Activation of Pyk2 by Stress Signals and Coupling with JNK Signaling Pathway

G. Tokiwa,* I. Dikic,* S. Lev, J. Schlessinger†

The c-Jun amino-terminal kinase (JNK) is activated by various heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors, inflammatory cytokines, and stress signals. Yet, upstream mediators that link extracellular signals with the JNK signaling pathway are currently unknown. The tyrosine kinase Pyk2 was activated by tumor necrosis factor α, by ultraviolet irradiation, and by changes in osmolarity. Overexpression of Pyk2 led to activation of JNK, and a dominant-negative mutant of Pyk2 interfered with ultraviolet light– or osmotic shock–induced activation of JNK. Pyk2 represents a cell type–specific, stress-sensitive mediator of the JNK signaling pathway.

Various cellular stimuli that control cell growth and differentiation use small guanine triphosphatases (GTPases) and kinase cascades to transmit signals from the cell surface to the nucleus. Activation of the small GTPase Ras is critical for stimulation of a kinase cascade composed of Raf, mitogen-activated protein kinase kinase (MAPKK), and mitogen-activated protein kinase (MAPK) (*1*). A related signaling pathway is activated by G protein–coupled receptors, inflammatory cytokines, and stress signals such as ultraviolet (UV) light and osmotic shock (*2–4*). Key elements of this pathway include the Rho-like GTPases, CDC42Hs and Rac (*3, 5, 6*), and a kinase cascade that phosphorylates and activates JNK, which is also known as the stress-activated protein kinase (SAPK) (*2*). Activated JNK phosphorylates c-Jun (*7, 8*), leading to stimulation of AP-1 transcriptional activity (*9*).

We recently discovered a protein tyrosine kinase, termed Pyk2, that is related to the focal adhesion kinase (*10*) and is activated by various extracellular signals that increase intracellular calcium concentrations (*11*). Pyk2 can tyrosine phosphorylate and modulate the action of ion channels as well as feed into and activate the Ras-MAPK signaling pathway (*11*). Pyk2 appears to function as an intermediate that links various calcium signals with both short- and long-term responses in neuronal cells.

We examined whether Pyk2 could be activated by tumor necrosis factor–α (TNF-α) and stress signals and whether it could function in the control of the JNK signaling pathway activated by these stimuli. We tested the effect of TNF-α on the status of Pyk2 phosphorylation in human promyelocytic leukemia (HL-60) cells and in rat pheochromocytoma (PC-12) cells (Fig. 1, A and B). Stimulation with TNF-α led to enhanced tyrosine phosphorylation of Pyk2 in HL-60 and PC-12 cells (Fig. 1).

We examined the status of Pyk2 phosphorylation in response to three other activators of JNK signaling. PC-12 cells were subjected to UV irradiation, sorbitol treatment to increase extracellular osmolarity, and anisomycin treatment to inhibit protein synthesis (*3, 4, 12*). Ultraviolet irradiation and sorbitol treatment of PC-12 cells induced tyrosine phosphorylation of Pyk2, whereas anisomycin treatment did not (Fig. 1B). Hence, not all stress signals stimulate activation of Pyk2.

Agonists such as carbachol stimulate Pyk2 activation by stimulation of extracellular calcium influx (*11*). To examine the effect of extracellular calcium influx on TNF-α–, sorbitol-, or UV-induced activation of Pyk2, we treated PC-12 cells with TNF-α, sorbitol, or exposed the cells to UV light in the presence or absence of the calcium chelator EGTA (3 mM) (*12*). JNK activity was also examined in the same lysates (*7, 13, 14*). As a control, the effect of EGTA on anisomycin activation of JNK was examined. Both UV light– and sorbitol-induced activation of Pyk2 were substantially reduced in cells that were stimulated in the presence of EGTA, whereas TNF-α–induced activation of Pyk2 was not affected by the presence of EGTA (Fig. 1B). This experiment demonstrates that UV light– or sorbitol-induced activation of Pyk2 can be mediated in part by the increase in intracellular calcium concentration that is induced by these stimuli.

In the presence of EGTA, JNK activity from UV-induced cells was reduced by approximately 20%, whereas JNK activity from sorbitol-induced cells was reduced by approximately 35% (Fig. 1C). In the presence of EGTA, however, stimulation of Pyk2 and JNK activity by TNF-α was not affected by EGTA, demonstrating that activation of Pyk2 and JNK is not mediated by TNF-α–induced influx of extracellular calcium (Fig. 1, B and C). Similarly, EGTA had no effect on anisomycin-induced activation of JNK in these cells.

