Phosphatidylinositol Ether Lipid Analogues That Inhibit AKT Also Independently Activate the Stress Kinase, p38α, through MKK3/6-independent and -dependent Mechanisms*

Received for publication, February 6, 2007, and in revised form, June 11, 2007 Published, JBC Papers in Press, July 13, 2007, DOI 10.1074/jbc.M701108200

Joell J. Gills†, S. Sianna Castillo†1, Chunyu Zhang‡, Pavel A. Petukhov§, Regan M. Memmott, Melinda Hollingshead, Noel Warfel, Jiahuai Han, Alan P. Kozikowski‡, and Phillip A. Dennis‡2

From the †Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois, Chicago, Illinois 60612; the ‡Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21701, and the §Department of Immunology, Scripps Research Institute, La Jolla, California 94080

Previously, we identified five active phosphatidylinositol ether lipid analogues (PIAs) that target the pleckstrin homology domain of Akt and selectively induce apoptosis in cancer cells with high levels of Akt activity. To examine specificity, PIAs were screened against a panel of 29 purified kinases. No kinase was inhibited, but one isoform of p38, p38α, was uniformly activated 2-fold. Molecular modeling of p38α revealed the presence of two regions that could interact with PIAs, one in the activation loop and a heretofore unappreciated region in the upper lobe that resembles a pleckstrin homology domain. In cells, two phases of activation were observed, an early phase that was independent of the upstream kinase MKK3/6 and inhibited by the p38 inhibitor SB203580 and a latter phase that was coincident with MKK3/6 activation. In short term xenograft experiments that employed immunohistochemistry and immunoblotting, PIA administration increased phosphorylation of p38 but not MKK3/6 in tumors in a statistically significant manner. Although PIAs rapidly activated p38 with similar time and dose dependence as Akt inhibition, p38 activation and Akt inhibition were independent events induced by PIAs. Using SB203580 or p38α−/− cells, we showed that p38α is not required for PIA-induced apoptosis but is required for H2O2- and anisomycin-induced apoptosis. Nonetheless, activation of p38α contributes to PIA-induced apoptosis, because reconstitution of p38α into p38α−/− cells increased apoptosis. These studies indicate that p38α is activated by PIAs through a novel mechanism and show that p38α activation contributes to PIA-induced cell death. Independent modulation of Akt and p38α could account for the profound cytotoxicity of PIAs.

Akt (or protein kinase B) is a serine/threonine kinase that regulates key cellular processes, such as transcription, proliferation, and survival, and is an emerging target in cancer (1). Developing methods to inhibit Akt has become a major effort within academic laboratories and the pharmaceutical industry. To date, most small molecule inhibitors of Akt have been identified through screening large compound libraries, which typically culminates in the isolation of compounds that target the ATP binding region of Akt (2). Other efforts, including our own, have focused on lipid-based inhibitors that do not target the ATP binding domain (3–5). We used molecular modeling to guide synthesis of phosphatidylinositol ether lipid analogues (PIAs) that were designed to inhibit the pleckstrin homology (PH) domain of Akt. PIAs rapidly inhibited Akt and selectively induced apoptosis in cell lines with high levels of constitutively active Akt (6). PIAs are broadly cytotoxic in cancer cells, their activity correlates with the presence of active Akt, and they are effective in vivo in a hollow fiber assay (7). Several observations in previous studies suggested that PIAs might have other targets in addition to Akt. First, in comparison with other inhibitors of the PI3K/Akt/mTOR pathway, such as LY294002 or rapamycin, PIAs equally inhibited Akt but induced more apoptosis than these compounds. Second, activation of members of the MAPK superfamily was observed with PIA administration (6). Third, a COMPARE analysis of PIAs using the NCI60 molecular targets and cancer screening data base revealed that other molecular targets correlated more highly with responsiveness to PIAs than phospho-Akt (7). The transition of PIAs (or any other approach targeting Akt) from preclinical compounds to useful therapeutics will depend in part upon identifying “off target” effects that could contribute to cellular responses.

Ascertainment of specificity not only provides a deeper understanding of the biologic activities of a given compound but also has practical implications for further development. Identification of off target effects could expand possible clinical applications or provide greater understanding of drug-associated toxicities, which is perhaps best illustrated by the experience with imatinib mesylate. Although it was designed to inhibit the pathogenic fusion protein Bcr/Abl in chronic

* This research was supported in part by the Intramural Research Program of the NCI, National Institutes of Health (NIH), Center for Cancer Research and in part with Federal funds from NCI, NIH, under Contract NO1-CO-12400. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† These two authors contributed equally to this work.

‡ To whom correspondence should be addressed: Bldg. 8, Rm. 5101, 8901 Wisconsin Ave., Bethesda, MD 20889. Tel.: 301-496-0929; Fax: 301-435-4345; E-mail: pdennis@nih.gov.

§ The abbreviations used are: PIA, phosphatidylinositol ether lipid analogue; PH, pleckstrin homology domain; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; NSCLC, non-small cell lung cancer; FBS, fetal bovine serum.
myelogenous leukemia and is approved for use in this disease, imatinib also inhibits PDGFR-b and c-Kit, which are highly relevant targets in other tumor types, such as gastrointestinal stromal tumors (8, 9). More recently, heart failure associated with use of imatinib in chronic myelogenous leukemia patients has been linked to inhibition of c-Abl in cardiac myocytes (10), thereby linking inhibition of the same target, Abl, to clinical efficacy and toxicity. Therefore, the identification of other biologic activities of PIAs could possibly extend future clinical applications to tumors that exhibit activities other than Akt activation. Moreover, the identification of deleterious off-target effects could facilitate choosing the best inhibitors of Akt that are most efficacious and least toxic.

