 (Δ MEKK1) were amplified by RT-PCR and cloned into Pet 9a (Novagen). The expressed proteins were purified using the TALON purification kit (Clontech). The quality and quantity of purified recombinant proteins were confirmed by SDS-PAGE and Coomassie staining.

In Vitro Kinase Assay. For the autophosphorylation assay, 1 μ g of GST-JNK isoform protein was incubated in 40 μ l of kinase reaction buffer [25 mM HEPES (pH 7.4), 25 mM MgCl₂, 2 mM DTT, 0.1 mM NaVO₄, and 25 mM β -glycerophosphate] containing 10 μ Ci of [γ -³²P]ATP and 20 μ M cold ATP at 30°C for 30 min. For the substrate kinase assay, 300 ng of GST-ATF2 or GST-c-jun were added in the kinase buffer mixture as the substrate. Reactions were terminated with Laemmli sample buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and the phosphorylated proteins were detected by autoradiography.

Western Blotting. Human glial tumor tissues and corresponding normal brain tissues were obtained under Institutional Review Board-approved protocols, and the diagnoses were confirmed by histological analysis as described previously (20). Tissue specimens were lysed with PBS/TDS buffer as de-

scribed (17) and centrifuged at 14,000 \times g to remove insoluble material. Equal amounts of protein were electrophoresed on 4–20% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBS or PBS and then incubated with primary antibody overnight at 4°C. The membranes were washed three times with PBS containing 0.3% Tween 20 or T-TBS and then incubated for 1 h with matched secondary antibodies. The membranes were detected using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). The scanned films were quantified by NIH Image software.

Immunofluorescence Microscopic Analysis. Immunofluorescence microscopic analysis was performed as described previously (19, 21). U87MG cells grown on coverslips were transfected with pEGFP-JNK isoform expression vectors using the Effectene reagent (Qiagen, Valencia, CA). After overnight incubation, the growing media were changed to serum-free DMEM and incubated for an additional 24 h. The cells were then fixed in 4% paraformaldehyde for 10 min, followed by 0.2% Triton X-100 for 5 min. For visualization of the nuclei, 4',6-diamidino-2-phenylindole staining was used. After washing with PBS, the coverslips were mounted with antifade reagent and visualized with an immunofluorescence microscope.

Two-dimensional Electrophoresis. Tissue lysates were prepared with the PlusOne two-dimensional cleanup kit (Amersham Biosciences) to remove materials that interfere with IEF. The prepared proteins were resolubilized with 8 M urea and 1% Triton-X and then mixed with 2 \times IEF sample buffer (Invitrogen, Carlsbad, CA). Protein samples (20 μ g) were electrophoresed on an IEF gel (pH 3–7) for 2.5 h according to the manufacturer's instructions, and the gel was then incubated with fixing solution (0.14 M sulfosalicylic acid and 0.7 M trichloroacetic acid). After washing with 20% ethanol, the IEF gel was trimmed into strips, equilibrated with 2 \times Laemmli sample buffer, and placed in a 4–20% SDS-PAGE gel. Western blotting was performed according to standard procedure as described above.

RESULTS

Several JNK Isoforms Have Autophosphorylation Activity Resulting in Constitutive Activation. We have noted previously that glial tumors frequently show activation of the M_r 55,000 isoform of JNK but not the M_r 46,000 isoform (17). To understand the differences between each JNK isoform, we constructed GST fusion proteins for each JNK isoform and examined their activity using *in vitro* kinase assays. All JNK isoforms are capable of phosphorylating GST-c-Jun in the presence of the MAPKK, either MKK4 or MKK7, and a recombinant active MAPKKK, Δ MEKK1, showing that we were able to reconstitute the kinase cascade *in vitro* (Fig. 1a). Surprisingly, several JNK isoforms showed kinase activity in the absence of any