Both UV- and sorbitol-induced JNK activation are largely calcium independent. It is likely that stress signals activate the JNK signaling pathway by means of several upstream mediators; Pyk2 may function as one but not the only upstream mediator of UV- or sorbitol-induced JNK activation. This hypothesis is consistent with the smaller effect of EGTA on UV- and sorbitol-in-

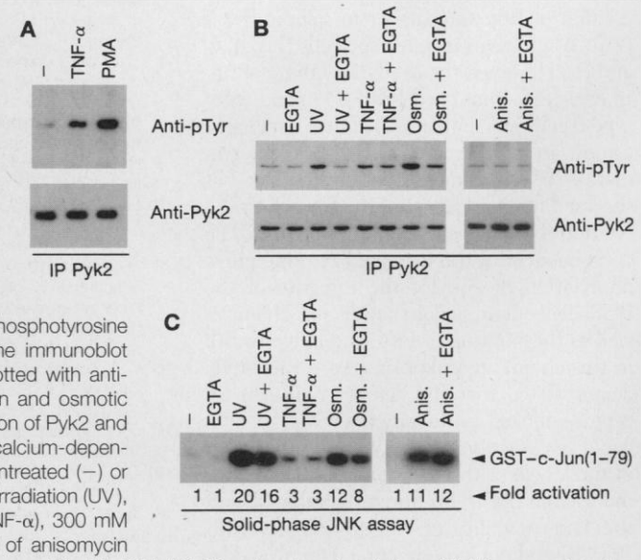

Fig. 1. Phosphorylation of Pyk2 in cells exposed to TNF-α and stress stimuli. (**A**) HL-60 cells were left unstimulated (−) or stimulated with human recombinant tumor necrosis factor (30 ng/ml) (TNF-α) or 1 μM phorbol 12-myristate 13-acetate (PMA) as a control (*11, 12*). The cells were lysed, Pyk2 was immunoprecipitated (IP) with anti-Pyk2, and analyzed by immunoblotting with anti-phosphotyrosine (anti-pTyr) (*11, 13*). The same immunoblot was stripped and immunoblotted with anti-Pyk2. (**B**) Ultraviolet irradiation and osmotic shock–induced phosphorylation of Pyk2 and JNK activation are partially calcium-dependent. PC-12 cells were left untreated (−) or exposed to 80 J/m^2 of UV-C irradiation (UV), murine TNF-α (50 ng/ml) (TNF-α), 300 mM sorbitol (Osm.), or 20 μg/ml of anisomycin (Anis.) in the presence or absence of 3 mM EGTA (*12*). Tyrosine phosphorylation of Pyk2 was analyzed as described in (A). (**C**) Solid-phase JNK assays were done with 2 μg of GST–c-Jun(1–79) immobilized on glutathione-agarose beads as the substrate (*7, 14*). The experiment was repeated three times with similar results. Fold activation of the experiment is indicated. Minus (−) indicates unstimulated cells.

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duced activation of JNK as compared to the effects of EGTA on stress signal–induced activation of Pyk2 in the same cells.

Overexpression of Pyk2 in human embryonic kidney 293T cells led to phosphorylation of the glutathione-S-transferase (GST)–c-Jun(1–79) fusion protein (*7*, *14*), revealing clear Pyk2-dependent activation of JNK in these cells (Fig. 2). The effect was dependent on the level of expression of Pyk2. We also analyzed the activity of epitope-tagged (*15*) JNK that was co-expressed with a Pyk2 expression vector (*16*). Overexpression of Pyk2 caused activation of co-expressed hemagglutinin (HA)-tagged JNK (*17*). JNK activity was monitored from cells that were incubated with conditioned medium taken from cells transfected with a Pyk2 expression vector. JNK was not activated in these cells, which ruled out activation of JNK by a Pyk2-induced autocrine loop (Fig. 2, B and C).

