Selective Activation of p38α and p38γ by Hypoxia

ROLE IN REGULATION OF CYCLIN D1 BY HYPOXIA IN PC12 CELLS*

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P. William Conrad, Randall T. Rust, Jiahuai Han‡, David E. Millhorn, and Dana Beitner-Johnson§

From the Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267-0576 and the §Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Hypoxic/ischemic trauma is a primary factor in the pathogenesis of a multitude of disease states. The effects of hypoxia on the stress- and mitogen-activated protein kinase signaling pathways were studied in PC12 cells. Exposure to moderate hypoxia (5% O2) progressively stimulated phosphorylation and activation of p38γ in particular, and also p38α, two stress-activated protein kinases. In contrast, hypoxia had no effect on enzyme activity of p38β, p38γ2, p38δ, or on c-Jun N-terminal kinase, another stress-activated protein kinase. Prolonged hypoxia also induced phosphorylation and activation of p42/p44 mitogen-activated protein kinase, although this activation was modest compared with nerve growth factor- and ultraviolet light-induced activation. Hypoxia also dramatically down-regulated immunoreactivity of cyclin D1, a gene that is known to be regulated negatively by p38 at the level of gene expression (Lavoie, J. N., L’Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) J. Biol. Chem. 271, 20608–20616). This effect was partially blocked by SB203580, an inhibitor of p38α but not p38γ. Overexpression of a kinase-inactive form of p38γ was also able to reverse in part the effect of hypoxia on cyclin D1 levels, suggesting that p38α and p38γ converge to regulate cyclin D1 during hypoxia. These studies demonstrate that an extremely typical physiological stress (hypoxia) causes selective activation of specific p38 signaling elements, and they also identify a downstream target of these pathways.

Mammalian cell function is critically dependent on a continuous supply of oxygen. Even brief periods of oxygen deprivation (hypoxia/ischemia) can result in profound cellular and tissue damage. Thus, it is vital that organisms meet changes in O2 tension with appropriate cellular adaptations; however, the specific intracellular pathways by which this occurs are not well delineated. The stress- and mitogen-activated protein kinases (SAPK1 and MAPK) pathways play a critical role in responding to cellular stress and promoting cell growth and survival (1, 2). We therefore investigated the effect of hypoxia on the SAPK and MAPK signaling pathways.

SAPKs and MAPKs are the downstream components of three-member protein kinase modules (3). Five homologous subfamilies of these kinases have been identified, and the three major families include p38/SAPK2/RK, JNK/SAPK, and p42/p44 MAPKs/ERKs (1–6). In general, the stress-activated protein kinases (p38 and JNK) are activated primarily by noxious environmental stimuli such as ultraviolet light, osmotic stress, inflammatory cytokines, and inhibition of protein synthesis (7–10). However, increasing evidence suggests that, at least under certain conditions, these pathways can also be activated by mitogenic and neurotrophic factors (11, 12). In contrast, p42/p44 MAP kinases are stimulated primarily by mitogenic and differentiative factors in a Ras-dependent manner (5, 13, 14), although these enzymes can also be activated by certain environmental stressors (1–3). Thus, we hypothesized that hypoxia, a prevalent physiological stressor in many disease states, may regulate the activity of the SAPK and MAPK signaling pathways.

The pheochromocytoma cell line PC12 is a catecholaminergic, excitable cell type that has been used widely as an in vitro model for neural cells (15). Upon prolonged exposure to nerve growth factor (NGF), PC12 cells decrease proliferation and extend neurite-like processes (15). It has also been shown that PC12 cells are an O2-sensitive cell type that provides a useful system to study the effects of hypoxia on catecholaminergic gene expression (16–18). PC12 cells are exquisitely sensitive to hypoxia in that very small reductions in atmospheric O2 (from 21 to 15% O2) dramatically induce tyrosine hydroxylase gene expression and mRNA stability (16, 17). Hypoxia also induces activation of the cAMP response element-binding protein (CREB) and c-fos transcription factors in this cell type (17, 20, 21). In addition, PC12 cells tolerate moderate hypoxia well in that they maintain greater than 95% cell viability for up to 72 h of exposure to hypoxia (5% O2, N2). Finally, PC12 cells also express hypoxia-regulated ion channels, as shown by the finding that PC12 cells depolarize during hypoxia via an oxygen-regulated K+ current (23, 24) and secrete dopamine and norepinephrine (25, 26). Thus, this cell type is an ideal system in which to study regulation of intracellular signaling systems by hypoxia.