The MAPK superfamily is composed of kinases that can be organized into three pathways that are either stimulated by growth stimuli or cellular stress. Each pathway has a prototypic MAPK and MAPK kinase or MKK (11). One of the pathways stimulated by stress is composed of MKK3 and MKK6, which are two closely related protein kinases, and their MAPK substrate, p38 MAPK (12). p38 is an important member of the MAPK superfamily and is activated in response to various cell stresses, such as changes in osmolarity, DNA damage, heat shock, ionizing radiation, inflammatory cytokines, and ischemia (13). Activation of the p38 pathway can induce a number of cellular responses, including alterations in transcription, the cell cycle, inflammation, and apoptosis. p38 exists as four isoforms (α, β, γ, and δ), all of which are normally activated by phosphorylation of the conserved Thr<sup>180</sup>-X-Tyr<sup>182</sup> residues by the upstream kinases MKK3 or MKK6 (14). Activation of p38 results in phosphorylation of many downstream substrates, such as MAPK-activated protein kinase 2 and ATF2, and the p38 signal can be further propagated to proteins such as hsp27 (15, 16), p38 activation has been observed in cancer cells following administration of different types of chemotherapy (17–19) and, in some cases, is a critical determinant of response to therapy (20).

In this study, we screened PIAs against a panel of purified kinases and show that active PIAs activate a single isoform of p38, p38α, in vitro and in vivo. p38α activation is independent of Akt inhibition and occurs directly through mechanisms probably based on structural features of p38α as well as indirectly through mechanisms involving MKK3/6. Although activation of p38α is not required, it does contribute to PIA-induced apoptosis. Because p38α activation occurs in cancer cells after chemotherapy and in normal cells during inflammatory processes, activation of p38α by PIAs could contribute to the efficacy and/or toxicity of PIAs.

**EXPERIMENTAL PROCEDURES**

**Materials**

The synthesis of the PIAs has previously been described (4). Phospho-p38 (Thr<sup>180</sup>/Tyr<sup>182</sup>), phospho-Akt (Ser<sup>473</sup>), phospho-ATF-2 (Thr<sup>23</sup>), phospho-ASK1 (Thr<sup>845</sup>), phospho-Hsp27 (Ser<sup>82</sup>), phospho-MAPK-activated protein kinase 2 (Thr<sup>334</sup>), phospho-MKK3/6 (Ser<sup>189</sup>/207), TAB1 and TAK1 antibodies, and the p38 kinase assay kit were purchased from Cell Signaling Technologies (Beverly, MA). α-Tubulin antibody was purchased from Sigma. SB203580 and LY294002 were from Calbiochem. Protease inhibitor mixture was obtained from Sigma, and protein assay materials were from Bio-Rad. All cell culture reagents were purchased from Invitrogen. Protran pure nitrocellulose membranes were purchased from Schleicher & Schuell. The TAB1 antibody used for immunoprecipitations and the FLAG-tagged p38α plasmid have been previously described (21).

**Methods**

**Cell Culture—**NSCLC lines were established at the NCI/Navy Medical Oncology. The p38α<sup>−/−</sup> mouse embryo fibroblasts were a kind gift of Dr. Michael Karin, University of California (San Diego). All cell lines were maintained in a 75-cm<sup>2</sup> flask in Dulbecco’s modified Eagle’s medium and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were incubated at 37 °C in a 7.0% CO<sub>2</sub> atmosphere.

**Molecular Modeling—**The ligand binding site of the PH domain of Akt (Protein Data Bank code 1H10 (22)) and a putative PIA binding site of p38 (Protein Data Bank code 1A9U (23)) were superimposed using the backbone atoms of Thr<sup>101</sup>-Asp<sup>103</sup> and Arg<sup>10</sup>-Trp<sup>19</sup> in p38 and Arg<sup>15</sup>-Arg<sup>32</sup> and Arg<sup>41</sup>-Pro<sup>41</sup> in Akt, respectively. The secondary structure of the proteins was rendered using Lithium 2.1 (Tripos Associates, St. Louis, MO). The putative binding sites were identified using the GRID algorithm available in the SiteID module in Sybyl 7.0. The GRID settings were set as default. The volumes of the grooves and cavities were estimated by Sybyl, and those smaller than 500 Å<sup>3</sup> (roughly the volume of the sugar part of the PIA molecule and a phosphate group) were discarded. The docking of PIA23 was performed using the FlexX module in Sybyl 7.0 (Tripos Associates). The FlexX settings were set as default. The top conformations of PIA23 are shown in Fig. 1, B and C. For clarity, the long lipophilic chain of PIA23 was truncated.

**Screening Purified Kinases—**Kinase assays were performed as previously described (24, 25).

**Pharmacological Treatments—**NSCLC cells were plated 2–2.5 × 10<sup>5</sup> cells/well in 6- or 12-well plates in Dulbecco’s modified Eagle’s medium containing 10% FBS and incubated for 24 h. The medium was then changed to Dulbecco’s modified Eagle’s medium with 0.1% FBS, and the cells were incubated overnight. Following overnight incubation, cells were treated with 10 μM PIAs dissolved in Me<sub>2</sub>SO for 2 h (immunoblotting/kinase assays), 18 h (immunoblotting experiments), or 24 h (apoptosis studies). In all experiments, Me<sub>2</sub>SO was added to control samples and had no effect on Akt activity. After incubation with PIAs, the cells were harvested for immunoblot analysis or for analysis of apoptosis as described below. For dose-response studies, NSCLC cells were plated at 2–2.5 × 10<sup>5</sup> cells/well in 6-well plates. After attachment, the medium was changed to Dulbecco’s modified Eagle’s medium containing 0.1% FBS overnight. Cells were treated with the indicated doses of PIAs for 2 h, and the cells were harvested for immunoblot analysis.

