p21-activated Protein Kinase
γ-PAK Is Activated by Ionizing Radiation and Other DNA-damaging Agents

SIMILARITIES AND DIFFERENCES TO α-PAK

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The p21-activated protein kinase γ-PAK is activated 2–5-fold in response to ionizing radiation (IR) in 3T3-L1 fibroblasts and U937 leukemia cells. γ-PAK is activated in a dose- and time-dependent manner. Doses from 1 to 100 Gy result in significant stimulation of activity at 30 min, whereas maximal stimulation is observed at 120 min after irradiation. UV (80 J/m²) and the DNA-damaging drugs cytosine β-d-arabinofuranoside (AraC) and cis-platinum(II)diammine dichloride (cisplatin) also induce γ-PAK activation. The activation of γ-PAK in response to IR or AraC is dependent on tyrosine kinase and phosphoinositide 3-kinase activity, as demonstrated by use of the inhibitors genistein and wortmannin; in contrast activation of γ-PAK by cisplatin and UV is not affected significantly by these inhibitors, suggesting that γ-PAK can be activated by more than one pathway in response to different types of DNA damage. In contrast to γ-PAK, α-PAK and JNK are activated only by cisplatin and UV in 3T3-L1 cells, suggesting differential regulation of the protein kinases. This is the first time that members of the Ste20/PAK family of protein kinases have been shown to be involved in the cellular response to IR and other DNA-damaging agents.

The p21-activated protein kinases (PAKs) are activated by the small G-proteins Rac and Cdc42 and Cdc42 (1–3). Three mammalian PAKs are known: α-PAK (PAK1, 68 kDa), β-PAK (PAK3, 65 kDa), and γ-PAK (PAK2, PAK1, 58–60 kDa). γ-PAK is unique among the mammalian members of the PAK family, as it is activated by caspase cleavage during apoptosis and can induce some of the changes involved in this death program (4–6). γ-PAK appears to have cytostatic properties, as injection of the active enzyme into frog embryos results in cleavage arrest at mitotic metaphase (7). γ-PAK is activated in response to cell stress (8) and serum starvation, in contrast to the related α-PAK, which appears to be involved in the control of the cytoskeletal architecture (9–11) and is activated by growth factors (12) and insulin (13). When 3T3-L1 cells are subjected to hyperosmotic stress, γ-PAK is activated by a two-step mechanism involving translocation to the particulate fraction and activation of the protein kinase in a phosphoinositide 3-kinase (PI3-kinase)-dependent manner (8). γ-PAK involvement in cell cycle regulation is supported by data showing that the protein kinase phosphorylates and regulates Raf-1 (14).

DNA damage is a major threat to cells; failure to repair the DNA before cell division causes genomic instability or lethality and a predisposition to cancer in multicellular organisms. Agents that induce DNA damage such as ionizing radiation (IR), ultraviolet light (UV), or chemotherapeutic drugs such as cis-platinum(II)diammine dichloride (cisplatin) or cytosine β-d-arabinofuranoside (AraC) lead to activation of the DNA repair machinery of the cell and of different signaling pathways that transiently inhibit the cell cycle. This cytostatic response allows DNA repair prior to cell division (15–18).

In this study, IR and other DNA-damaging agents have been used to examine whether γ-PAK could be involved in the cytostatic response to DNA damage. It is shown that γ-PAK is activated in response to IR in a dose- and time-dependent manner. UV and the DNA-damaging drugs cisplatin and AraC also activate γ-PAK, suggesting that different types of DNA damage trigger the activation of the protein kinase. The possible relationship between the observed γ-PAK activation and its described cytostatic properties is discussed.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and reagents were purchased from Life Technologies, Inc. Okadaic acid was from LC Laboratories. Histone 4, leupeptin, pepstatin, and aprotinin were from Roche Molecular Biochemicals. Wortmannin, AraC, cisplatin, and myelinar basic protein were from Sigma. Genistein was from Alexis Co. Ly 294002 was from Calbiochem. RR-1 anti-PAK antibody was obtained as described previously (7). Antibodies against γ-PAK (N19, C20) and α-PAK (N20), peroxidase-coupled secondary antibodies, and protein G-agarose were purchased from Santa Cruz Biotechnology. The enhanced chemiluminescent (ECL) detection kit was from Amersham Pharmacia Biotech.