In the current studies, we have used this cell line to investigate the effect of hypoxia on the SAPK and MAPK signaling pathways. We show that hypoxia selectively activates the p38γ and p38α isoforms of the p38 pathway in this cell type. The p38γ subtype in particular is most strongly targeted by hypoxia. Furthermore, we identify cyclin D1, a gene that has been

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‡ To whom correspondence should be addressed: Dept. of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, P.O. Box 67-0576, Cincinnati, OH 45267-0576. Tel.: 513-558-6099; Fax: 513-558-5738; E-mail: dana.johnson@uc.edu.

§ To whom correspondence should be addressed: Dept. of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, P.O. Box 67-0576, Cincinnati, OH 45267-0576. Tel.: 513-558-6099; Fax: 513-558-5738; E-mail: dana.johnson@uc.edu.

1 The abbreviations used are: SAPK, stress activated protein kinase; MAPK, mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropane-sulfonic acid; PDZ, PSD-95, Discs-Large, ZO-1; RK, reactivating kinase; MAPKAP, mitogen-activated protein kinase activated protein.
shown previously to be regulated by p38 (27), as a downstream target of hypoxia-induced activation of both p38 and p38.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—**SB203580 and NFG were obtained from Calbiochem. Anisomycin, sorbitol, and anti-FLAG M2 antibody were obtained from Sigma. Anti-p38 (C-20), anti-JNK1 (C-17), and anti-ERK2 (C-14) antibodies, protein G-coupled agarose for immunoprecipitations, and anti-cyclin D1 (C-20) antibodies for Western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A-coupled Sepharose was obtained from Amersham Pharmacia Biotech. MAPKAP kinase-2 assay kits and myelin basic protein were from Upstate Biotechnology, Inc. (Lake Placid, NY), and c-Jun (1–79) was from Santa Cruz Biotechnology. Phospho- and total p38 and phospho- and total p42/p44 MAPK antibodies were obtained from New England Biolabs (Beverly, MA). All cell culture media and reagents were obtained from Life Technologies, Inc.

**Cell Culture—**PC12 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 20 mM HEPES (pH 7.4), 10% fetal bovine serum, and with penicillin (100 units/ml) and streptomycin (100 μg/ml). Prior to experimentation, cells were grown to approximately 85% confluence in 35- or 60-mm tissue culture dishes (Corning). Cells were then transfected with p38 or control plasmid. Hypoxia was achieved by exposing cells to 5% O₂, 5% CO₂, balanced with N₂ for various times in an O₂-regulated incubator (Forma Scientific, Marietta, OH). In previous studies, we have shown that the partial pressure of O₂ in the media of cells exposed to 5% O₂ is in the range of 50–55 mm Hg (16).

Stable PC12 cell lines were created by transfecting cells with either FLAG-tagged p38ΔYAP in pCDNA3 (28) or the empty pCDNA3 vector, using Trans-Fast reagent (Promega, Madison, WI), at a charge ratio of 1:1, according to the manufacturer's recommended conditions. Individual clones expressing the kinase-inactive form of p38δ (p38δ-YAP) were selected in the presence of G418 (0.4 mg/ml). Clones were screened for p38δ-YAP expression by immunoblotting whole cell lysates with an anti-FLAG antibody, as described below.

Western Blotting—Whole cell exposure to hypoxia, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested by adding 0.2 ml/35-mm dish of a lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 0.2 mM sodium vanadate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Lysates were sonicated for 1 s with a microultrasonic cell disruptor (Contes, Vineland, NJ) and then centrifuged for 10 min at 14,000 × g at 4 °C to remove the Triton-insoluble fraction. Total protein concentration was determined by the method of Bradford (Bio-Rad), and gel samples were prepared by adding sample buffer containing final concentrations of 50 mM Tris (pH 6.7), 2% SDS, 2% β-mercaptoethanol, and bromphenol blue as a marker. Gel samples were boiled for 2 min, and then 20–100 μg of protein was loaded on 7.5% or 9% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) using standard electroblotting procedures. Nitrocellulose membranes were blocked with 5% nonfat dry milk or 5% bovine serum albumin, for phosphotyrosine immunoblots. Blocking solutions were prepared in a buffer containing 10 mM sodium phosphate (pH 7.2), 140 mM NaCl, and 0.1% Tween 20 (PBST).