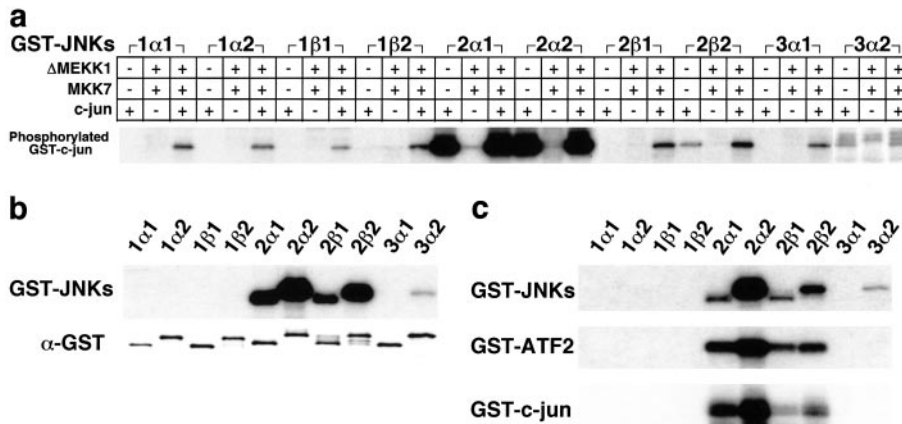


Fig. 1. Autophosphorylation activity and kinase activity of recombinant JNK isoforms. *a*, kinase activity of recombinant JNK isoforms incubated with the upstream kinases. Each purified GST-JNK fusion protein was incubated with or without GST-c-jun, MKK7, and Δ MEKK1 as indicated in kinase buffer containing [γ -³²P]ATP. *b*, autophosphorylation activity of recombinant JNK isoform protein. Each fusion protein was incubated in kinase buffer but in the absence of upstream kinases. The blot with anti-GST antibody in the bottom panel reveals that the same amount of fusion proteins was present in each lane. *c*, kinase activity of recombinant JNK isoforms in the absence of the upstream kinases. Purified GST-JNK proteins were incubated with GST-ATF2 (middle panel) or GST-c-jun (bottom panel) and in kinase buffer containing [γ -³²P]ATP. After terminating the reaction, the phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography.

activating upstream kinase, including JNK2 α 1, JNK2 α 2, JNK2 β 2, and JNK3 α 2, although the activity of the latter was very weak. Because it is thought that MAP kinases require phosphorylation to become active, we asked if these enzymes showed any evidence of autophosphorylation activity or were instead unique in possessing endogenous activity without modification. GST-JNK fusion proteins were incubated in kinase buffer in the absence of any other protein. This revealed that all JNK2 isoforms, including JNK2 β 1, and JNK3 α 2 were indeed capable of autophosphorylation activity (Fig. 1b). JNK2 α 2 showed the highest autophosphorylation activity, followed by JNK2 β 2, JNK2 α 1, JNK2 β 1, and JNK3 α 2. Next, we examined any relative differences in substrate activity. GST-JNK fusion proteins were incubated with either GST-ATF2 or GST-c-jun in kinase buffer. This revealed that JNK2 α 2 showed the highest kinase activity toward both substrates and then in order JNK2 α 1, JNK2 β 2, and JNK2 β 1 (Fig. 1c). Interestingly, JNK2 α 1 showed higher kinase activity than JNK2 β 2, although its autophosphorylation activity appeared lower, suggesting that it has a higher specific activity for these substrates. JNK2 β 1, which only shows weak activity toward c-jun, shows more substantial phosphorylation of ATF-2. All JNK1 and JNK3 isoforms, including JNK3 α 2, which did show autophosphorylation activity, did not show any substrate kinase activity in this assay. Overall, these results demonstrate that the JNK2 gene products, especially JNK2 α 2, have a high basal kinase activity that does not require phosphorylation by an upstream kinase.

The T-P-Y Motif Is Important for Autophosphorylation and Kinase Activity. The activation of MAP kinase family members requires the phosphorylation of a threonine and tyrosine residue located in a Thr-Xaa-Tyr motif (where Xaa is Pro in the case of JNK) found in the activation loop. We examined the requirement for autophosphorylation of this motif by the constitutively active JNK isoforms. We generated JNK2 α 2 constructs containing mutations of threonine to alanine and tyrosine to phenylalanine (JNK2 α 2APF). This mutant recombinant protein lacking dual phosphorylation sites did not show either autophosphorylation activity or substrate kinase activity (Fig. 2). These results indicate that the constitutive active isoform of JNK does indeed autophosphorylate on these sites in the activation loop and that these sites alone are required for this constitutive activation. Furthermore, it appears that autophosphorylation is required for activation.

