Induction of Apoptosis by Protein Inhibitor of Activated Stat1 through c-Jun NH$_2$-terminal Kinase Activation*

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Members of the protein inhibitor of activated signal transducer and activator of transcription (STAT) family (PIAS family) of proteins act as negative regulators of STATs in cytokine signaling. We report here that PIAS proteins have proapoptotic activity. PIAS1 induced apoptosis in both human 293T cells and human osteosarcoma U2OS cells. PIAS1 is localized in the nucleus as distinct nuclear dots. Ectopic expression of PIAS1 in U2OS cells activated JNK1 (c-Jun NH$_2$-terminal kinase). A dominant-negative JNK1, capable of inhibiting PIAS1-induced JNK1 activation, blocked PIAS1-mediated apoptosis. Furthermore, a mutant PIAS1, lacking the first 9 amino acid residues, failed to repress Stat1-mediated apoptosis. These results suggest that the inability of PIAS1 to repress Stat1-mediated apoptosis is independent of its ability to repress Stat1-mediated gene activation although it retained its ability to activate JNK and to trigger apoptosis. Our results identify a novel function of PIAS1 in the induction of JNK-dependent apoptosis, independent of the previously known inhibitory activity of PIAS1 in STAT-mediated gene activation.

The c-Jun NH$_2$-terminal kinase (JNK)$^1$ is activated by a variety of cellular signals including growth factors, inflammatory cytokines, and environmental stress (1). Multiple signaling pathways can lead to JNK activation. It has been demonstrated that UV radiation results in the rapid activation of JNK (2–4). The activation of JNK by UV is mediated through the aggregation of cell surface receptors, which triggers a series of cytoplasmic events to activate JNK (5, 6). The activation of JNK by nuclear DNA damage signals such as γ-irradiation is not clearly understood. Ectopic expression of the tumor suppressor BRCA1 in the nucleus has been shown to activate JNK (7). It has been reported that BRCA1-triggered JNK activation is coupled with the induction of gadd45, a growth arrest and DNA damage-induced gene (8, 9). In another study, Takekawa and Saito (10) showed that GADD45 can interact with MTK1 to activate JNK. It was proposed that GADD45 may be involved in stress-induced JNK activation. However, recent studies indicate that stress-induced activation of JNK is not altered in gadd45-null embryonic fibroblasts (11, 12). Apoptosis is a physiological cell death process fundamentally important for development and homeostasis maintenance in multicellular organisms (13, 14). It has been shown that JNK can elicit either proapoptotic or antiapoptotic signals depending on different cell types (15, 16). Studies from targeted disruption of jnk genes in primary murine embryonic fibroblasts indicate that JNK is required for UV-induced apoptosis, which involves cytochrome c release and the mitochondrial death pathway (17).

The signal transducer and activator of transcription (STAT) family is a family of latent cytoplasmic transcription factors that play important roles in cytokine signaling (18, 19). Stat1 is activated by interferon (IFN) stimulation and is required for IFN-mediated gene activation. Recently, we have identified a family of proteins named protein inhibitor of activated STAT (PIAS), which consists of four mammalian members: PIAS1, PIAS3, PIASx (PIASxα and PIASxβ as two splicing variants), and PIASy (20). PIAS1 and PIAS3 have been shown to be specific inhibitors for activated Stat1 and Stat3, respectively (21–23). Recently, PIASy has been identified as a corepressor of Stat1 (24). The role of PIASx in STAT signaling has not been illustrated.

In untreated cells, Stat1 does not interact with PIAS1. Upon IFN stimulation, PIAS1 binds to tyrosine-phosphorylated Stat1 dimer and inhibits Stat1-mediated gene activation by blocking the DNA binding activity of Stat1 (22, 25). Most recently, methylation of Stat1 has been suggested to modulate PIAS1-Stat1 interaction (26). PIAS1 is almost identical to Gu-RH-II-binding protein (GBP) except that GBP lacks the first 9 amino acid (aa) residues present in PIAS1 (27). An NH$_2$-terminal truncated version of PIASxβ (from aa 134 to 621), known as Miz1, was shown to interact with a homeobox DNA-binding protein Mxs2 (28). Recently, the rat homologue of PIASxα named ARIP3 (for androgen receptor interaction protein 3), has been suggested to act as a coregulator of androgen receptor (AR) (29). A PIAS homologue in Drosophila named Zimp has also been described (30). Genetic studies indicate that the Drosophila PIAS protein negatively regulates the Drosophila Jak_STAT pathway (41).