**Immunoblotting—**Cell extracts were prepared by washing cells with phosphate-buffered saline and adding 2× LSB supplemented with protease inhibitor mixture as described previ-
Nitrocellulose membranes were washed three times in wash mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na$_2$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin) was added to the cells for 10 min. Lysates were cleared and allowed to immunoprecipitate for 2–3 h at 4 °C with anti-p38 antibody. Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer. Kinase reaction was performed for 30 min at 30 °C in kinase buffer supplemented with 200 mM ATP and 1 mg of ATF2 fusion protein. Reactions were terminated with 3× SDS buffer. The samples were heated at 100 °C for 5 min and loaded onto a 12% SDS-polyacrylamide gel. Kinase assays were repeated at least three times.

**Analysis of p38 and M KK3/6 in Vivo—PC3 prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA). These were grown in RPMI 1640 plus 10% FBS. Male 6-week-old athymic nude mice (Athymic NCr-nu/nu) were inoculated subcutaneously (1 × 10$^7$ cells) in each rear flank. When tumors reached 200 mm$^3$, the mice were randomized into 10 groups of three mice each. PIA23 was dissolved in a vehicle of 10% Me$_2$SO in saline/Tween 80. The control group received intraperitoneal injections of the vehicle once a day, two times a day (every 12 h), three times a day (every 8 h), 4 times a day (every 6 h), or once a day for five consecutive days. The treatment groups received injections of 40 mg/kg PIA23 intraperitoneally on the same schedules. Four hours after the last dose, the mice were sacrificed, the tumors were removed, and half of each tumor was snap-frozen and half was fixed in 10% formalin. For immunoblotting, frozen tumors were thawed and homogenized in radioimmune precipitation buffer containing protease and phosphatase inhibitors. Protein was quantified with the DC protein assay (Bio-Rad). Densitometry was performed using NIH Image software. For immunohistochemistry, fixed tumors were paraffin-imbedded, sectioned, and placed on slides (Histoserv Inc., Gaithersburg, MD). Antigen retrieval was performed in target retrieval solution (Dako-Cytomation California, Inc., Carpinteria, CA). Phospho-p38 antibody (catalog number 4631; Cell Signaling) was used at a dilution of (1:50), and phospho-MKK3/6 antibody (catalog number 9231;
Cell Signaling) was used at a dilution of (1:100). Detection was performed by standard avidin-biotin complex methods.

**Immunoprecipitation**—H157 cells were treated with PIA5 for 0 min, 15 min, or 1h. To harvest, cells were washed with ice-cold phosphate-buffered saline and then scraped into lysis buffer (20 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Roche Applied Science), 25 mM NaF, 1 mM Na3VO4, and 25 mM β-glycerol phosphate. Cells were allowed to incubate on ice for 10 min and then centrifuged at 13,000 × g for 15 min at 4 °C to clear the lysate. TAB1 was immunoprecipitated from 500 mg of protein using 2 μl of TAB1 antibody and protein A/G PLUS-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Transient Transfections**—1 × 10^6 p38α−/− cells were nucleofected with 2 μg of p38α plasmid or pc3DNA vector using nucleofector technology (Amaxa, Cologne, Germany). Following nucleofection, 4 × 10^5 cells/well were plated in 6-well plates in RPMI plus 10% FBS. 72 h after nucleofection, the medium was removed and replaced with RPMI plus 0.1% FBS containing either Me2SO or 10 μM PIA5, followed by incubation and processing for Western blot or apoptosis assay.

**Apoptosis Assays**—Cells were treated with PIAs for 18–24 h as described above. Floating cells were collected, and adherent cells were harvested by trypsinization and then centrifuged at 1000 × g for 5 min. Cells were fixed in ice-cold 70% methanol added dropwise and then incubated at −20 °C for 30 min. Cells were centrifuged and incubated with propidium iodide (25 μg/ml) supplemented with RNase A (30 μg/ml) for 30 min at room temperature. Quantification of sub-2N DNA was determined by flow cytometry analysis using a BD Biosciences FACSsort and by manual gating using CellQuest software. Apoptosis experiments were performed in triplicate and were repeated at least three times.

**RESULTS**

PIAs Activate Purified p38α in Vitro—To examine the specificity of the PIAs, five active PIAs (PIA5, -6, -23, -24, and -25) and a negative control (PIA7, which is composed of only an ether lipid backbone and lacks the inositol head group) were screened against a panel of purified kinases (25) (Table 1). Individual PIAs caused inhibition of select kinases (such as SGK and protein kinase A), but the inhibition of these kinases was not uniform and was not investigated further, because all active
PIAs have similar cytotoxic properties in vitro (6). In contrast, the largest change in activity for all of the active PIAs was a 2-fold induction of activity in one isoform of p38 MAPK, p38α (SAPK2). An approximately 20% increase in p38β activity was also observed. Direct activation of p38α by phosphoinositides or analogues has not been described previously. Therefore, to examine whether there was a structural basis for activation of p38α by PIAs, molecular modeling was performed.