Blots were immunolabeled overnight at 4 °C with antibodies recognizing the dual phosphorylation motif of Thr180 and Tyr182 of p38 and Thr180 and Tyr182 of p38 (1:500) or with an antibody that equally recognizes phospho-and dephospho-p38 (1:3,000). The phosphorylation state of p42/p44 MAPK was evaluated using an antibody that specifically recognizes phospho-Tyr204 MAPK (1:1,000) or an antibody that equally recognizes phospho- and dephospho-MAPK (1:1,000). Cyclin D1 expression was analyzed using an anti-cyclin D1 antibody (1:2,500). FLAG-tagged p38 protein kinases were detected with an anti-FLAG M2 monoclonal antibody (1:500). Immunoblots were washed in several changes of PBST at room temperature and then incubated with anti-rabbit Ig linked to horseradish peroxidase or, for FLAG and cyclin D1, an anti-mouse Ig linked to horseradish peroxidase (Amersham Pharmacia Biotech). Immunoreactivity was detected with enhanced chemiluminescence (Amersham Pharmacia Biotech). Immunoreactivity was detected with enhanced chemiluminescence (Amersham Pharmacia Biotech). Immunoreactivity was detected with enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Flow Cytometry—** Flow cytometry was performed as described previously (31). PC12 cells were grown to approximately 70% confluence on 35-mm tissue culture dishes. After normoxic or hypoxic treatment for 24 h, cells were harvested by adding 150 μl of 0.05% trypsin, 1 ml of a solution containing 10% fetal bovine serum in PBS was added to quench the trypsin. Cells were then centrifuged and resuspended in 100 μl of a freezing buffer containing 250 mM sucrose, 5% dimethyl sulfoxide, and 40 mM sodium citrate. Cells were stored at –80 °C until preparation for flow cytometry. 50 μl from each cell sample was aliquoted and then lysed by the addition of 30 μl of a solution containing 0.5% Nonidet P-40 and 0.5 mM EDTA in PBS. 1 μl of RNase (10 mg/ml), Giaggen, Santa Clarita, CA) was also added, and the cell mixture was then rocked for 15 min at room temperature. The samples from each tube were added to 1 ml of a solution containing 50 μg/ml propidium iodide in PBS. Samples were analyzed on a Coulter Epics XL (Beckman-Coulter Co., Miami, FL) and analyzed using a WinCycle software package (Phoenix Flow Systems, San Diego).

**RESULTS**

Hypoxia is an extremely common physiological stressor. To investigate the effects of hypoxia on the stress- and mitogen-activated signaling pathways, PC12 cells were exposed to 5% O₂ for various times, between 20 min and 6 h. Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and then immunoblotted with an antibody specific for Thr180 and Tyr182–phosphorylated p38α. Phosphorylated at these sites is both necessary and sufficient for enzymatic activation of p38α (5). It can be seen in Fig. 1A that exposure to hypoxia progressively induced phospho-p38α immunoreactivity in two closely migrating bands. Phospho-p38α blots were then stripped and reblotted with an antibody that recognizes phospho- and dephospho-p38α (i.e. total p38α). Fig. 1B shows that the lower phospho-p38α immunoreactive protein shown in Fig.
Illustrating the effect of hypoxia on phospho-p38 activity was quantified by densitometry (*p < 0.01, by χ² test).

Phosphorylation of p38 activity (data not shown). Phosphorylation of p38 occurred at 6 h, where there was an average 4.5-fold increase in p38 phosphoimmunoreactivity (Fig. 1C). The upper phospho-p38 immunoreactive band was identified as the p38α isoform, as described below. Phosphoimmunoreactivity of p38γ was increased more strongly by hypoxia, with an average of 12.7-fold increase over control levels by a 6-h exposure to hypoxia (Fig. 1C). These results suggest that both p38α and p38γ are activated by hypoxia. Phosphorylation of p38α and p38γ declined somewhat but was still elevated above control levels up to 24-h exposure to hypoxia (data not shown).