We report here that the PIAS family of proteins has proapoptotic activity. PIAS1 induced apoptosis in both human 293T cells and human osteosarcoma cells. Ectopic expression of PIAS1 activates JNK kinase, which is required for PIAS1-mediated apoptosis. In addition, the proapoptotic activity of PIAS1 is independent of the ability of PIAS1 to repress Stat1-mediated transcription. Taken together, our results reveal a novel function of PIAS1 to trigger JNK-dependent apoptosis.

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‡‡ The abbreviations used are: JNK, c-Jun NH$_2$-terminal kinase; aa, amino acid(s); STAT, signal transducer and activator of transcription; PIAS, protein inhibitor of activated signal transducer and activator of transcription; FBST, phosphate-buffered saline plus Tween 20; Dax, doxycycline; PI, propidium iodide; IFN, interferon; GBP, Gu/RH-II-binding protein; AR, androgen receptor; GST, glutathione S-transferase; GFF, green fluorescence protein; PAGE, polyacrylamide gel electrophoresis; MEKK1, mitogen-activated protein kinase kinase kinase 1.
EXPERIMENTAL PROCEDURES

**Plasmids and Antibodies**—Flag-PIAS1 and Flag-PIAS1Δ(1–9) were constructed by insertion of the murine PIAS1 wild-type cDNA or PIAS1 cDNA lacking the first 27 nucleotides in the coding region into the BamHI and SalI sites of pCMV-Flag vector, respectively. Bcl-xL and a dominant-negative caspase 9 mutant construct, which carries a Cys 287 to Ala mutation, were from J. Yuan (31). TRE-Flag-PIAS1 and TRE-PIAS1Δ(1–9) were constructed by insertion of either the wild-type murine PIAS1 cDNA or the murine PIAS1 cDNA lacking the first 27 nucleotides in the coding region into the BamHI and XhoI sites of pTRE-Flag vector, respectively (a gift from D. Chang). The dominant-negative JNK1 construct pcDNA3-Flag-JNK1(APF), which contains mutations at the phosphorylation sites (Thr183 to Ala and Tyr 185 to Phe), is a gift from R. Davis (2). The mammalian expression construct pEBG-SEK1, which encodes the GST-SEK1 fusion protein, is provided by Dr. L. Zon (32). The plasmid MEKK1*, which encodes a constitutively active form of mitogen-activated protein kinase kinase kinase (MEKK1) by deleting the NH₂-terminal 351 amino acids of MEKK1, was a gift from Dr. M. Karin (33). The plasmid GST-JNK1 was constructed by inserting JNK1 cDNA into the SalI and NotI sites of pGEX-4T-1 (Amersham Pharmacia Biotech).

The following antibodies were purchased and used according to manufacturers’ instructions: anti-Flag (Sigma), anti-tubulin (Sigma), anti-JNK1 (c-17, Santa Cruz Biotechnology), anti-phosphorylated JNK1 (New England Biolabs), anti-SEK1 (k18, Santa Cruz Biotechnology).

**Cell Lines**—Utf, Utf-PIAS1, and Utf-PIAS1Δ(1–9) were established by transfecting UTA6, a tet-off U2OS cell line (34), with a puromycin vector alone or together with pTRE-Flag-PIAS1 or with pTRE-Flag-PIAS1Δ(1–9), respectively. Cells were selected in the presence of doxycycline (Dox; 20 ng/ml), puromycin (2.5 μg/ml), and G418 (100 μg/ml). Cell lines 1–6 and 24–48 were constructed by transfecting Utf-PIAS1 cells with a hygromycin vector alone (1–6) or together with the JNK1 dominant-negative plasmid pcDNA3-Flag-JNK1 (APF) (2–48). Cells were selected in the presence of Dox (20 ng/ml), puromycin (2.5 μg/ml), hygromycin (200 μg/ml), and G418 (100 μg/ml). Calcium phosphate method was used for transfection as described (35).