Molecular Modeling of p38α and Akt—PH domains are phosphoinositide-binding protein regulatory domains, and the PH domain of Akt was the intended target of PIAs (4). Molecular modeling of p38α revealed two regions that could possibly interact with PIAs (Fig. 1). Similarities were identified between the secondary and tertiary structures of the upper lobe of p38α and the PH domain of Akt. First, both proteins have a positively charged groove that is large enough to accommodate the PIAs. Second, the ligand binding site in Akt and the putative binding site in p38α are located between two pairs of antiparallel β-strands; the one pair is connected by a short β-hairpin loop, and the other is connected by a long β-hairpin loop (Fig. 1A). Specifically, two antiparallel β-strands β1 and β2 and the short loop β1/β2 (also known as VL1) in Akt match two antiparallel β-strands β1 and β2 and the short loop β1/β2 in p38α; the two β-strands β3 and β4 and the long loop β3/β4 (also known as VL2) in Akt match the β-strands β6 and β7 and the loop β6/β7 in p38α. In the case of Akt, the long hairpin loop β3/β4 is interrupted by a short α-helix. Third, the ligand binding site in Akt and the putative binding site in p38 contain amino acids (e.g. Arg, Asn, Tyr, and Lys) that are able to form strong electrostatic interactions with ligands such as phosphatidyl-inositol and PIAs (Fig. 1B). These data are consistent with the hypothesis that the upper lobe of p38α has a PH-like binding site/ regulatory domain that might mediate interaction with PIAs.

In addition to the putative PH-like domain, we identified another region of p38α in the activation loop near the ATP binding site at His174 that is predicted to interact with PIAs (Fig. 1A, A and C).

![Figure 2. PIAs induce p38 activation in an MKK3/6-independent and -dependent manner in intact cells. A, PIAs induce p38 phosphorylation and kinase activity in intact cells. H157 and PC3 cells were treated with the indicated PIAs (10 μM) for 2 h prior to immunoblotting for p38 phosphorylation at Thr180/Tyr182 (top panels). p38 kinase activity was assessed in H1703 cells after treatment with PIAs (10 μM) for 2 h prior to immunoprecipitation of p38 and performing in vitro kinase assays using an ATP2 peptide as the substrate. Fast green staining shows equal loading. B, phosphorylation of p38 induced by PIAs, but not H2O2, is inhibited by SB203580 and is independent of MKK3/6 at early time points. H157 cells were incubated with PIAs (10 μM) (top left panels) or H2O2 (300 μM) (top right panels) in the presence or absence of SB203580 (10 μM) for the indicated time periods prior to immunoblotting for phospho-MKK3/6, phospho-p38, phospho-hsp27, and total p38. H157 cells were also treated with PIAs (10 μM), H2O2 (300 μM), or LY294002 (10 μM) for the indicated time periods prior to immunoblotting with phospho-ASK1 and α-tubulin antibodies (bottom panels).](https://www.jbc.org/content/282/37/27024/F1)
observed with the combination of SB203580 and PIA5, which could have contributed to the increased phosphorylation of MKK3/6 (data not shown) (29). Therefore, at times at or beyond 1 h, PIAs cause p38 activation through activation of upstream kinases. In contrast, H$_2$O$_2$-induced p38 activation correlated with MKK3/6 activation at 15 min (Fig. 2B, right panels). Rapid, coincident induction of MKK3/6 and p38 phosphorylation by anisomycin was also observed (data not shown). These data show that activation of p38 by PIAs is different from that induced by H$_2$O$_2$ or anisomycin, because PIAs activate p38 independently of MKK3/6 at early time points. Moreover, they suggest that in intact cells PIAs induce p38 activation through direct (MKK3/6-independent) and indirect (MKK3/6-dependent) mechanisms.

To confirm that other proximal activators of p38 are not involved in PIA-induced p38 activation, we assessed the activation state of ASK1 (apoptosis signal-regulating kinase 1), a MAPK kinase that can phosphorylate MKK3 and MKK4 and promote stress-induced apoptosis (30) (Fig. 2B, lower panels). Although H$_2$O$_2$ treatment increased phosphorylation of ASK1 at T845 (a site that correlates with kinase activity) within 15 min (31), PIA5 did not increase ASK1 phosphorylation at any time point tested. Thus, PIA-induced activation of p38 does not involve activation of ASK1, even at time points when MKK3/6 are activated.

Because p38 activation at early time points did not seem to depend on upstream kinases, we studied whether PIAs could induce binding of TAB1 (transforming growth factor-β-activated protein kinase 1-binding protein 1) to p38, which has previously been shown to induce p38 autophosphorylation in an MKK3/6-independent manner (21). TAB1 was immunoprecipitated from control or PIA5-treated H157 cells, and although a known binding partner, Tak1, was associated with TAB1, p38 was not associated with TAB1, either in the absence or presence of PIA5 (data not shown). This indicates that PIA-induced p38 activation was not mediated through the binding of TAB1 to p38.

**Activation of p38 in Vivo**—To determine whether short term administration of PIAs could induce p38 activation in vivo, groups of nude mice bearing PC3 human prostate cancer xenografts were given intraperitoneal injections of vehicle or PIA23 (40 mg/kg) on varying schedules over 24 h (once, every 12 h × 2, every 8 h × 3, or every 6 h × 4), or once a day for 5 days. (PIA23 was chosen for these studies because of limited supply of other PIAs and because it had been most recently shown to have activity in short term hollow fiber assays (7).) Subse-

---

**PIAs Activate p38**

**Mechanisms of p38 Activation by PIAs**—Because the data from the purified kinase screen and molecular modeling suggested that PIAs could directly activate purified p38αε in the absence of other kinases, we assessed the p38 pathway at early time points in H1703 cells in the absence or presence of SB203580, an inhibitor of p38-mediated autophosphorylation and phosphorylation of downstream substrates (Fig. 2B, upper left panels). Within 10 min, PIA5 increased phosphorylation of p38 and hsp27 in the absence of MKK3/6 phosphorylation. Concurrent incubation with SB203580 completely inhibited PIA-induced p38 phosphorylation up to 1 h, a time during which MKK3/6 activation did not occur with PIA5 alone. This suggests that PIAs cause p38 activation through autophosphorylation at early times. Beginning at 1 h, MKK3/6-mediated phosphorylation was increased by PIA5 alone, and the inhibition of PIA-induced p38 phosphorylation by SB203580 was diminished. The fact that SB203580 increased MKK3/6 activation beyond that observed with PIA5 alone is consistent with earlier observations that a negative feedback loop exists between p38 and MKK3 such that direct inhibition of p38 increases MKK3 activation (28). In addition, increased phosphorylation of TAK-1 was
PIAs Activate p38