The upper phospho-p38 immunoreactive band shown in Fig. 1A corresponded to p38α, as determined by alignment of films using luminescent markers affixed directly to the blot. As shown in Fig. 1B, hypoxia did not alter the total amount of p38α protein. Of the time points examined, maximal hypoxia-induced phosphorylation of p38α occurred at 6 h, where there was an average 4.5-fold increase in p38α phosphoimmunoreactivity (Fig. 1C). The upper phospho-p38 immunoreactive band was identified as the p38γ isoform, as described below. Phosphoimmunoreactivity of p38γ was increased more strongly by hypoxia, with an average of 12.7-fold increase over control levels by a 6-h exposure to hypoxia (Fig. 1C). These results suggest that both p38α and p38γ are activated by hypoxia. Phosphorylation of p38α and p38γ declined somewhat but was still elevated above control levels up to 24-h exposure to hypoxia (data not shown).

To characterize further the effects of hypoxia on p38 enzyme activity, PC12 cells were transfected with FLAG epitope-tagged versions of p38α, p38β, p38β2, p38γ, or p38δ. Cells were then exposed to either normoxia (21% O₂) or hypoxia (5% O₂, 6 h). The various kinases were then immunoprecipitated with an anti-FLAG antibody, and immune complex kinase assays were performed, as described under “Experimental Procedures.” As shown in Fig. 2A, hypoxia stimulated both p38α and p38γ enzyme activity. In contrast to these results, hypoxia did not significantly alter p38β, p38β2, or p38δ enzyme activity. Hypoxia-induced changes in enzyme activity were not the result of differences in transfection efficiency as cell lysates blotted with anti-FLAG show equal amounts of the transfected protein (Fig. 2B). It can be seen that the effect of hypoxia on the p38γ isoform is by far the most robust (average 5.9-fold activation, Fig. 2C).

We next evaluated the effect of hypoxia on JNK, another SAPK. PC12 cells were exposed to hypoxia for various times, from 20 min to 6 h, and JNK enzyme activity was measured in an immune complex kinase assay, as described under “Experimental Procedures.” Unlike its effects on p38, hypoxia did not alter JNK enzyme activity significantly, whereas exposure of cells to UV light increased JNK activity markedly (Fig. 3).

To determine the effect of hypoxia on p42/p44 MAPK, PC12 cells were again exposed to either normoxia (21% O₂) or hypoxia (5% O₂) for various times, between 20 min and 6 h. Samples of whole cell lysates were immunoblotted with either an antibody specific for tyrosine-phosphorylated (activated) p42/p44 MAPK or an antibody that equally recognizes phospho- and dephospho-p42/p44 MAPK (total MAPK). Hypoxia had no significant effect on the levels of phospho-p42/p44 MAPK at the earliest time points studied. However, exposure to hypoxia for 6 h caused an increase in the tyrosine phosphorylation of p42/p44 MAPK (Fig. 4A). MAPK enzyme activity was measured directly by immune complex kinase assay. Fig. 4C shows that p42 MAPK enzyme activity, like the MAPK phosphorylation state, increased after 6 h of hypoxia. To compare the effects of hypoxia with the prototypical activators of MAPK, we also evaluated p42/p44 MAPK phosphorylation in response to NGF and UV light. In contrast to the rather modest effect of hypoxia, these stimuli caused a robust phosphorylation of p42/p44 MAPK (Fig. 4D).

The downstream transcription factors and protein kinases...
FIG. 3. Lack of effect of hypoxia on JNK activity. PC12 cells were exposed to either hypoxia (5% O2) for various times between 0 and 6 h, as indicated, or to 300 J/m2 UV light for 30 min. JNK was immunoprecipitated by the addition of 1 μg of anti-JNK1 polyclonal antibody as described under “Experimental Procedures.” JNK enzyme activity was determined in an immune complex kinase assay by the amount of 32P incorporation into c-Jun as quantified by PhosphorImager. Similar results were found in three separate experiments representing three dishes in each group.