JNK2 Isoforms Are Predominantly Localized in the Nucleus and Are Phosphorylated *in Vivo*. It has been shown that after activation, MAP kinases subsequently translocate from the cytoplasm to the nucleus. We wished to establish that JNK2 isoforms are

constitutively active within cells and to rule out any artifacts from using bacterially expressed proteins. We examined the subcellular localization of JNK1 and JNK2 isoforms in the human glioblastoma cell line, U87MG. We generated GFP-JNK isoform expression vectors and transiently transfected these constructs into U87MG. Under serum depletion culture conditions, JNK1 α 1 and JNK1 α 2 showed a predominantly cytoplasmic localization, whereas all JNK2 isoforms showed a predominantly nuclear localization (Fig. 3a). We also examined subcellular localization of these JNK isoforms under normal culture conditions (with 10% serum) and after transfection into normal human astrocytes, and the results were identical (data not shown). To further test constitutive activation of these isoforms in cells, we examined whether these GFP-tagged JNK1 and JNK2 proteins are phosphorylated using Western blotting with antiphospho-JNK antibody (Fig. 3b). All JNK2 isoforms showed evidence of activation, but the two JNK1 isoforms did not show any phosphorylation. Collectively, these results are consistent with the *in vitro* kinase assays and demonstrate that the JNK2 isoforms are indeed constitutively active *in vivo* and localize to the nucleus.

The M_r 55,000 Form of JNK2 Is Predominantly Expressed in Primary Glial Tumors. As we have demonstrated, the JNK2 α 2 and JNK2 β 2 isoforms have the highest autophosphorylation activity. In combination with our previous observation that the M_r 55,000 form is preferentially phosphorylated in glial tumors, we speculated that these specific isoforms of JNK are the prevalent forms found in primary tumors. Accordingly, we examined the level of expression of each JNK isoform in glial tumor tissue.

First, we evaluated the isoform specificity of commercially available antibodies using the 10 recombinant proteins for each JNK isoform (Fig. 4a). JNK2-specific antibodies were found to be specific for all four JNK2 isoforms with no cross-reactivity toward JNK1 or JNK3. JNK1-specific antibodies were relatively specific for the four JNK1 isoforms but did show some cross-reactivity with the two JNK3 proteins. The anti-JNK3 antibody could recognize all isoforms, but because the JNK2 reactivity was preabsorbed by the manufacturer, its reactivity toward this isoform is relatively lower. However, this anti-JNK3 antibody recognized the JNK1 isoform to the same extent as JNK3.

Taking into account these cross-reactivities, we examined the isoform expression of JNK in 25 primary glial tumors (19 glioblastoma, 2 anaplastic astrocytoma, 3 low-grade astrocytoma, and 1 pilocytic astrocytoma) and five normal brain tissues. Each JNK gene product (either JNK1, 2, or 3) was detected at various levels of expression. There was preferential expression of the M_r 46,000 JNK1 isoforms, JNK1 α / β 1, over the M_r 55,000 isoforms, JNK1 α / β 1, in 64% of tumor specimens (16 of 25) and all five normal brain tissue specimens, revealing no statistically significant difference ($P > 0.1$). Fig. 4b shows 15 representative tumor cases and three normal specimens. On the other hand, JNK2 expression revealed a very different expression pattern between normal brain and glial tumors. The JNK2 α / β 1 (M_r 46,000) form is predominantly expressed in all five normal brain tissues, whereas the JNK2 α / β 2 (M_r 55,000) is predominantly expressed in all 25 tumor samples. Statistically, there is a highly significant difference with respect to M_r 46,000 versus M_r 55,000 JNK2 expression between glial tumor tissue and normal brain tissues ($P < 0.05$). The JNK3 expression pattern varied between normal brain and tumor, where some cases preferentially expressed the M_r 46,000 form (2 of normal brain and 9 of the tumor samples) and others expressed M_r 55,000 forms (3 of normal brain and 16 of the tumor samples).

For these specimens, we also examined the pattern of expression with antiphospho-JNK antibody, which recognizes the active form of JNK (Fig. 4b). The M_r 55,000 isoform of JNK isoform appeared to be