**Apoptosis Assays**—Apoptosis in 293T cells transfected with green fluorescence protein (GFP) was analyzed by the detection of apoptotic bodies under a fluorescence microscope. Hoechst staining was performed by staining cells with 1 μg/ml bisbenzimide (Hoechst no. 33342, Sigma) in the culture medium at 37 °C incubator for 5 min, followed by analyzing under a fluorescence microscope. Annexin V and propidium iodide (PI) staining was performed as described by the manufacturer, using an annexin V-fluorescein isothiocyanate apoptosis detection kit (R&D Systems). After flow cytometry, cells were divided into four distinct populations using the control cells as a reference; the lower left quadrant of the dot plot represents the viable cells, whereas the lower and upper right quadrants represent the early and late apoptotic cells.

**Fig. 1. Induction of apoptosis by PIAS proteins in 293T cells.** A, induction of apoptosis by PIAS1. 293T cells were transfected with an empty vector (a, c, e, g, and i) or Flag-PIAS1 (b, d, f, h, and j) together with GFP (a–h) or Bcl-xL (c and d) or a dominant negative mutant of caspase 9 (Casp 9 dn) (a and f). Z-VAD (50 μM) was added 6 h after transfection (g and h). Three days after transfection, the morphology of the cells was examined either directly (a–h) or after cells were stained with Hoechst dye (i and j). B, same as in A, except Flag-PIAS3 or Flag-PIASy was used for transfection as indicated.
by FACS analysis. The except cells were stained with annexin V and propidium iodide followed by Hoechst dye and analyzed under a fluorescence microscope.

**JNK Kinase Assay**—Cells were lysed in WL buffer (50 mM Tris, pH 8.0, 0.4 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 0.1 mM Na3VO4). 200 μg of cell lysates were subjected to immunoprecipitation in 500 μl of WL buffer (same as above except using 0.2 mM NaCl) with 1.5 μg of anti-JNK1 (c-17, Santa Cruz Biotechnology) polyclonal antibody. The immunoprecipitates were washed four times with WL buffer with 0.4 mM NaCl, then once with the kinase buffer (25 mM Tris, pH 7.5, 25 mM MgCl2, 2 mM dithiothreitol, 25 mM β-glycerol phosphate, 0.5 mM Na3VO4). The beads were then resuspended in 30 μl of kinase buffer containing 15 μM ATP, 1 μg of GST-Jun-(1-79) fusion protein (Santa Cruz Biotechnology), and 3 μCi of [γ-32P]ATP. The kinase reaction was carried out at 30 °C for 30 min. 10 μl of 4X SDS loading buffer was then added to the reaction mixture. After boiling at 95 °C for 5 min, samples were subjected to 10% SDS-PAGE, followed by autoradiography.

**In Vitro Two-step Kinase Assay**—In vitro two-step kinase assay was performed as described (36). Human 293T cells were transiently transfected with either Flag-PIAS1 alone, or pEBG-SEK1 together with MEKK1*. Flag-PIAS1 was recovered by immunoprecipitation with anti-Flag monoclonal antibody. Briefly, cells were lysed in WL buffer (same as above) 30 h after transfection. Approximately 2 mg of cell lysates were incubated with 10 μg of anti-Flag monoclonal antibody (M2, Sigma) in 500 μl of WL buffer (same as above except using 0.2 mM NaCl). The immunoprecipitates were then washed four times with WL buffer with 0.4 mM NaCl, once with the kinase buffer described above, and subjected to the kinase reaction. GST-SEK1 was recovered using glutathione-agarose. Cells were lysed in G buffer containing 20 mM Tris, pH 8.0, 0.2 mM EDTA, 50 mM β-glycerol phosphate, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, and 1 mM leupeptin. Approximately 2 mg of cell lysates were incubated with 30 μl of glutathione-agarose at 4 °C for 30 min with rotation. The beads were then washed four times with G buffer, and once with the kinase buffer. The first kinase reaction was carried out by resuspending the beads in 20 μl of kinase buffer containing 50 μM ATP with or without 50 ng of GST-JNK1 fusion protein and incubating at 30 °C for 30 min. The second reaction was started by adding 2.5 μl of substrate mixture containing 50 μM ATP, 1 μg of GST-Jun-(1-79) fusion protein (Santa Cruz Biotechnology), and 3 μCi of [γ-32P]ATP. The kinase reaction was incubated at 30 °C for another 30 min and stopped by adding 7 μl of 4X SDS loading buffer. After boiling at 95 °C for 5 min, samples were subjected to 10% SDS-PAGE followed by autoradiography.