Cell Culture and Treatment—3T3-L1 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. U937 leukemia cells were grown in RPMI 1640 medium supplemented with glutamine and 10% fetal calf serum. Exponentially growing cells were irradiated in a 32P3Cs γ irradiator (MARK I, J. L. Shepherd & Associates, San Fernando, CA) at dose rates of 325 Gy/min (for 100 Gy dose), 16 Gy/min (for 5 Gy dose), or 39.8 Gy/min (for 1 Gy dose). Control cells were treated exactly without irradiation. The DNA-damaging drugs AraC and cisplatin were added to the culture medium for the indicated times at a concentration of 50 μM. UV irradiation was carried out in a UV cross-linker (FB-UVXL-1000; Fisher); the culture medium was removed during irradiation. Control cells were treated exactly the same without irradiation. Cells were incubated for the indicated times, harvested, frozen in liquid nitrogen, and stored at −70 °C. When indicated, the cells were preincubated for 15 min with 100 μM genistein or 200 μM wortmannin (stock solutions in Me2SO) before treatment. Control cells were mock-treated with Me2SO.

Preparation of Total Extracts—Cells were thawed in 0.5 ml of freshly prepared lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 10 μM β-mercaptoethanol, phosphatase inhibitors (50 mM NaF, 5 mM Na3P04, 2 mM NaVO4, 10 mM okadaic acid) and protease inhibitors (40 μg/ml leupeptin, 40 μg/ml pepstatin, 40 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride)) plus 1% Nonidet P-40. After 10 min on ice, the lysate was centrifuged at 16,000 × g for...
RESULTS

To examine whether γ-PAK activity was affected by IR, exponentially growing 3T3-L1 mouse fibroblasts and U937 human leukemia cells were exposed to 5 and 100 Gy of γ-radiation using a 137Cs source. After irradiation, the cells were incubated for 120 min in a CO2 incubator, and lysed in buffer containing 1% Nonidet P-40. γ-PAK activity was determined following immunoprecipitation of the protein kinase from cell extracts with histone 4 as substrate. Fig. 1A shows that 120 min after irradiation, γ-PAK activity in 3T3-L1 cells was increased about 2-fold at doses of 5 and 100 Gy. In U937 cells, a dose of 100 Gy induced a 4–5-fold increase in γ-PAK activity, whereas 5 Gy had no observable effect. As U937 cells undergo rapid apoptosis in response to IR, 3T3-L1 cells were chosen as a model for this study.

The observed increase in γ-PAK activity in response to IR was both dose- and time-dependent (Fig. 1B) with significant stimulation of activity observed at 30 min with doses of 1, 5, and 100 Gy, suggesting a physiologically modulated response. Maximum stimulation was observed 120 min after irradiation, as longer incubation times after irradiation did not result in further increase of γ-PAK activity (data not shown).

When immunoprecipitates of γ-PAK were analyzed by Western blotting, the same amount of γ-PAK was detected in control and irradiated cells (Fig. 1C, left panel), indicating a stimulation of γ-PAK activity in irradiated cells. This was supported further by the use of an anti-γ-PAK antibody (RR1), which reacted preferentially with the regulatory domain (6) of highly autophosphorylated active γ-PAK. Although in response to IR no changes in total γ-PAK protein were detected with the N19 antibody (which recognizes both active and inactive γ-PAK), a higher level of γ-PAK immunoreactivity was observed with the RR1 antibody in irradiated cells as compared with control cells (Fig. 1C, right panel). This indicated that IR stimulated γ-PAK autophosphorylation and activation.