PIAs Activate p38 in a Dose- and Time-dependent Manner—Although PIA5, -6, -23, -24, or -25 activated p38 at a given dose (10 μM) and time (2 h), additional studies were conducted to determine the minimal dose required as well as the time course of p38 activation. When H1703 or H157 cells were treated with different concentrations of PIA5, -6, or -24 for 2 h, increased p38 phosphorylation was observed at concentrations of 5 or 10 μM (Fig. 4A). Correspondingly, these concentrations of PIAs also resulted in the inhibition of Akt phosphorylation in both cell lines. These results show that the dose dependence of PIAs for Akt inhibition and p38 activation is similar.

To determine the time course of p38 activation by PIAs, cells were treated with PIA5 or -6 for varying times, and changes in Akt phosphorylation and p38 phosphorylation were assessed (Fig. 4B). Although the results using purified p38α indicated that activation of p38α by PIAs was independent of Akt inhibition (Table 1), we included an inhibitor of the upstream PI3K, LY294002, for comparison. PIA5 or -6 activated p38 and inhibited Akt within 15 min in H1703 cells. Activation of p38 was maintained throughout the course of the experiment, with marked increases in phosphorylation of p38 present 18 h after administration. Similar rapid activation of p38 and inhibition of Akt was observed with H157 cells. In contrast to the dual activities of PIAs, LY294002 did not increase p38 phosphorylation in either cell line, despite rapid and sustained inhibition of Akt. Taken together, these experiments show that PIAs activate p38 within minutes at concentrations in the low micromolar range.

FIGURE 4. PIA-induced p38 activation is independent of Akt inhibition. A, similar dose dependence of Akt inhibition and p38 activation by PIAs. H1703 or H157 cells were treated with PIAs (10 μM) for 2 h prior to immunoblotting for levels of phosphorylated and total Akt and p38. Akt phosphorylation and p38 phosphorylation exhibit an inverse relationship. B, similar time dependence of Akt inhibition and p38 activation by PIAs. Akt and p38 phosphorylation were assessed by immunoblotting in the absence or presence of 10 μM PIA5, PIA6, PIA7, or LY294002 in H1703 and H157 cells. C, p38 activation does not depend upon Akt inhibition. H157 cells were pretreated or not with the PI3K inhibitor LY294002 for 30 min and then incubated with either 10 μM PIA5 or 10 μM LY294002 for an additional 30 min or 2 h. Immunoblotting was performed as above. D, Akt inhibition does not depend upon p38 activation. Wild type (WT) or p38α−/− cells were treated with 10 μM PIA5 for the indicated times, and immunoblotting was performed for phosphorylated and total levels of Akt and p38. Immunoblotting experiments were repeated three times.

Pammently, the mice were sacrificed, and activation of p38 and MKK3/6 in the tumors was assessed using immunohistochemistry and immunoblotting. Immunohistochemistry for phospho-p38 and phospho-MKK3/6 showed that PC3 cells exhibit a basal level of cytoplasmic and nuclear staining for each epitope (Fig. 3). Treatment with PIA23 increased the intensity of staining for phospho-p38 and phospho-MKK3/6 in each subcellular compartment. Immunoblotting was performed to quantify differences in active p38 and MKK3/6. Levels of phospho-p38 or phospho-MKK3/6 varied within each group, but when vehicle-treated tumors and PIA23-treated tumors were compared for each treatment schedule, a trend toward increased p38 and MKK3/6 activation was noted in the PIA-treated tumors. Maximal induction of MKK3/6 activation occurred in every 6 h × 4 group, and maximal induction of p38 activation occurred in the 8 h × 3 group. When all treatment schedules were combined and compared against all control animals, a statistically significant increase in p38 activation was observed with PIA treatment. A trend toward increased activation of MKK3/6 was also observed. These data suggest a correlation between frequency of dosing of PIAs and increased phosphorylation of p38 and MKK3/6 in tumors and that p38 could serve as a biomarker to monitor PIA administration in vivo.
PIAs Activate p38

Activation of p38 and Inhibition of Akt Are Independent Activities of PIAs—To confirm the independence of Akt inhibition and p38 activation in intact cells, H157 cells were pretreated with LY294002 to inhibit Akt activation, and then PIA5 was added to activate p38α (Fig. 4C). Pretreatment with LY294002 completely inhibited Akt phosphorylation but did not increase p38 phosphorylation by itself and did not inhibit PIA-induced p38 activation. These studies show that PIA-induced p38 activation is not a consequence of Akt inhibition. To demonstrate the converse, that Akt inhibition does not depend on p38 activation, we employed mouse embryo fibroblasts that were derived from p38α−/− mice (32). p38α−/− cells or p38α wild type cells were treated with PIA5 for various times, and Akt inhibition was assessed (Fig. 4D). PIA5 decreased Akt phosphorylation in both cell types, with more rapid inhibition observed in the p38α−/− cells. As expected, p38α activation was only observed in the p38α wild type cells. Akt inhibition therefore did not depend upon p38α activation. These studies establish that activation of p38 and inhibition of Akt are two distinct activities of PIAs.