**Northern Blot Analysis**—Total RNAs were prepared from cells growing in the presence or absence of doxycycline (20 ng/ml) at various time points using RNA STAT60 reagent (Tel-Test). 10 μg of total RNA was subjected to electrophoresis on a 1% agarose gel, followed by capillary transfer to a Hybond N+ membrane (Amersham Pharmacia Biotech). The filter was baked in a vacuum oven at 80 °C for 90 min to immobilize RNA and was prehybridized in SLOP solution containing 4× SSC, 20 mM Tris, pH 7.4, 1× Denhardt’s, 40% formamide, 10% dextran sulfate, 1% SDS, 100 μg/ml sonicated salmon sperm DNA. The filter was then incubated with 32P-labeled DNA probe in SLOP solution at 42 °C for 16 h and washed once in 5× SSC, 0.1% SDS at 42 °C for 5 min; once in 0.5× SSC, 0.1% SDS at 50 °C for 20 min; and once in 0.1× SSC, 0.1% SDS at 80 °C for 15 min. The blot was then analyzed on a PhosphorImager (Molecular Dynamics).

**RESULTS**

The PIAS Family of Proteins Has Proapoptotic Activity—Our initial experimental attempts to establish a stable cell line overexpressing PIAS1 had failed. 2 To examine the effect of PIAS1 on cell growth, human embryonic kidney 293T cells were transfected with Flag-tagged PIAS1, together with an expression vector encoding GFP. Three days after transfection,
the morphology of transfected cells (green cells) was analyzed under a fluorescence microscope. In samples transfected with PIAS1, apoptosis was detected as indicated by the presence of apoptotic bodies (Fig. 1A, panel b). When cells were stained with Hoechst dye, an enriched population of cells with condensed nuclei, characteristic of apoptosis, were observed in samples transfected with PIAS1 (Fig. 1A, panel j). The PIAS1-induced cell death was inhibited in the presence of Z-VAD, a caspase inhibitor (Fig. 1A, panel h). The coexpression of Bcl-xl, an antiapoptotic protein of the Bcl-2 family, which can block the cytochrome c release from the mitochondria (Fig. 1A, panel d), or a dominant-negative caspase 9 mutant (Fig. 1A, panel f) also inhibited PIAS1-induced cell death. These results suggested that PIAS1 has proapoptotic activity and PIAS1-mediated apoptosis requires caspase activation and the mitochondrial death pathway.

We next examined if other PIAS proteins can induce apoptosis. Expression vectors encoding PIAS3 or PIASy were introduced into 293T cells, and apoptosis analysis was performed as detailed above. Apoptotic bodies and nuclear condensation were detected in cells overexpressing PIAS3 or PIASy, suggesting that PIAS3 and PIASy can also induce apoptosis (Fig. 1B). Similarly, PIASSxα or PIASSxβ induced apoptosis when overexpressed in 293T cells, although their apoptotic activity was weaker as compared with that of PIAS1, PIAS3, or PIASy as indicated by less apoptotic body formation as well as less nuclear condensation. These results suggest that the PIAS family of proteins has proapoptotic activity.