FIG. 4. Hypoxia modestly activates p42/p44 MAPK. PC12 cells were exposed to hypoxia (5% O2) for various times between 0 and 6 h, as indicated. In panels A and B, lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for either Tyr204-phosphorylated p42/p44 MAPK or total (phospho- and dephospho-) MAPK, as described under “Experimental Procedures.” Panel A, representative immunoblot showing phospho-p42/p44 MAPK enzyme activity at the various time points studied. Panel B, representative immunoblot showing total MAPK at the various time points studied. Results similar to those shown in panels A and B were observed in three separate experiments. Panel C, MAPK enzyme activity was determined in an immune complex kinase assay by the amount of 32P incorporation into myelin basic protein as quantified by PhosphorImager. Data shown are representative of those obtained in two separate experiments and represent six dishes in each group. Panel D, representative immunoblot of Tyr204-phosphorylated p42/p44 MAPK immunoreactivity in lysates of PC12 cells exposed to normoxia (C, 21% O2), hypoxia (H, 5% O2), NGF (50 ng/ml), or 300 J/m2 UV light (30 min). Similar results were found in two separate experiments representing six dishes in each group.

that are targeted by the p38 family are beginning to be elucidated (1–3, 94–43); however, very little is known about the specific genes that are regulated in response to activation of the p38 pathways. The cyclin D1 gene is one known target of p38, as Lavoie et al. (27) have shown that cyclin D1 gene expression is regulated negatively by p38 in CC339 cells. We therefore tested whether hypoxia regulated cyclin D1 levels in PC12 cells. We found that exposure to hypoxia (0, 3, 6, or 24 h at 5% O2) progressively down-regulated cyclin D1 levels, with an 81% decrease of cyclin D1 from control levels observed at 24 h (Table I). Pretreatment of cells with SB203580, a relatively selective inhibitor of p38 (43, 44), was able to reverse in part the down-regulation of cyclin D1 by hypoxia in a dose-dependent manner (Fig. 5A). These results are expressed quantitatively in Fig. 5B, where it can be seen that pretreatment with SB203580 resulted in a partial, but statistically significant, recovery of cyclin D1 toward control levels. The inhibitory effect of SB203580 on hypoxic regulation of cyclin D1 was observed at low doses (0.3–1 μM) as was its inhibitory effect on anisomycin-activated MAPKAP kinase-2. MAPKAP kinase-2 is a protein kinase that is specifically phosphorylated and activated by the p38 family of protein kinases (Fig. 5C). The fact that SB203580 only partially reversed the effects of hypoxia may be because this drug does not inhibit the p38γ isoform (45–47), as discussed further below.

Cyclin D1 is a G1 cyclin whose synthesis and associated cyclin-dependent kinase activity are generally required for progression through the G1 phase of the cell cycle (48, 49). Our finding that hypoxia induces a down-regulation of cyclin D1 suggested that hypoxia may cause cells to accumulate in the G1/G0 phase of the cell cycle. We therefore evaluated the relative percentage of cells in the various phases of the cell cycle in PC12 cells that were exposed to either normoxia or hypoxia for 24 h. Cells were stained with propidium iodide and analyzed by flow cytometry. It can be seen in Fig. 5D that hypoxia caused a 17.4% increase in the number of cells in G0/G1. Furthermore, pretreatment with SB203580 followed by a 24-h exposure to hypoxia was able to reverse in part this accumulation in G0/G1.

Our results show that hypoxia activates both p38α and p38γ; however, the p38γ isoform is insensitive to inhibition by SB203580 (45–47). This raised the possibility that p38γ might also contribute to the inhibition of cyclin D1 by hypoxia (i.e. the portion of the effect that was not inhibited by SB203580). To test this hypothesis, we generated stably transfected PC12 cell lines that express p38AF, a kinase-inactive mutant of p38γ. Overexpression of a similar mutant (Y185F) has been shown previously to inhibit endogenous p38γ enzyme activity effectively (32). Fig. 6 shows that, compared with vector-transfected cells, the hypoxia-induced decrease in cyclin D1 is partially reversed in the p38γAF cell line. These results were confirmed in two separate clones and show that p38γ, like p38α, is involved in the down-regulation of cyclin D1 during hypoxia; however, pretreatment of p38γAF-expressing cells with SB203580 did not result in a further impairment of the effect of hypoxia on cyclin D1 expression (data not shown).