Activation and Propagation of the p38 Signal—To assess propagation of the p38 signal after PIA administration, we compared the phosphorylation of p38 pathway components in H157 and H1703 cells after administration of PIAs or two different types of cellular stressors, H2O2 or anisomycin. Treatment of each cell line for 2 h with PIA5, PIA6, or anisomycin (but not PIA7) increased phosphorylation of the upstream kinases, MKK3/6, as well as p38 itself and the p38 downstream substrates MAPK-activated protein kinase 2, hsp27, and ATF2. Apoptosis assays were repeated three times. H157 cells were pretreated with 10 μM SB203580 for 30 min, followed by PIA6 (10 μM) or H2O2 (300 μM) for 24 h, and the percentage of cells with sub-2N DNA content was measured by propidium iodide staining and flow cytometry, as described under “Experimental Procedures.” C, PIAs induce apoptosis and decrease Akt phosphorylation in p38α−/− cells. p38α−/− cells were treated with PIAs (10 μM) for 24 h, and the percentage of apoptotic cells was analyzed by flow cytometry, as described under “Materials and Methods.” Apoptosis assays were repeated three times. D, PIA treatment, but not H2O2 or anisomycin treatment, induces morphologic changes in p38α−/− cells. Cells were treated for 24 h. Representative photomicrographs are shown. E, PIA6, but not H2O2, or anisomycin, increases apoptosis and decreases Akt phosphorylation in p38α−/− cells. p38α−/− cells were treated with 10 μM PIA6, 300 μM H2O2, or 100 ng/ml anisomycin for 24 h, and parallel samples were processed for apoptosis and immunoblotting. Immunoblotting was performed for activation of components of the p38 pathway and Akt. F, reconstituting wild type p38α into p38α−/− cells increases PIA-induced apoptosis. p38α−/− cells were transfected with vector or a wild type p38α plasmid as described under “Experimental Procedures.” After 72 h, cells were treated with Me2SO (C) or 10 μM PIA5 for 12 h, and the amount of apoptosis was measured. Transfection experiments were repeated three times.

FIGURE 5. p38α is not necessary but contributes to PIA-induced apoptosis. A, activation of the p38 pathway in NSCLC cell lines. H157 or H1703 cells were treated with PIAs (10 μM), H2O2 (300 μM), or anisomycin (100 ng/ml) for 2 h, and immunoblotting was performed as described under “Experimental Procedures.” Phosphorylation of the upstream kinase MKK3/6, p38 itself, and three downstream substrates (MAPK-activated protein kinase 2, hsp27, and ATF2) are shown. α-Tubulin is included as a loading control. B, SB203580 inhibits H2O2-induced apoptosis but not PIA-induced apoptosis. H157 cells were pretreated with 10 μM SB203580 for 30 min, followed by PIA6 (10 μM) or H2O2 (300 μM) for 24 h, and the percentage of cells with sub-2N DNA content was measured by propidium iodide staining and flow cytometry, as described under “Experimental Procedures.” C, PIAs induce apoptosis and decrease Akt phosphorylation in p38α−/− cells. p38α−/− cells were treated with PIAs (10 μM) for 24 h, and the percentage of apoptotic cells was analyzed by flow cytometry, as described under “Materials and Methods.” Apoptosis assays were repeated three times. D, PIA treatment, but not H2O2 or anisomycin treatment, induces morphologic changes in p38α−/− cells. Cells were treated for 24 h. Representative photomicrographs are shown. E, PIA6, but not H2O2, or anisomycin, increases apoptosis and decreases Akt phosphorylation in p38α−/− cells. p38α−/− cells were treated with 10 μM PIA6, 300 μM H2O2, or 100 ng/ml anisomycin for 24 h, and parallel samples were processed for apoptosis and immunoblotting. Immunoblotting was performed for activation of components of the p38 pathway and Akt. F, reconstituting wild type p38α into p38α−/− cells increases PIA-induced apoptosis. p38α−/− cells were transfected with vector or a wild type p38α plasmid as described under “Experimental Procedures.” After 72 h, cells were treated with Me2SO (C) or 10 μM PIA5 for 12 h, and the amount of apoptosis was measured. Transfection experiments were repeated three times.
PIAs Activate p38

results suggest that p38 activation is not required for PIA-induced apoptosis.

To extend these studies, we employed mouse embryo fibroblasts that were derived from p38α−/− mice (32). p38α−/− cells were treated for 18 h with PIA5, -6, or -25 as well as with the PIA that lacks an inositol ring, PIA7. As shown in Fig. 5C, PIA5, -6, or -25 induced apoptosis in 40–50% of p38α−/− cells after 18 h, which is comparable with what had been previously observed in NSCLC and breast cancer cells (6). In parallel samples, we performed immunoblot analyses to confirm inhibition of Akt phosphorylation by PIA treatment as well as the absence of p38 protein. Treatment of the p38α−/− cells for 2 h with PIA5, -6, or -25 decreased phosphorylation of Akt, thereby correlating induction of apoptosis with inhibition of Akt. p38 protein and p38 phosphorylation were not detected. (Very long exposures of the total p38 blot revealed a low signal, indicating the presence of other p38 isoforms (data not shown).) These experiments confirm that p38α activation is not required for the induction of apoptosis by PIAs.