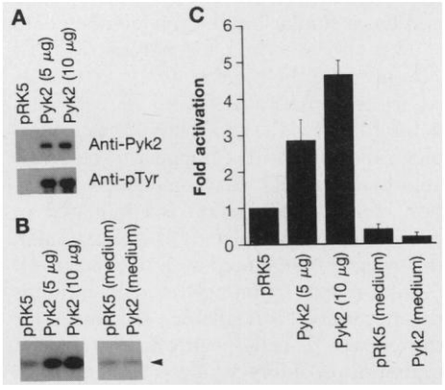

Fig. 2. Activation of JNK by overexpression of Pyk2. (**A**) 293T cells were transiently transfected with expression vector alone (pRK5) or with 5 or 10 μg of a Pyk2 expression vector (*16*). Pyk2 was immunoprecipitated from cell lysates after 48 hours and analyzed as described (*11*, *13*). (**B**) Solid-phase JNK assays were performed (*7*, *14*) from lysates in (A). Also, medium was taken from cells transfected with pRK5 or Pyk2 (10 μg) after 48 hours and incubated for 20 min with untransfected 293T cells. These cells were lysed and assayed for JNK activation. The arrowhead in (B) indicates GST–c-Jun(1–79). The experiment was repeated four times and (**C**) shows fold activation in JNK assays (± SD).

To test whether Pyk2 is required as an upstream regulator of the JNK signaling pathway, we used a catalytically inactive mutant of Pyk2 (PKM) that acts as a dominant-negative inhibitor of wild-type Pyk2 in PC-12 cells (*11*). PC-12 cells were transiently transfected with a PKM expression vector (*16*). After 48 hours, cells were irradiated with UV light or treated with sorbitol, lysed, and analyzed for the activation of JNK (*7*, *13*, *14*). Expression of PKM inhibited both UV- and sorbitol-induced JNK activation (Fig. 3A). However, overexpression of PKM did not affect UV- and sorbitol-induced JNK activation in 293T or COS cells, which do not express Pyk2 (Fig. 3B).

These results suggest that Pyk2 can act as a cell type–specific and stress-sensitive mediator of the JNK signaling pathway. Recent studies demonstrated that dominant-negative mutants of the small GTPases, CDC42Hs and Rac1, can block JNK activation induced by stress responses, inflammatory cytokines, or tyrosine kinases (*3*, *5*, *6*, *18*). We examined whether Pyk2 acts upstream of the small GTPases, CDC42Hs, Rac1, RhoA, and Ras. Expression of dominant-negative mutants of CDC42Hs(N17), Rac1(N17), or Ras(N17) efficiently blocked Pyk2-induced activation of JNK. Expression of a dominant-negative mutant of RhoA(N19), however, did not influence Pyk2-induced activation of JNK (*17*).

The protein tyrosine kinase Pyk2 is activated by the inflammatory cytokine TNF-α, by UV irradiation, and by changes in osmolarity and can function as an upstream mediator of the JNK signaling pathway. The mechanism by which Pyk2 activates the JNK signaling pathway is currently unknown. Pyk2 can activate the Ras-MAPK signaling pathway by both direct and indirect recruitment of the adapter proteins Grb2 and Shc (*11*). These two adapter proteins may also play a role in the activation of JNK because a dominant-negative mutant of Ras conferred partial inhibition of tyrosine kinase–induced activation of JNK (*19*) as well as partial inhibition of Pyk2-induced activation of JNK in 293T cells. The molecular link or links between Pyk2 and CDC42Hs (or Rac1) are also not known. The primary structure of Pyk2 contains putative binding sites for phosphatidylinositol (PI) 3-kinase (*11*); PI 3-kinase may serve as an intermediate between protein tyrosine kinases and Rho-like GTPases (*20*). Pyk2 can be activated by a variety of G protein–coupled receptors that are able to activate the MAPK signaling pathway (*11*, *21*). Hence, Pyk2 has the potential to activate the MAPK signaling pathway, the JNK signaling pathway, or both signaling pathways in the context of different cells and in response to different extracellular stimuli.