To further characterize PIAS1-mediated apoptosis, we established an inducible cell line expressing PIAS1 under the control of tetracycline from human osteosarcoma U2OS cells. In Utf-PIAS1 cells, the expression of Flag-tagged PIAS1 (Flag-PIAS1) was induced upon the removal of Dox (Fig. 2A). Cells growing in the presence or absence of Dox for 48 h were analyzed for apoptosis by three independent assays: Hoechst staining followed by fluorescence microscopy to detect chromatin condensation, annexin V and PI staining followed by flow cytometry to measure membrane changes, or the determination of caspase 3 activity using the chromogenic caspase-3 substrate, aspartate-glutamate-valine-aspartate-p-nitroanilide. The induction of PIAS1 by the removal of Dox clearly resulted in nuclear condensation (Fig. 2B), the appearance of an increased population of apoptotic cells (from 4 to 36% (19 and 17% of early and late stages of apoptotic cells, respectively)) (Fig. 2C), and an increased level of caspase-3 activity (Fig. 2D). In contrast, withdrawal of Dox had no effect on the growth of theUtf control cells. These assays confirmed that inducible expression of PIAS1 causes apoptosis.

**PIAS1 Is Localized to the Nucleus**—To study the molecular mechanism of PIAS1-mediated apoptosis, we first characterized the localization of PIAS1 by immunofluorescence staining analysis. Utf or Utf-PIAS1 cells growing in the presence or absence of Dox for 16 h (no apoptosis occurs at this time point) were stained with anti-Flag antibody (for the detection of PIAS1) and Hoechst dye (for nuclear staining). Although no apparent PIAS1 staining was detected in the Utf control cells, a strong nuclear immunostaining was observed when PIAS1 was induced by withdrawal of Dox (Fig. 3). Interestingly, PIAS1 was found to form distinct nuclear dotlike structures. A very weak Flag-PIAS1 staining was also observed in Utf-PIAS1 cells growing in the presence of Dox, probably resulting from the slight leakage of the tetracycline-inducible system. These results suggest that PIAS1 may trigger a nuclear signaling event to induce apoptosis.

**Induction of PIAS1 Expression Activates JNK1 Kinase**—The JNK pathway has been implicated in apoptosis. To test whether JNK is activated by PIAS1, JNK1 kinase assay was performed using the Utf-PIAS1 cell line. Cells growing in the presence or absence of Dox for 12 or 24 h were lysed and utilized for immunoprecipitation using a specific antibody against JNK1 (α-JNK1 (c17), Santa Cruz Biotechnology). The immunoprecipitates were then washed extensively and subjected to kinase reactions using GST-Jun-(1–79) (Santa Cruz Biotechnology) as a substrate. The parental UTF6 cells either untreated or treated with UV at a dose of 200 J/m² were included as controls (Fig. 4, top panel, lanes 1 and 2). As shown in Fig. 4, after inducing PIAS1 expression for 12 h, JNK1 kinase activity was clearly activated as indicated by the phosphorylation of the JNK1 substrate, GST-Jun-(1–79) fusion protein (Fig. 4, top panel, lanes 3 and 4). Further induction of PIAS1 for 24 h resulted in increased JNK1 kinase activation (Fig. 4, top panel, lane 5). In the same assay, a portion of JNK1 immunoprecipitates was subjected to SDS-PAGE, followed by Western blotting against the same JNK1 antibody to show that similar amounts of JNK1 were present in each lane (Fig. 4, bottom panel). These data indicate that induction of PIAS1 expression can activate JNK1 kinase.

**JNK1 Activation Is Required for PIAS1-mediated Apoptosis**—To determine whether JNK1 kinase activity is required for PIAS1-mediated apoptosis, a dominant-negative mutant JNK1 (JNK1dn) encoded by pcDNA3-Flag-JNK1(APF) (2) was stably introduced into the Utf-PIAS1 cell line to block JNK1 activation. Two stable clones, 1–6 and 2–48, without or with JNK1dn expression, respectively, were obtained and used for further analysis (Fig. 5A, top panel). In the control Utf cells, the removal of Dox did not affect JNK1 kinase activity, as indicated both by Western blotting using an antibody against phosphorylated JNK1, and by JNK1 kinase assay using GST-Jun-(1–79) as the substrate (Fig. 5A, middle and lower panels). In 1–6 cell line, which does not express JNK1dn, the removal of Dox for 24 h induced PIAS1 expression, which triggered JNK1 activation (Fig. 5A). In contrast, in 2–48 cell line, which expresses JNK1dn, the induction of PIAS1 by Dox removal failed to activate JNK1 (Fig. 5A). Thus, JNK1dn expression can block PIAS1-triggered JNK1 activation.