TABLE I

| Time in hypoxia (5% O2) | Cyclin D1 immunoreactivity* |
|------------------------|----------------------------|
| h                      | %                         |
| 0                      | 100 (5)                   |
| 3                      | *45.9 ± 3.4 (5)           |
| 6                      | *29.2 ± 3.0 (5)           |
| 24                     | *18.8 ± 2.0 (8)           |

* Values are the average percent of control ± S.E. (n). * indicates p < 0.05 by χ2 test.

regulation of cyclin D1 by hypoxia in a dose-dependent manner. These results are expressed quantitatively in Fig. 5B, where it can be seen that pretreatment with SB203580 resulted in a partial, but statistically significant, recovery of cyclin D1 toward control levels. The inhibitory effect of SB203580 on hypoxic regulation of cyclin D1 was observed at low doses (0.3–1 μM) as was its inhibitory effect on anisomycin-activated MAPKAP kinase-2. MAPKAP kinase-2 is a protein kinase that is specifically phosphorylated and activated by the p38 family of protein kinases (Fig. 5C). The fact that SB203580 only partially reversed the effects of hypoxia may be because this drug does not inhibit the p38γ isoform (45–47), as discussed further below.

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DISCUSSION

The signaling pathways involved in cellular responses and adaptations to hypoxia are very poorly understood. The PC12 cell line is a neural-like cell line that has been shown to respond to very small reductions in O2 levels with changes in ion conductances (23, 24), protein phosphorylation (20, 22), and gene expression (16–21). These studies were aimed at identifying specific intracellular signaling pathways that are regulated by hypoxia in this cell type. We have shown that moder-
ate hypoxia (5% O₂) selectively activates p38γ and p38α, but not other isoforms of the p38 family of SAPKs. Furthermore, activation of both p38γ and p38α is involved in the down-regulation of cyclin D1 during hypoxia. In contrast, another major SAPK, JNK, was not affected by hypoxia.

The p38 family of protein kinases consists of several isoforms, including p38α, p38δ, p38β, p38γ/SAPK3/ERK6, and p38δ/SAPK4 (4, 10, 28–30, 32, 33, 45, 50, 51). These kinases are activated by a variety of stressors, including osmotic stress, UV light, inhibition of protein synthesis, and inflammatory cytokines; however, the mechanism by which these diverse stimuli activate p38 kinases is not known. Our results demonstrate, for the first time, that physiological levels of hypoxia selectively activate p38γ and p38α. Phosphorylation of p38 has been shown to occur after ischemia in heart and kidney (52). Taken together with our findings, it is possible that the hypoxic component of ischemia, rather than the other types of substrate depletion (glucose, ATP, etc.), results in the activation of p38α and p38γ.

The p38γ isoform was most strongly targeted by hypoxia in PC12 cells. The molecular basis of this selectivity is not known, and in general, previous studies have found the closely related isoforms to be activated coordinately by various stressors (29, 46, 50, 51). Recent evidence suggests, however, that there may be unique physiological roles for p38γ and p38δ.

The other major stress-activated signaling pathway acts through the JNK family of protein kinases (1–3). Like p38, the JNK family is activated by a number of stressors but is distinctive in its ability to phosphorylate the transcription factor c-Jun (6, 8). It has been reported previously that ischemia/reperfusion in the kidney and hypoxia/reoxygenation in cardiac myocytes induce activation of JNK (52, 56). These groups found

FIG. 5. Hypoxia inhibits cyclin D1 and causes accumulation at G1/G0, via a partially p38-dependent mechanism. PC12 cells were exposed to either normoxia (C, 21% O₂) or hypoxia (H, 5% O₂) for 24 h in the presence or absence of increasing amounts of SB203580, as indicated. Panel A, representative immunoblot showing the effects of hypoxia and p38 inhibition on cyclin D1 immunoreactivity. Panel B, immunoreactivity levels of cyclin D1 are expressed as the average percent change from control ± S.E. and represent seven dishes in each group performed in at least two separate experiments. * indicates significant difference from control-pcDNA3, p < 0.05, by χ² test, and # indicates significant difference from hypoxia-pcDNA3, p < 0.05, by χ² test.