Doses of 1 and 5 Gy of IR resulted in a temporal slowing of the growth of 3T3-L1 cells; for doses of 20 and 100 Gy, the cells stopped dividing and accumulated in the G2/M phase of the cell cycle. None of the doses of IR used in the experiments induced significant apoptosis in 3T3-L1 cells, even 48-h post-irradiation. Accordingly, no γ-PAK activation by caspase cleavage was observed by Western blot analysis using antibodies against either the C or the N terminus of γ-PAK (data not shown).

Although the primary cellular effect of IR is the induction of double strand DNA breaks, it also causes other changes (16). To examine if DNA damage was the trigger for γ-PAK activation in response to IR, 3T3-L1 cells were incubated with two different DNA-damaging drugs or irradiated with increasing doses of UV, and γ-PAK activity was determined with histone 4 as substrate after immunoprecipitation. Cisplatin, which produces interstrand cross-links in the DNA (20), was a potent activator of γ-PAK, with a 2.5- and 5-fold stimulation of activity after incubation for 30 and 120 min, respectively (Fig. 2). The antimitabolite AraC, which is incorporated into the DNA and produces termination of DNA synthesis (21), activated γ-PAK around 2.5-fold at both 30 and 120 min of incubation. UV, which induces mainly pyrimidine dimers in the DNA (18), activated γ-PAK up to 3-fold 120 min after irradiation at 80 J/m², whereas 10 J/m² had no effect on the protein kinase activity (Fig. 2). Neither DNA-damaging drugs nor UV resulted in cleavage of γ-PAK by caspase, as determined by Western blotting (data not shown). Thus, different types of DNA damage result in activation of γ-PAK, supporting the idea that DNA damage is the trigger of γ-PAK activation in response to IR.

To study further the physiological mechanisms of γ-PAK activation by DNA damage, 3T3-L1 cells were preincubated with or without the tyrosine kinase inhibitor genistein or the PI3-kinase inhibitor wortmannin before irradiation; DNA-damaging drugs were present in the culture medium throughout the experiments (Fig. 3). Short incubation times were used to avoid a direct effect of wortmannin or genistein on γ-PAK activity. A significant inhibition of γ-PAK activation by IR (5 Gy) was observed when genistein or wortmannin were present (60 and 90%, respectively). Genistein and wortmannin also abolished activation of γ-PAK by AraC (65 and 100%, respectively). The PI3-kinase inhibitor Ly 294002 also inhibited...
γ-PAK activation by IR or AraC (data not shown), further supporting the involvement of PI3-kinase activity in the activation of γ-PAK. Cisplatin activation of γ-PAK was not affected by wortmannin and only slightly affected by genistein, whereas neither compound inhibited activation of γ-PAK by UV.

The fact that both genistein and wortmannin interfered with the activation of γ-PAK by IR and AraC indicated that tyrosine kinase and PI3-kinase activity are essential upstream activators of γ-PAK in response to these DNA-damaging treatments. In contrast, UV and cisplatin activation of γ-PAK is independent of tyrosine kinase and PI3-kinase activity. This suggests the involvement of more than one pathway in the control of γ-PAK activity in response to different types of DNA damage.

To determine whether γ-PAK activation in response to DNA damage was a general feature of other members of the PAK family, α-PAK activity was assayed after immunoprecipitation from control and treated 3T3-L1 cells (Fig. 4A). No activation of α-PAK in response to IR or AraC was observed, indicating that α-PAK and γ-PAK were differentially regulated. In contrast, α-PAK was activated 2.4- and 1.8-fold, respectively, by cisplatin and UV.

It has been suggested that the stress-activated protein kinase JNK acts downstream of PAK (5, 22, 23), even though this has been questioned (24–26). Thus, we examined whether JNK was activated in response to the different DNA-damaging treatments that were able to activate γ-PAK. No activation of JNK was observed in 3T3-L1 cells 120 min after irradiation with doses of 1, 5, or 100 Gy of IR, as determined by phosphorylation of c-Jun (Fig. 4B). Longer incubation times after irradiation (up to 6 h) did not induce JNK activation (data not shown). The DNA-damaging drugs cisplatin and AraC also failed to activate JNK significantly; in contrast, JNK was activated by UV (80 J/m²) in 3T3-L1 cells (Fig. 4B). The data is consistent with results previously reported for NIH 3T3 cells, wherein IR doses up to 100 Gy failed to activate JNK, although inducing c-Abl activation or p53 accumulation (27). It is interesting to note that in 3T3-L1 cells, stress-inducing agents like the drugs anisomycin and arsenite are able to activate JNK without any observable effect on γ-PAK activity (data not shown).