To test whether JNK1 activation is required for PIAS1-mediated apoptosis, annexin V and PI staining analysis was performed. In the control Utf cell line, withdrawal of Dox did
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FIG. 4. Inducible expression of PIAS1 activates JNK1 kinase. Cell lysis from Utf-PIAS1 cells growing in the presence or absence of Dox for 12 or 24 h were subjected to JNK1 kinase assay using a GST-Jun-(1–79) fusion protein as the substrate. Cell lysis from the parental UTA6 cells either left untreated or treated with UV (200 J/m²), and then harvested 30 min later were included as controls. Two hundred μg of cell lysis was subjected to immunoprecipitation in each reaction using an anti-JNK1 antibody (c17, Santa Cruz Biotechnology) (top). A portion (~10%) of each immunoprecipitate (IP) was subjected to SDS-PAGE, followed by Western blotting (WB) with the same anti-JNK1 antibody to show equal loading in each lane (bottom).

FIG. 5. JNK1 activation is required for PIAS1-mediated apoptosis. A, top panel, Western blot was performed with cell lysates from the control cell line (Utf) and two Utf-PIAS1 derived cell lines without (1–6) or with (2–48) stably expressed Flag-tagged JNK1dn growing in the presence or absence of Dox for 24 h. Anti-Flag and anti-tubulin antibodies were used. PIAS1, JNK1dn, and tubulin were indicated by arrows. Tubulin was used as a control to show equal loading in each lane. JNK1dn, JNK1 dominant-negative mutant encoded by pcDNA3-Flag-JNK1 (APP). Middle panel, the same lysates as in top panel were subjected to Western blot with an antibody specific for phosphorylated JNK1 (pJNK1). Bottom panel, the same cell lysates as in top panel were subjected to JNK1 kinase assay using GST-Jun(1–79) fusion protein (Santa Cruz Biotechnology) as the substrate. 200 μg of the cell lysate was used in each reaction. B, the same cell lines as in A were assayed for apoptosis by annexin V and PI staining analysis. After removing Dox for 24 h, JNK1 was clearly activated as indicated by JNK1 kinase assay as well as protein blotting analysis with an antibody specific for phosphorylated JNK1 (pJNK1), or anti-JNK1 as indicated. The same lysates were subjected to JNK1 kinase assay using GST-Jun(1–79) fusion protein as the substrate. B, the same cell lines as in A were assayed for apoptosis by annexin V and PI staining analysis. After removing Dox for 24 h, JNK1 was clearly activated as indicated by JNK1 kinase assay as well as protein blotting analysis with an antibody specific for phosphorylated JNK1 (pJNK1), or anti-JNK1 as indicated. The same lysates were subjected to JNK1 kinase assay using GST-Jun(1–79) fusion protein as the substrate.

FIG. 6. The NH2-terminal 9 amino acid residues of PIAS1 are not required for PIAS1 to trigger JNK1 activation and apoptosis. A, cell lysates were prepared from Utf, Utf-PIAS1, and Utf-P1A-(1–9) growing in the presence or absence of Dox for 24 h and subjected to Western blot (WB) analysis with anti-Flag, anti-tubulin, anti-phosphorylated JNK1 (pJNK1), or anti-JNK1 as indicated. The same lysates were subjected to JNK1 kinase assay using GST-Jun(1–79) fusion protein as the substrate. B, the same cell lines as in A were assayed for apoptosis by annexin V and PI staining (see “Experimental Procedures”) 40 h after Dox withdrawal. The percentage of apoptosis is the sum of early and late apoptotic populations.

not cause apoptosis, whereas the induction of PIAS1 expression in clone 1–6 for 40 h resulted in ~40% cells undergoing apoptosis (Fig. 5B). In contrast, in clone 2–48 expressing JNK1dn, apoptosis triggered by the induction of PIAS1 was greatly reduced (~10%) after Dox removal (Fig. 5B). These results suggest that JNK1 kinase activity is required for PIAS1-mediated apoptosis.