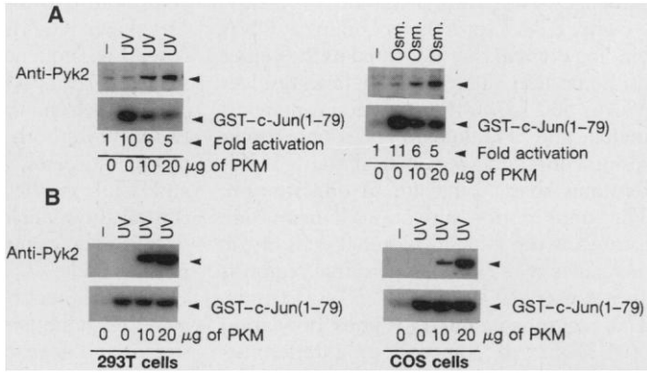

Fig. 3. Effects of dominant-negative PKM on JNK activation. (**A**) PC-12 cells were transiently transfected with expression vector alone (pRK5) or various amounts of a dominant-negative Pyk2 mutant expression vector (PKM) (*11*, *16*). After 48 hours, transfected cells were either exposed to 80 J/m^2 of UV-C irradiation (left) or osmotically shocked in 300 mM sorbitol containing medium and lysed (right) (*12*, *13*). Total cell lysates were used for immunoblot analysis with anti-Pyk2 to monitor PKM expression (upper panel) (*11*). The top arrowhead indicates the total contribution of endogenous Pyk2 and PKM identified by anti-Pyk2. Solid-phase JNK assays were done as described (*7*, *14*). The experiment was repeated three times with similar results. Fold increases for the experiment are indicated. (**B**) 293T cells (left) and COS cells (right) were transiently transfected with various amounts of PKM or pRK5 (*16*). After 48 hours, transfected cells were exposed to UV-C irradiation and treated as in (A). Total cell lysates were used for PKM immunoblot analysis (top arrowhead), and solid-phase JNK assays were done as described (*7*, *14*).

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12. PC-12, COS, and 293T cells were grown as described (*5*, *11*). For UV irradiation (80 J/m^2 UV-C), osmotic-shock activation (with 300 mM sorbitol) as well as treatments with TNF-α (50 ng/ml), PMA (1 μM), or with anisomycin (20 μg/ml) were performed as described (*4*, *7*, *11*). Untreated cells (–) had the medium removed and then immediately replaced. When appropriate, cells were also treated as described in the presence of 3 mM EGTA (*11*) [F. M. Mitchell, M. Russell, G. L. Johnson, *Biochem. J.* **309**, 381 (1995)]. HL-60 cells were grown to 1 × 10^7 to 2 × 10^7 cells per milliliter in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were collected by centrifugation and treated with human recombinant

TNF-α (30 ng/ml) or with PMA (1 μM).
13. Cell lysis, Pyk2 immunoprecipitations, and immunoblotting were carried out as previously described (*11*). Polyclonal antibodies to amino acids 684 to 1009 of human Pyk2 cDNA (anti-Pyk2) expressed as a GST fusion protein in pGEX-2T (Pharmacia Biotech) and phosphotyrosine antibodies were used. Equal amounts of total cell lysate (500 to 700 μg) as determined by Bradford assay (Bio-Rad) were subjected to immunoprecipitation.
14. GST–c-Jun(1–79) fusion proteins were produced and bound to agarose beads as described (*7*). Bound protein (2 μg) was used in solid-phase JNK assays. Lysates were incubated with the GST fusion protein, and after JNK proteins were bound, the beads were washed extensively, [γ-^{32}P]adenosine triphosphate was added, and the phosphorylation of the probe was analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. JNK activity was determined by quantitation of the amount of GST–c-Jun(1–79) phosphorylation with a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics).
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16. 293T cells grown on 10-cm plates were transiently transfected [lipofectamine reagent (Gibco BRL)] with various amounts (5, 10, or 15 μg) of expression vector alone (pRK5); 5 or 10 μg of Pyk2 expression vector together with various amounts of pRK5 (5 or 10 μg); or 5 μg of Pyk2 together with 10 or 20 μg of a dominant-negative mutant of Pyk2 (PKM). COS and PC-12 cells on 10-cm plates were transiently transfected with 10 or 20 μg of pRK5 and 10 or 20 μg of PKM. After 48 hours, the cells were treated as described (*11*, *13*).
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Cooperative DNA Binding and Sequence-Selective Recognition Conferred by the STAT Amino-Terminal Domain

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STAT proteins (signal transducers and activators of transcription) activate distinct target genes despite having similar DNA binding preferences. The transcriptional specificity of STAT proteins was investigated on natural STAT binding sites near the interferon-gamma gene. These sites are arranged in multiple copies and required cooperative interactions for STAT binding. The conserved amino-terminal domain of STAT proteins was required for cooperative DNA binding, although this domain was not essential for dimerization or binding to a single site. Cooperative binding interactions enabled the STAT proteins to recognize variations of the consensus site. These sites can be specific for the different STAT proteins and may function to direct selective transcriptional activation.