Because SB203580 inhibited apoptosis caused by H2O2 or anisomycin in NSCLC cells (anisomycin data not shown), we compared the response of p38α−/− cells to PIA6, H2O2, or anisomycin. Within hours, PIA6 induced profound morphological changes in p38α−/− cells, whereas H2O2- or anisomycin-treated cells showed no morphological changes (Fig. 5D). When apoptosis was measured at 24 h, PIA6 induced apoptosis in 68% of p38α−/− cells (Fig. 5E). Induction of apoptosis by H2O2 or anisomycin was negligible in p38α−/− cells. PIA6-induced apoptosis correlated with decreased activation of Akt, which was not observed with H2O2 or anisomycin. The PI3K inhibitor LY294002 also caused inhibition of Akt phosphorylation, yet it failed to cause apoptosis in these cells. Together, these data show that H2O2- or anisomycin-induced apoptosis is p38α-dependent, whereas PIA-induced apoptosis is not.

p38α Activation Contributes to PIA-induced Cell Death—Although p38α activation is not required for PIA-induced apoptosis, activation of p38α could still play a role in the cellular response to PIAs by promoting or inhibiting PIA-induced cytotoxicity. To determine whether p38 activation contributes to PIA-induced cell death, we reconstituted wild type p38α into p38α−/− cells and assessed the response to PIA5 (Fig. 5F). In vector-transfected p38α−/− cells, PIA5 caused 45% of the p38α−/− cells to undergo apoptosis at 12 h, which was similar to the level of apoptosis observed when untransfected wild type mouse embryo fibroblasts were treated with PIA5 (data not shown). When p38α was reconstituted into p38α−/− cells, PIA5 administration nearly doubled the amount of apoptosis to ~84% (p < 0.0001). Increased apoptosis correlated with p38α activation, because PIA5 increased the phosphorylation of the exogenously introduced wild type p38α (data not shown). These experiments demonstrate that whereas p38 activation is not necessary for apoptosis, p38α activation contributes to PIA-induced apoptosis.

Discussion

The usefulness of a targeted agent will depend upon modulation of its desired target as well as other unintended off target effects. Previously, we showed that PIAs inhibit activation and translocation of their desired target, the serine/threonine kinase Akt (6). In this study, we report that PIAs are equipotent, independent activators of p38. In a screen of purified kinases, PIAs selectively activated a single member of the MAPK superfamily, p38α, independently of MKK3/6.

Activation of p38α by MKK3/6-independent mechanisms has been reported previously. Ge et al. (21) showed that TAB1 (transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein 1) can directly bind to p38α and cause p38α autophosphorylation. TAB1 does not appear to mediate PIA-induced p38 activation in our studies, because we saw no evidence of association of TAB1 with p38 in the absence or presence of an active PIA. Salvador et al. have shown that in T cells, initial phosphorylation of p38α at Tyr323 by Zap70 leads to p38 autophosphorylation (33). Since we employed different cell types (epithelia-derived cancer cell lines) and PIAs caused ex vivo activation of p38α in the absence of Zap70 or TAB1, it is likely that PIAs induce p38α activation in a novel manner that might be related to disruption of key residues that maintain the kinase in an inactive conformation, as described by Diskin et al. (34).

In their studies, Diskin et al. (34) designed and constructed active mutants of p38 based on activating mutations found in yeast p38/Hog1. These authors found that mutating Asp176 or Phe327 in p38α (either single or double mutants) led to kinase activation through autophosphorylation. The high resolution crystal structure of these active p38 mutants suggested that the L16 loop of p38α acts as a molecular switch (35). In wild type p38, the L16 loop is normally stabilized by a hydrophobic core consisting of Trp327 in the L16 helix, Phe327 in the L16 loop, and Tyr69 in the C-terminal helix, and these interactions maintain a low basal activity of p38. In the Phe327 mutant, the L16 loop is displaced by 1–1.5 Å, and p38 becomes phosphorylated on Thr180.

This region in p38 is relevant to our modeling studies that identified two regions within p38α that could bind PIAs. One region around His174 corresponds to the hydrophobic core region around the L16 loop identified by Diskin et al. (34). Our modeling identified His174, Tyr200, Arg149, Glu328, Arg70, and Gln325 as PIAs-interacting residues in this putative binding site. Four of these residues nearly overlap the key residues identified by Diskin et al. (Asp176 in the activation loop and Trp327, Tyr69, and Phe327 in the hydrophobic core). Therefore, it is possible that PIAs interact with p38 in this region and disrupt the molecular interactions that stabilize the L16 loop, which then could lead to activation of p38 through autophosphorylation. The other region that was identified in our modeling studies is structurally similar to the PH domain of Akt. Such a region in p38 has not been reported previously. Whether this PH-like domain functions as a typical PH domain and controls translocation of p38α in response to phosphoinositides is unknown. Although mechanisms related to conformational changes induced by PIAs probably occur within cells because PIAs induce p38 activation independently of MKK3/6 at early time points and this activation was inhibited by SB203580, these mechanisms are either lost or obscured by MKK3/6-mediated activation of p38 at later time points.

Despite similar kinetics and dose dependence for Akt inhibition and p38 activation by PIAs, activation of p38 was not observed with other inhibitors of the Akt pathway, such as the PI3K inhibi-
PIAs Activate p38