The Ability of PIAS1 to Activate JNK and Apoptosis Is Independent of Its Inhibitory Effect on Stat1-mediated Gene Activation—PIAS1 can inhibit Stat1-mediated gene activation (22). PIAS1 is almost identical to GBP (27). The major difference between PIAS1 and GBP is that GBP lacks the first 9 aa residues present at the NH2 terminus of PIAS1. We wanted to examine the role of the NH2-terminal 9 aa residues of PIAS1 in its proapoptotic activity as well as in its inhibitory effect on Stat1-mediated gene activation. A mutant PIAS1, PIAS1Δ(1–9), which lacks the first NH2-terminal 9 aa residues, was generated. To compare the activity of PIAS1Δ(1–9) with that of wild-type PIAS1, a PIAS1Δ(1–9) tet-off cell line derived from U2OS cells, Utf-P1A-(1–9), was established. The ability of PIAS1Δ(1–9) to activate JNK1 and trigger apoptosis was examined. After inducing the expression of PIAS1Δ(1–9) by removing Dox for 24 h, JNK1 was clearly activated as indicated by JNK1 kinase assay as well as protein blotting analysis with an antibody specific for phosphorylated JNK1 (Fig. 6A). The level of JNK1 activation was slightly lower in Utf-P1A-(1–9) as compared with that of Utf-PIAS1, which is correlated to the slightly lower expression of PIAS1Δ(1–9) protein as compared with wild-type PIAS1 (Fig. 6A, top panel).

We next examined the ability of PIAS1Δ(1–9) to trigger apoptosis by annexin V and PI staining analysis. After remov-
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**Fig. 7.** The NH$_2$-terminal 9 amino acid residues of PIAS1 are required for PIAS1 to repress Stat1-mediated gene activation. **A**, human 293T cells were transiently transfected with Flag-tagged Stat1 expression vector, 3×Ly6E luciferase reporter construct containing three copies of Stat1-binding sites, together with or without increasing amounts of wild-type (PIAS1) or mutant PIAS1 (PIAS1Δ(1–9)) as indicated. 24 h after transfection, cells were either left untreated or treated with IFN-γ (5 ng/ml) for 6 h, then harvested for luciferase assays. The cotransfected β-galactosidase was used as an internal control to correct for differences in transfection efficiency. The results are representative of several independent experiments. **Luc.**, luciferase. **B**, Northern blot was performed with total RNA (10 μg/lane) isolated from Utf, Utf-PIAS1, and Utf-PIAS1Δ(1–9) cells growing in the presence or absence of doxycycline for 12 h with or without subsequent IFN-γ (5 ng/ml) treatment for 6 h. The filter was probed with Stat1 or actin gene. Quantitative analysis was performed on a PhosphorImager (Molecular Dynamics). -Fold induction of the Stat1 gene as indicated by the bar graph (lower panel) was normalized against actin gene. Shown is a representative of three independent experiments.

We next examined the ability of the Stat1Δ(1–9) mutant to repress Stat1-mediated gene activation. Human 293T cells were transiently transfected with Flag-tagged Stat1 expression construct and a Stat1 luciferase reporter (3×Ly6E), together with or without Flag-PIAS1 or Flag-PIAS1Δ(1–9) mutant construct as indicated. Transfection of Flag-Stat1 into 293 cells resulted in ~10-fold activation of luciferase activity upon IFN-γ treatment (Fig. 7A). Stat1-mediated gene activation was inhibited by wild-type PIAS1 in a dose-dependent manner. However, PIAS1Δ(1–9) failed to repress Stat1-mediated gene activation. In fact, a weak dominant negative effect of PIAS1Δ(1–9) was observed when it was expressed at higher levels (Fig. 7A). Protein blot analysis showed comparable expression levels of PIAS1 and PIAS1Δ(1–9) in the same extracts (Fig. 7A, bottom panel). Thus, PIAS1Δ(1–9) lacks the ability to repress Stat1-mediated gene activation.