**DISCUSSION**

γ-PAK is activated up to 5-fold during the early response to IR in 3T3-L1 fibroblasts and U937 leukemia cells. This activation is time- and dose-dependent, and correlates with an increase in the amount of phosphorylated γ-PAK present in the cells. Caspase cleavage is not involved in the observed activation of γ-PAK, because no fragments of γ-PAK are detected by Western blotting. γ-PAK activation by IR is dependent on tyrosine kinase and PI3-kinase activity, as shown by the use of the inhibitors genistein and wortmannin. A direct involvement of PI3-kinase (perhaps through activation of Cdc42) in the response to IR cannot entirely be ruled out, but wortmannin could also act through inhibition of the PI3-kinase-related enzymes DNA-dependent protein kinase or ATM. These protein kinases are known to have a central role in the early response to agents that induce DNA damage, such as IR (18). Other candidates for upstream activators of γ-PAK are the tyrosine kinases c-Abl and Lyn, which have been shown to be activated in response to IR (28, 29). Because γ-PAK activation in vitro requires autophosphorylation of both the regulatory and catalytic domains of the enzyme (30–32), the data suggest that autophosphorylation and phosphorylation by other kinases may be involved in activation in vivo.
the cell cycle, with the G2/M transition checkpoint playing a role in the cellular response to DNA damage. In eukaryotic cells, all specific DNA-damaging agents, but activation of different members of the PAK family can be activated by specific DNA-damaging agents, after irradiation ([30278–30281]). We propose that PAK activation by IR and DNA damage is important in the cytostatic response elicited by these agents, as PAK has been shown previously to induce cytostasis ([7, 38]). Additional studies are underway in our laboratory to determine the requirements for activation of PAK in response to IR and to define the role of the protein kinase in the early response to DNA damage.

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and ATM, whereas the pathway activated by UV is not dependent on PI3-kinase-related enzymes (19). Note that IR and AraC also activate the tyrosine kinase c-Abl, which is not activated by UV (27, 28, 33), and cispasin activates the tyrosine kinase Abl (28), but not DNA-dependent protein kinase (34).

Because all the studied DNA-damaging treatments stimulate γ-PAK activity, γ-PAK activation may be a common event in the cellular response to DNA damage. In eukaryotic cells, all types of DNA damage result in delayed progression through the cell cycle, with the G2/M transition checkpoint playing a major role in preventing cell division (15, 17, 18). Both γ-PAK and X-PAK (a γ-PAK Xenopus homologue) inhibit G2/M transition and mitotic progression in frog oocytes and embryos (7, 35, 36). We propose that γ-PAK activation in response to IR and other DNA-damaging treatments is related to the cytostatic properties of the kinase and is part of the common cytostatic response activated by different types of DNA damage.

Currently γ-PAK is the only mammalian PAK that, like the yeast PAK homologue Ste20, has been related to cell cycle arrest (7, 37). α-PAK is not activated by IR or treatment with AraC, but it is activated by UV and cispasin. Thus, the two different members of the PAK family can be activated by speciﬁc DNA-damaging agents, but activation of α-PAK is not a general response to DNA damage, as is γ-PAK activation. In contrast to γ-PAK, JNK is activated primarily by UV.

This is the first time that members of the Ste20/PAK family of protein kinases have been shown to be involved in the cellular response to irradiation or treatment with DNA-damaging drugs. It is suggested that γ-PAK activation by IR and DNA damage is important in the cytostatic response elicited by these agents, as γ-PAK has been shown previously to induce cytostasis (7, 38). Additional studies are underway in our laboratory to determine the requirements for activation of γ-PAK in response to IR and to define the role of the protein kinase in the early response to DNA damage.