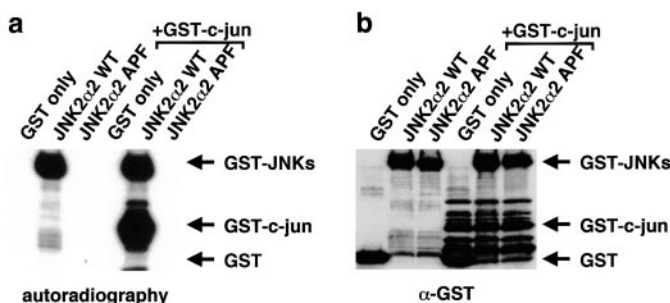


Fig. 2. The T-P-Y motif is essential for autophosphorylation activity and substrate kinase activity. Site-directed mutagenesis was performed to mutate the Thr and Tyr in the activation motif of JNK2 α 2 to Ala and Phe (JNK2 α 2 APF), respectively. In *a*, purified GST-JNK fusion proteins were incubated with GST-c-jun and [γ - 32 P]ATP in kinase buffer. After terminating the reaction, the phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. In *b*, a duplicate blot was incubated with anti-GST antibody as a loading control. Arrows, GST-JNK, GST-c-jun, and GST protein, respectively. Several premature termination and stop codon readthrough-contaminating proteins are seen in the lanes containing GST-c-jun.

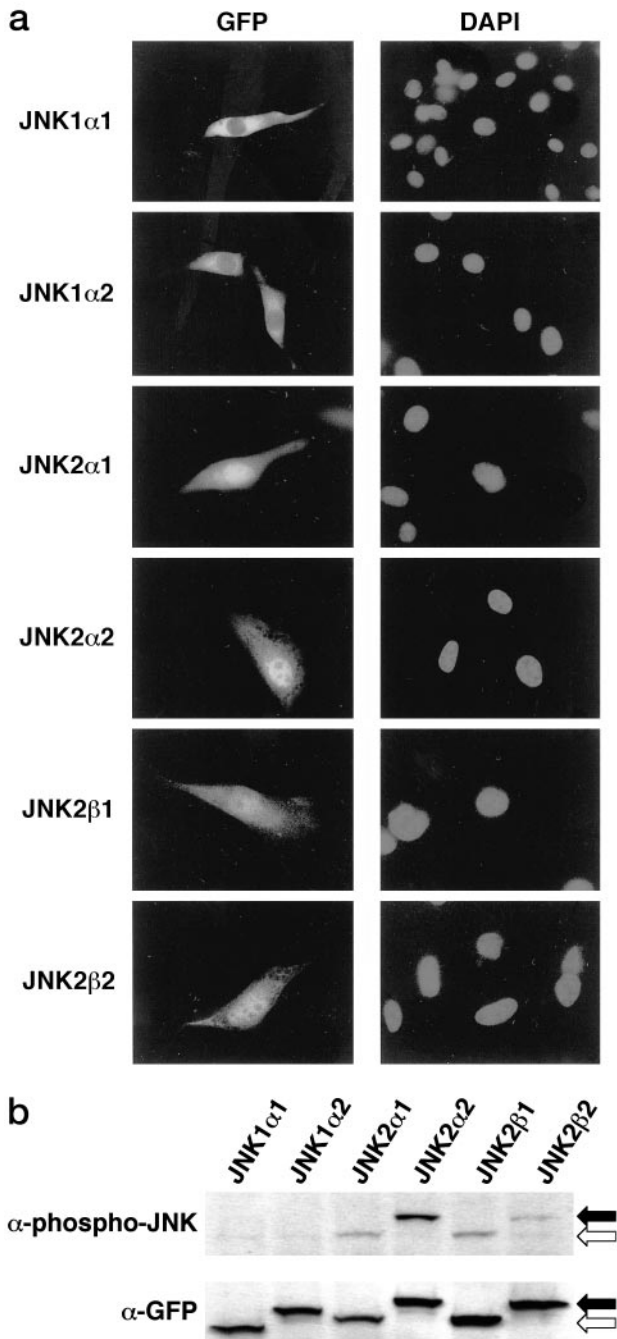


Fig. 3. JNK2 isoforms are constitutively activated *in vivo*. *a*, subcellular localization of JNK isoforms. Plasmid constructs were generated that fused GFP to various JNK isoforms (from top: JNK1 α 1, JNK1 α 2, JNK2 α 1, JNK2 α 2, JNK2 β 1, and JNK2 β 2) and then transfected into U87MG cells on coverslips. After serum depletion, the cells were fixed with paraformaldehyde. Staining with 4',6-diamidino-2-phenylindole was used to visualize the nuclei (right panels). Pictures are representative of three separate experiments with >100 transfected cells examined. *b*, JNK2 isoforms are phosphorylated *in vivo*. Cells transfected with the same constructs were prepared for Western blotting with antiphospho-JNK antibody or anti-GFP antibody to demonstrate expression of constructs. Black arrow, the M_r 81,000 form of GFP fusion JNK protein; white arrow, the M_r 72,000 form, respectively.

the major active form present in primary glial tumor tissues, which is in agreement with our previous study (17). Interestingly, this expression pattern resembled that seen for JNK2. The M_r 46,000 form was the predominant phosphorylated form found in normal brain tissue, which correlates with previous findings that JNK is active in neurons (9). Overall, these results suggest that tumors specifically up-regulate the constitutively active M_r 55,000 form of JNK2.