FIG. 6. Role of p38γ in the hypoxia-induced decrease in cyclin D1. PC12 cells stably transfected with a kinase-inactive form of p38γ or the empty expression vector pcDNA3 were exposed to hypoxia for 24 h, as indicated. Panel A, representative immunoblot showing the effect of p38γ inhibition on the hypoxia-induced decrease in cyclin D1. Panel B, immunoreactivity levels of cyclin D1 are expressed as the average percent change from control ± S.E. and represent six dishes in each group performed in two separate experiments * indicates significant difference from control-pcDNA3, p < 0.05, by χ² test, and # indicates significant difference from hypoxia-pcDNA3, p < 0.05, by χ² test.
Activation of Specific Isoforms of p38 by Hypoxia

JNK activity to be activated by the reoxygenation event but not during the initial hypoxia or ischemia. It has also been reported recently that severe hypoxia (pO2 ≤ 0.01%) transiently activated JNK in human squamous carcinoma cells (57). In contrast, we found that neither hypoxia nor hypoxia plus reoxygenation (data not shown) between 20 min and 6 h stimulated JNK enzyme activity in PC12 cells. Clearly, various stressors can have different effects, depending on the specific cell type and its environment. The differential effects of hypoxia on p38 and JNK contribute to a small but growing number of stimuli that selectively activate p38 but not JNK (58).

Hypoxia (6 h, 5% O2) also caused a modest activation of p42/p44 MAPK in PC12 cells. It has been reported previously that HeLa cells respond to severe hypoxia with a rapid (within 15 min) but transient activation of p42/p44 MAPK (59). In PC12 cells, hypoxia induced a relatively small and delayed activation of p42/p44 MAPK compared with the robust and rapid activation induced by NGF or UV exposure.

It is of considerable interest to determine which downstream genes are regulated by p38α and p38γ in response to hypoxia. A number of downstream kinases, including MAPKAP kinase-2/3 (34, 35), MAPK signal-integrating kinase (MKIK) (36), and p38-regulated/activated protein kinase (Prkak) (37), as well as transcription factors and ternary complex factors, including CREB-homologous protein (Chop), switch-activating protein (Sap1), myocyte-enhancer factor 2A (MEF2A), and MEF2C have been shown to be phosphorylated and activated by the p38 family of protein kinases (38–42); however, the specific genes that are regulated in response to activation of p38α and these transcription factors remain largely unknown. One gene that has been shown to be regulated by p38α is cyclin D1 (27). Activation of p38α strongly inhibits cyclin D1 gene expression in CCL39 cells (27). Likewise, hypoxia down-regulates cyclin D1 expression in PC12 cells. We showed further that p38α is involved in this hypoxia-induced decrease in cyclin D1 levels, as the effect is partially blocked by low doses of SB203580, a relatively selective inhibitor of p38 (43, 44). The failure of SB203580 to reverse this effect completely may be because of activation of p38γ, which is insensitive to inhibition by SB203580 (45–47). p38γ is also involved in the regulation of cyclin D1, as overexpression of a kinase-inactive mutant (p38γAF) partially reverses the decrease in cyclin D1 during hypoxia. However, pretreatment of PC12 cells overexpressing p38γAF with SB203580 did not result in a further reversal of the effects of hypoxia on cyclin D1 expression (data not shown).

It is not clear why SB203580 would be ineffective in this cell line, but it is possible that p38γAF expression could impair both p38α and p38γ function. Because both p38α and p38γ have been shown to have identical upstream activators (46), p38γAF may sequester activated MAP kinase kinase-3 (MKK3) and/or MKK6, thereby impairing the activity of any of its downstream p38 kinases. Alternatively, the stably transfected p38γAF cells, because they are cultured in the presence of the selection drug (G418) may differ from the parental cell line in a number of ways that are difficult to assess.

Cyclin D1 has been implicated in regulating progression through the G1 phase of the cell cycle (48, 49). The hypoxia-induced inhibition of cyclin D1 correlates with an increased accumulation of cells in G0/G1 after exposure to hypoxia. This accumulation was also shown to be partially blocked by cotreatment of cells with SB203580. It is important to note that although there is a relative increase in the accumulation of cells in the G0/G1 phase, we did not observe a corresponding decrease in cell cycle progression during hypoxia. In fact, preliminary findings suggest that hypoxia may induce prolifera-

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