To further confirm that wild-type PIAS1, but not the mutant PIAS1Δ(1–9), acts as an inhibitor of Stat1-mediated gene activation in vivo, we used the tet-off cell lines to examine the IFN-mediated induction of the endogenous Stat1 gene, which is a known IFN-responsive gene. Utf, Utf-PIAS1, or Utf-PIAS1Δ(1–9) cells growing in the presence or absence of Dox for 12 h were either left untreated or treated with IFN-γ for 6 h. Total RNA was prepared and subjected to Northern blot analysis using Stat1 cDNA as a probe. Wild-type PIAS1 caused ~40% inhibition of Stat1 transcription (Fig. 7B, compare lanes 6 and 8), whereas the PIAS1Δ(1–9) mutant did not inhibit Stat1 gene activation in response to IFN-γ (Fig. 7B, compare lanes 10 and 12). As a control, Dox treatment did not affect Stat1 induction in response to IFN in Utf cells (Fig. 7B, compare lanes 2 and 4). These results are consistent with the in vitro luciferase reporter data, indicating that wild-type PIAS1, but not the mutant PIAS1Δ(1–9), can repress Stat1-mediated gene activation in response to IFN stimulation. We conclude that the ability of PIAS1 to activate JNK and to induce apoptosis is independent of its inhibitory effect on Stat1-mediated gene expression.

The Induction of GADD45 by PIAS1 Is Not Sufficient to Activate JNK or to Trigger Apoptosis—The data presented above indicate that PIAS1 can trigger a nuclear signal to activate JNK to induce apoptosis. GADD45, a growth arrest and DNA-damage-inducible protein, has been reported to interact with MTK1 to trigger JNK/p38 activation (10). Furthermore, overexpression of the tumor suppressor BRCA1 (a nuclear protein) in U2OS cells has been shown to activate JNK and to induce JNK-dependent apoptosis coupled with up-regulation of gadd45 gene (7). We next examined if PIAS1 can induce
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**DISCUSSION**

In this paper, we have provided strong evidence to demonstrate a novel function of PIAS1 in the activation of JNK and apoptosis. We showed that PIAS1-induced apoptosis is dependent on JNK activation. Four different types of apoptotic assays were used to confirm the proapoptotic activity of PIAS1: the detection of apoptotic bodies, the examination of nuclear condensation by Hoechst staining, annexin V analysis, and the measurement of caspase 3 activity. In addition, PIAS-induced cell death was inhibited by the caspase inhibitor Z-VAD-fmk, the expression of the antiapoptotic protein Bcl-xL, or a dominant negative caspase 9 mutant protein.

Several lines of evidence indicate that PIAS1-induced apoptosis is mediated through JNK1 activation. First, JNK1 activation was observed after inducing PIAS1 expression for 12 h, whereas PIAS1-triggered apoptosis could be detected only after PIAS1 induction. Thus, PIAS1-induced JNK1 activation precedes the apoptotic phenotype caused by PIAS1. Second, the level of JNK activation by PIAS1 is significantly higher than that observed after UV treatment at 200 J/m², a dose significantly higher than physiological settings, suggesting that JNK acti-
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viation may be important in PIAS1-triggered apoptosis. The most convincing data were derived from studies with the dominant-negative JNK. After introducing JNK1dn into the PIAS1 tet-off cell line, JNK1dn sufficiently blocked both PIAS1-induced JNK1 activation and PIAS1-mediated apoptosis, indicating that JNK1 is required for PIAS1-triggered apoptosis.

Our data indicate that PIAS1 is a nuclear protein and that it triggers a nuclear signal to activate JNK. Previous studies suggest that JNK activation by UV radiation involves a series of cytoplasmic events (5, 6). The mechanism of JNK activation by nuclear signals such as DNA damage is not well understood. p53 can cause transcriptional up-regulation of gadd45 in response to genotoxic stress (37, 38). It has been proposed that GADD45 and its related proteins can bind MTK1 to activate apoptosis coupled with up-regulation of gadd45 and its related proteins can bind MTK1 to activate JNK-mediated JNK activation and apoptosis. Since our in vitro signals that can activate the PIAS1-JNK pathway. It will be of great interest in the future to identify genes that can activate the PIAS1-JNK pathway.

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