Cytokines activate intracellular signaling pathways during immune and inflammatory responses. One of the signal-transduction pathways activated by these ligands involves the STAT family of proteins (*1*). STAT proteins are selectively recruited to various cytokine receptors by virtue of their peptide-binding specificities mediated by their SRC homology 2 (SH2) domains (*2*). Thus, part of the specificity of STAT activation relies on selective recognition of phosphotyrosine-containing peptides of cytokine receptors. After they are tyrosine phosphorylated, the STAT proteins dimerize and migrate to the nucleus where they bind to specific DNA sequences and elicit various programs of gene activation. The mechanisms that determine STAT specificity in transcriptional regulation are unclear because most of the STAT proteins have similar DNA binding preferences (*3*, *4*).

The STAT proteins contain a DNA binding domain that is located in the center of the protein, approximately from residues 350 to 500 (*3*). Other functional domains include the SH2 domain and a phosphotyrosine-containing region near the COOH-terminus that participate in dimerization. The transcription-activation domains are located at the COOH-terminal ends of the molecules (*5*). The NH_2-terminal region is also conserved among the STAT family. This region is required for phosphorylation of STAT2 in response to interferon-α (IFN-α) and may be required for receptor binding (*6*). It is not known whether this region plays a similar role in other STAT proteins or whether the NH_2-terminal domain takes part in the nuclear function of these proteins as transcription factors.

We investigated the mechanisms of STAT regulation in response to interleukin-12 (IL-12), a key cytokine that regulates cell-mediated immunity (*7*). Treatment of cells with IL-12 results in tyrosine phosphorylation and activation of STAT4 (*8*). A full-length STAT4 cDNA was cloned and expressed in baculovirus-infected insect cells (*9*). The DNA binding preference of STAT4 was determined by the random binding site selection method (*10*), and the optimal sequence for STAT4 was TTCCGGGAA (*11*). The sequence selected by STAT4 is identical to that of optimal sites for STAT1 and STAT3 (*3*). STAT5 also has a similar binding preference (*12*).

We also searched for natural STAT4 sites near the IFN-γ gene. IFN-γ is activated in response to IL-12 in T cells and natural killer cells, and many of the physiological effects of IL-12 appear to be mediated by IFN-γ (*7*). IL-12–mediated activation of IFN-γ expression is eliminated in STAT4-deficient mice (*13*). The human IFN-γ gene is organized on four exons (*14*). A 9-kb region spanning the gene contains the necessary cis-regulatory sequences for the correct T cell–specific and inducible transcription of IFN-γ (*15*). The first intron contains a deoxyribonuclease I (DNase I)–hypersensitive site, ~0.5 to 1 kb downstream of the transcription start site (*16*). Using DNase I footprinting (*17*), we detected binding sites for STATs 1, 4, 5, and 6 in the first intron (Fig. 1A). On the basis of our binding site selection results, we expected that STATs 1, 4, and 5 might have the same site preferences. Instead, we observed that each of the STAT proteins bound to a distinct pattern of adjacent sites in the first intron. The protected regions contain multiple sites that are variations of STAT consensus binding sequence (Fig. 1B).

The STAT4 footprint spans ~30 base pairs (bp) of DNA and contains two adjacent binding sites, designated 2 and 3 (Fig. 1B). Each binding site is a variant of the consensus sequence, and the two sites are spaced by 10 bp (*18*). The STAT4 binding sites vary from the optimum sequence in two ways. In both cases the sites are imperfect palindromes, with TTC in one half-site and TTA in the other half-site, and the central three nucleotides are different from those in the optimal site, which has CGG in the middle.

STAT1 also bound to site 3. STAT1 protected a large region that extends downstream and appears to contain at least four additional sites, 4, 5, 6, and 7. The other STAT proteins tested did not bind well to sites 4 through 7 (Fig. 1A). STAT5 bound to sites 2 and 3 and also to a third site that was immediately upstream, site 1. STATs 1 and 4 did not bind to site 1, and STAT6 bound only to site 1. The results indicate that STAT proteins can have selective

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