1. Vivanco, I., and Sawyers, C. L. (2002) Nat. Rev. Cancer 2, 489–501
2. Yang, L., Dan, H. C., Sun, M., Liu, Q., Sun, X. M., Feldman, R. I., Hamilton, A. D., Polokoff, M., Nicosia, S. V., Herlyn, M., Sebti, S. M., and Cheng, J. Q. (2004) Cancer Res. 64, 4394–4399
3. Meuillet, E. J., Mahadevan, D., Vankayalapati, H., Berggren, M., Williams, R., Coon, A., Kozikowski, A. P., and Powis, G. (2003) Mol. Cancer Ther. 2, 389–399
4. Kozikowski, A. P., Sun, H., Brognard, J., and Dennis, P. A. (2003) J. Am. Chem. Soc. 125, 1144–1145
5. Meuillet, E. J., Ikle, N., Baker, A. F., Bard, J. M., Stamper, C., Williams, R., Coon, A., Mahadevan, D., George, B. L., Kirkpatrick, L., and Powis, G. (2004) Oncol. Rep. 14, 513–527
6. Castillo, S. S., Brognard, J., Petukhov, P. A., Zhang, C., Tsurutani, J., Granville, C. A., Li, M., Jung, M., West, K. A., Gills, J. G., Kozikowski, A. P., and Dennis, P. A. (2004) Cancer Res. 64, 2782–2792
7. Gills, J. J., Holbeck, S., Hollingshead, M., Hewitt, S. M., Kozikowski, A. P., and Dennis, P. A. (2006) Mol. Cancer Ther. 5, 713–722
8. Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A. (2002) Nat. Rev. Drug Discov. 1, 493–502
9. Heinrich, M. C., Corless, C. L., Demetri, G. D., Blanke, C. D., von Mehren, M., Joensuu, H., McGreevey, L. S., Chen, C. J., Van den Abbeele, A. D., Druker, B. J., Kiese, B., Eisenberg, B., Roberts, P. J., Singer, S., Fletcher, C. D., Silberman, S., Dimitrijevic, S., and Fletcher, J. A. (2003) J. Clin. Oncol. 21, 4342–4349
10. Kerkela, R., Gazzette, L., Yacobi, R., Iliescu, C., Patten, R., Beahm, C., Walters, B., Shvetsov, S., Pesant, S., Clubb, F. J., Rosenzweig, A., Salmon, R. N., Van Etten, R. A., Alroy, J., Durand, J. B., and Force, T. (2006) Nat. Med. 12, 908–916
11. Raman, M., and Cobb, M. H. (2003) Curr. Biol. 13, R886–R888
12. Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–685
13. Johnson, G. L., and Lapadat, R. (2002) Science 298, 1911–1912
14. New, L., and Han, J. (1998) Trends Cardiovasc. Med. 8, 220–228
15. Zarubin, T., and Han, J. (2005) Cell Res. 15, 11–18
16. Rouse, J., Cohen, P., Trigdon, S., Morange, M., Alonso-Llamazaeres, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) Cell 78, 1027–1037
17. Olson, J. M., and Hallahan, A. R. (2004) Trends Mol. Med. 10, 125–129
18. Losa, J. H., Cobb, C. P., Viniegra, J. G., Sanchez-Arevalo Lobo, V. J., Ramon y Cajal, S., and Sanchez-Prieto, R. (2003) Oncogene 22, 3998–4006
19. Habiro, A., Tanno, S., Koizumi, K., Izawa, T., Nakano, Y., Osanai, M., Mizukami, Y., Okamura, T., and Kohgo, Y. (2004) Biochem. Biophys. Res. Commun. 316, 71–77
20. Varfol, N. A., Lepper, E. R., Zhang, C., Figg, W. D., and Dennis, P. A. (2006) Clin. Cancer Res. 12, 3502–3509
21. Ge, B., Gram, H., Di Padova, F., Huang, B., New, L., Ulevitch, R. J., Luo, Y., and Han, J. (2002) Science 295, 1291–1294
22. Thomas, C. C., Deak, M., Alessi, D. R., and van Aalten, D. M. (2002) Curr. Biol. 12, 1256–1262
23. Wang, Z., Canagarajah, B. J., Boehm, J. C., Kassisa, S., Cobb, M. H., Young, P. R., Abdel-Meguid, S., Adams, J. L., and Goldsmith, E. J. (1998) Structure 6, 1117–1128
24. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Biochem. J. 351, 95–105
25. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) Biochem. J. 371, 199–204
26. Canman, C. E., Wolff, A. C., Chen, C. Y., Fornace, A. J., Jr., and Kastan, M. B. (1994) Cancer Res. 54, 5054–5058
27. Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992) Cell 71, 587–597
28. Xiao, Y. Q., Malcolm, K., Worthen, G. S., Gardai, S., Schiemann, W. P., Fadok, V. A., Bratton, D. L., and Henson, P. M. (2002) J. Biol. Chem. 277, 14884–14893
29. Cheung, P. C., Campbell, D. G., Nebreda, A. R., and Cohen, P. (2003) EMBO J. 22, 5793–5805
30. Matsuzaawa, A., and Ichijo, H. (2001) J. Biochem. (Tokyo) 130, 1–8
31. Tobiume, K., Matsuzaawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) EMBO Rep. 2, 222–228
32. Tamura, K., Sudo, T., Sentfleben, U., Dadak, A. M., Johnson, R., and Karin, M. (2000) Cell 102, 221–231
33. Salvador J. M., Mittelstadt, P. R., Gusczynski, T., Copeland, T. D., Yamaguchi, H., Appella, E., Fornace, A. J., Jr., and Ashwell, J. D. (2005) Nat. Immunol. 6, 390–395
34. Diskin, R., Askari, N., Capone, R., Engelberg, D., and Livnah, O. (2004) J. Biol. Chem. 279, 47040–47049
35. Diskin, R., Lebendiker, M., Engelberg, D., and Livnah, O. (2007) J. Mol. Biol., 365, 66–76
36. Gratton, J. P., Morales-Ruiz, M., Kureishi, N., Ashwell, J. D., and Fornace, A. J., Jr. (2006) Trends Mol. Med. 12, 713–722
37. Gillis, J. J., Holbeck, S., Hollingshead, M., Hewitt, S. M., Kozikowski, A. P., and Dennis, P. A. (2006) Mol. Cancer Ther. 5, 713–722
38. Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A. (2002) Nat. Rev. Drug Discov. 1, 493–502

Acknowledgments—We thank Jenny Bain for technical assistance and Drs. Philip Cohen and Dario Alessi for helpful discussions. We are grateful to members of the Dennis laboratory for critical reading of the manuscript.

REFERENCES