The JNK2 α / β 2 Form Is the Primary Phosphorylated Form Found in Glial Tumors. To directly address which isoform was phosphorylated in glial tumor tissue, we applied two-dimensional electrophoresis to analyze the relative amounts of active JNK isoforms in these specimens. These blots have the advantage of separating according to phosphorylation status and isoform type, as well as requiring less lysate than immunoprecipitation-based methods. The antiphospho-JNK antibody revealed only a M_r 46,000 spot in all three normal brain tissues, much as was found by standard Western blots (Fig. 5*a*). In glial tumors, the phospho-JNK antibody detected a relatively strong M_r 55,000 spot toward the anode side that was only found in tumor samples. A total of five glial tumor samples was examined. This M_r 55,000 spot was observed in all these samples, and its position was highly reproducible. Quantitation showed that this anode M_r 55,000 spot was 98.2% of the total phospho-JNK signal in case 2159, 96.2% in case 1729, 91.8% in case 1043, 77.2% in case 1133, and 40.3% in case 2082 (Fig. 5*a*), indicating that this spot is the major active isoform present. Next, we asked which isoform corresponded to this phospho-JNK spot. Duplicate two-dimensional gels were blotted with antibodies that recognized each JNK gene product (Fig. 5*b*). This revealed that this spot completely coincided with JNK2. On the other hand, all JNK1 and the majority of the JNK3 isoforms migrated near the cathode side, with only a faint spot for JNK3 that migrated in the same position detected by the phospho-JNK, which is most likely because of cross-reactivity of this antibody with JNK2. These results demonstrate that the M_r 55,000 form of JNK2, JNK2 α / β 2, is the primary phosphorylated form found in primary glial tumors.

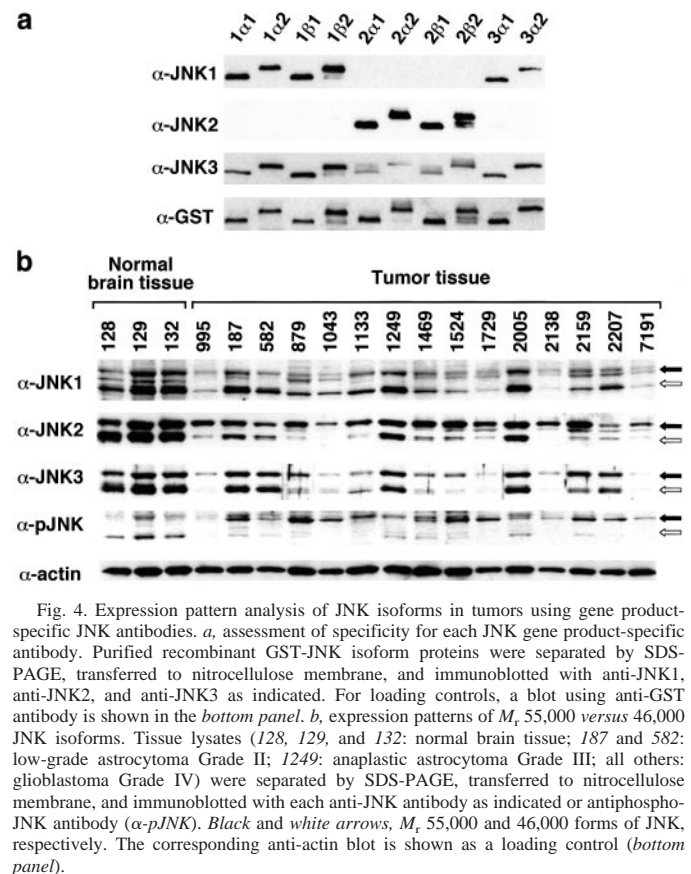


Fig. 4. Expression pattern analysis of JNK isoforms in tumors using gene product-specific JNK antibodies. *a*, assessment of specificity for each JNK gene product-specific antibody. Purified recombinant GST-JNK isoform proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-JNK1, anti-JNK2, and anti-JNK3 as indicated. For loading controls, a blot using anti-GST antibody is shown in the bottom panel. *b*, expression patterns of M_r 55,000 versus 46,000 JNK isoforms. Tissue lysates (128, 129, and 132: normal brain tissue; 187 and 582: low-grade astrocytoma Grade II; 1249: anaplastic astrocytoma Grade III; all others: glioblastoma Grade IV) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with each anti-JNK antibody as indicated or antiphospho-JNK antibody (α -pJNK). Black and white arrows, M_r 55,000 and 46,000 forms of JNK, respectively. The corresponding anti-actin blot is shown as a loading control (bottom panel).

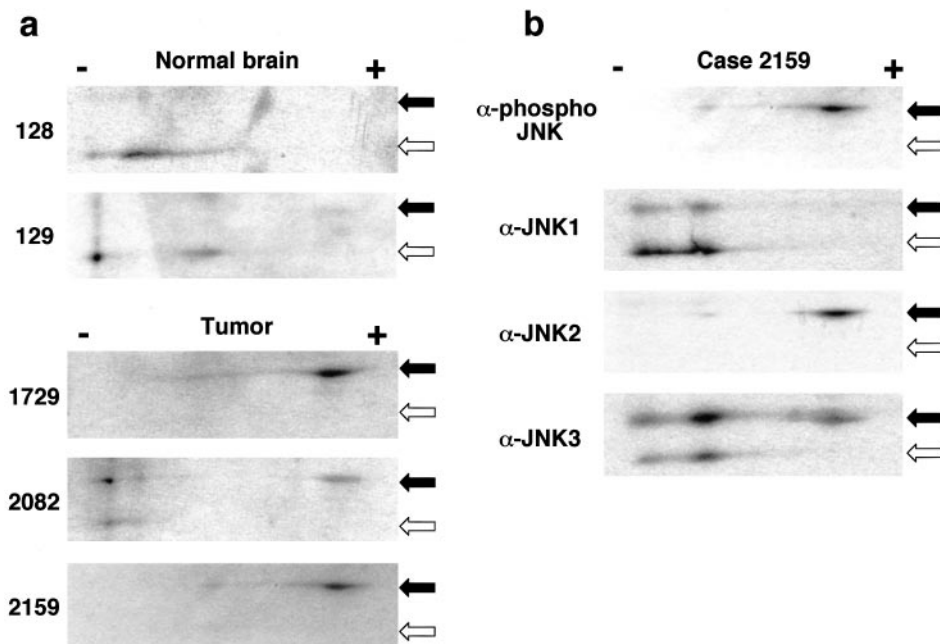


Fig. 5. Two-dimensional analysis of phospho-JNK and JNK isoform expression. Tissue lysates were separated by IEF gel for the first dimension, then separated by SDS-PAGE, followed by transfer to nitrocellulose membranes. Anode side as + and cathode side as - is indicated on the top of the column; black arrow, M_r 55,000 form of JNK; white arrow, M_r 46,000 form, respectively. In a, the blots were incubated with antiphospho-JNK antibody. Two normal brain and three tumor samples are shown. In b, the membrane was incubated with each JNK antibody as indicated.

DISCUSSION

The MAP kinase family, which includes ERK, JNK, and p38, are known as major effectors for growth factor receptors in normal and transformed cells. Elevated ERK activation has been associated with several human cancers (22, 23). However, some tumors may not rely on the ERK pathway for tumorigenesis. Astrocytic tumors of various histological grades have been shown to have elevated levels of phosphorylated ERK, but these levels varied significantly (24), and enhanced ERK activation occurs at a lower frequency in brain tumors than in other types of cancers (25). Our previous studies have shown that ERK activation was seen in only 38% of astrocytic tumors. In contrast, JNK activation was seen in 86% of primary glioblastoma (17).

Several lines of evidence indicate that the JNK pathway plays a role in cellular transformation and tumor cell growth. JNK activation is required for cellular transformation by certain oncogenes, such as Met, human T-cell lymphotropic virus, type I, and Bcr-Abl (5-7). The inhibition of the JNK pathway can reverse a transformed phenotype (5, 8), and it has also been shown that JNK is constitutively active in a variety of tumor cell lines (8, 9). Human brain tumors frequently show increased expression of the normal EGF receptor and the mutant EGF receptor, EGFRVIII, but not significantly elevated ERK activity (26). Instead, the activated form of JNK is highly prevalent in brain tumors, and JNK is constitutively activated by EGFR vIII (10, 17). Therefore, understanding JNK signal transduction will provide new insights into tumor formation and therapy.

In this study, we have shown that several of the JNK isoforms, particularly the JNK2 isoforms, have autophosphorylation activity and constitutive substrate kinase activity in the absence of upstream kinase. There is the possibility that these observations may result from some contaminant in the prokaryotic expression system used for some assays. However, this possibility is unlikely for several reasons. First, each isoform shows intrinsic differences with respect to autophosphorylation and substrate kinase activity, whereas all JNK1 isoforms showed no such activity. Next, GFP-tagged JNK2 proteins expressed in mammalian cells are constitutively localized to the nucleus, whereas JNK1 isoforms are not, reflecting endogenous activation of the JNK protein. Finally, JNK2 isoforms expressed in cells also

showed constitutive phosphorylation. Collectively, this is the first demonstration that specific JNK isoforms, especially the M_r 55,000 isoforms of JNK2, have constitutive activation in the absence of the upstream kinase. These results are supported by a recent study where insect cells were transfected with several JNK isoforms. Although no direct comparison among the isoforms was made, the basal activity of recombinant JNK2 α 2 appeared to be \sim 10 times higher than JNK1 α 1 or JNK3 α 2 (27).

Interestingly, previous work among the *JNK* genes has demonstrated a specific role for *JNK2* in tumorigenesis. It has been reported that specifically disabling *JNK2* isoforms using antisense reagents, but not *JNK1* isoforms, impairs the EGF-stimulated growth of A549 cells (8). Moreover, inhibition of *JNK2* enhances apoptosis and impairs cell cycle progression in glial tumor cell lines (16). The induction of papillomas by 12-*O*-tetradecanoylphorbol-13-acetate is greatly impaired in *JNK2*^{-/-} mice but not in *JNK1*^{-/-} mice (13). We speculate that the JNK2 isoforms are favored for tumorigenesis because of their constitutive activation properties. However, specific differences among isoforms, especially between the M_r 46,000 and the M_r 55,000 isoforms, have not been reported.

We have found that the M_r 55,000 JNK2 isoforms have the highest activity *in vitro*, and this may also have an important correlation with expression in tumors. We have shown previously that the M_r 55,000 form, but not the M_r 46,000 form, of JNK is activated in primary brain tumors (17), suggesting that these specific isoforms of JNK play a critical role in these tumors. Therefore, we examined the expression level of each JNK isoform and found that although the JNK2 α / β 1 (M_r 46,000) form is predominantly expressed in all five normal brain tissue samples examined, the JNK2 α / β 2 (M_r 55,000) is predominantly expressed in all 25 tumor samples examined. Our *in vitro* kinase assay results show that the M_r 55,000 isoforms have greater autophosphorylation, and this provides a tantalizing explanation for the prevalence of the JNK2 α / β 2 form in primary tumors. Intriguingly, a recent study has suggested that the distribution of active forms of JNK may result from differential expression of JNK isoforms within cells and tissues as opposed to a selective activation of isoforms (28).

It is worth noting that normal brain tissue showed detectable expression of the M_r 55,000 forms of JNK2 but did not show signif-

icant phosphorylation using antiphospho-JNK antibody. Recent studies have demonstrated the involvement of phosphatases in the down-regulation of the JNK signaling pathway (29, 30). We speculate that the mechanisms for inactivating JNK signaling may function more efficiently in normal tissues and/or are disrupted in tumor tissues.

The identification of constitutively active isoforms of JNK has several implications for the study of normal physiological processes and the design of therapeutics. Because up-regulation of certain isoforms can activate the JNK pathway, our results show that JNK isoform expression must be taken into account when studying JNK activation in tissues or cells. As certain isoforms are constitutively active, any decreased activation of these particular isoforms *in vivo* suggests that the role of phosphatases must also be considered. Certain JNK pathway inhibitors are currently in clinical trials, such as CEP-1347, for the treatment of neurodegenerative disorders, including Alzheimer's or Parkinson's disease (31, 32). Because these drugs principally target upstream kinases in the JNK cascade, our results suggest that these drugs may not be of benefit if constitutively active JNK isoforms are expressed. In general, the greatest therapeutic benefit would result from the inhibition of JNKs directly.

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