Role of Glycogen Synthase Kinase-3 in the Phosphatidylinositol 3-Kinase/ Akt Cell Survival Pathway*

(Received for publication, November 4, 1997, and in revised form, June 1, 1998)

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Growth factor-dependent survival of a variety of mammalian cells is dependent on the activation of phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream effector, the protein kinase Akt. Glycogen synthase kinase-3 (GSK-3) has been previously identified as a physiological target of Akt, which is inhibited by phosphorylation, so we have investigated the role of GSK-3 in cell survival. Overexpression of catalytically active GSK-3 induced apoptosis of both Rat-1 and PC12 cells, whereas dominant-negative GSK-3 prevented apoptosis following inhibition of PI 3-kinase. GSK-3 thus plays a critical role in regulation of apoptosis and represents a key downstream target of the PI 3-kinase/Akt survival signaling pathway.

Although many types of mammalian cells are dependent upon growth factors for survival (1), the intracellular signaling pathways that control cell survival by preventing apoptosis have only begun to be elucidated. A role for PI3 3-kinase in the regulation of cell survival was first indicated by experiments showing that PI 3-kinase was required to prevent apoptosis of PC12 rat pheochromocytoma cells maintained in nerve growth factor (NGF) (2). These findings have been extended by observations demonstrating that PI 3-kinase is required for survival of several other growth factor-dependent cell types, including fibroblasts, epithelial cells, hematopoietic cells, and primary neurons (3–12). In addition, the protein kinase Akt has been identified as a key effector of PI 3-kinase in signaling cell survival (5, 13, 14).

The principal characterized physiological substrate of Akt is glycogen synthase kinase-3 (GSK-3) (15), which was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (16). GSK-3 is a ubiquitously expressed protein-serine/threonine kinase whose activity is inhibited by Akt phosphorylation in response to growth factor stimulation. In addition to glycogen synthase, GSK-3 phosphorylates a broad range of substrates, including several transcription factors and translation initiation factor eIF2B (16). GSK-3 has also been implicated in the regulation of cell fate in Drosophila (17) and is a component of the Wnt signaling pathway required for Drosophila and Xenopus development (18–21). These studies suggest that GSK-3 is involved in multiple cellular processes, including metabolism, proliferation, and differentiation. Here we show that GSK-3 is also involved in the regulation of apoptosis, identifying it as a critical downstream element of the PI 3-kinase/Akt cell survival pathway.

EXPERIMENTAL PROCEDURES
Cell Culture—PC12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 5% horse serum. Rat-1 cells were grown in DMEM supplemented with 10% calf serum.

Assay of GSK-3—PC12 cells were plated in 100-mm culture dishes (3 × 106 cells/plate) in DMEM containing 10% fetal bovine serum and 5% horse serum. On the next day the medium was changed to DMEM containing 0.5% horse serum. One day later, cells were washed by addition of 100 ng/ml of NGF (Life Technologies, Inc.) for 5, 15, or 30 min with or without preincubation with 50 μM LY294002 (Biomol) or 100 nM wortmannin (Sigma). Cells were washed with phosphate-buffered saline and lysed in extraction buffer (100 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM EDTA, 0.1% Triton X-100, 1 mM benzamidine, 0.1 mM Na3VO4, 1 mg/ml glycogen, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, 100 mM sodium orthovanadate, 0.375 μg of GSK-3β antibody (Transduction Laboratories) for 2 h at 4 °C with rotation. 5 μg of rabbit anti-mouse IgG (Upstate Biotechnology Inc.) was added for 30 min. Protein A-Sepharose (60 μl of a 30% suspension) was then added, and the incubation was continued for 1 h at 4 °C with rotation. Immune complexes were recovered by centrifugation at 4 °C and washed once with extraction buffer and twice with immunoprecipitation buffer (50 mM sodium glycerophosphate, pH 7.3, 1 mM EGTA, 1 mM benzamidine, 1 mM diethiothreitol, 0.1 mM Na3VO4, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 mM sodium orthovanadate). GSK-3 was assayed in a total volume of 20 μl containing 250 mM sodium glycerophosphate, pH 7.4, 1 mM NaN3, 100 mM MgCl2, 5 mM EGTA, 5 mM benzamidine, 5 mM diethiothreitol, 0.5 mM Na3VO4, 100 mM oakad酸, 20 μM phosphoglycogen synthase peptide-2 (Upstate Biotechnology), and 50 μM [γ-32P]ATP (1 μCi). After 10 min of incubation at 30 °C, reaction mixtures were centrifuged for 1 min, and 20 μl of the supernatant was spotted onto Whatman P81 phosphocellulose paper. Filters were washed in four changes of 175 mM phosphoric acid for a total of 20 min, rinsed in aceton, dried, and counted in a liquid scintillation counter.

Transient Transfection—3 × 105 cells were plated on poly-l-lysine treated cover-slips in 35-mm plates 24 h before transfection. Transient transfections were performed with 1 μg of expression vectors using the LipofectAMINE Reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Wild-type rat GSK-3 (pSGSK3β) and catalytically inactive GSK-3 (pRSGSK3β) (21) are transcribed from the EF1α promoter (22); wild-type p53 (pWTP53) and dominant-negative p53 (p145p53) are transcribed from the cytomegalovirus promoter (23); Bcl-2 (pSG5-bcl-2) and Bcl-xL (pSG5-Bcl-xL) are transcribed from the early SV40 promoter (provided by C. Nalin, Sandoz Pharmaceuticals); dominant-negative PI-3-kinase (p85) (24) is transcribed from the SV40 promoter (provided by C. Rudd, Dana-Farber Cancer Institute). Cells were cotransfected with 1 μg of a green fluorescent protein (GFP) expression construct (pEGFP-C1) (CLONTECH). After 2 days cells were fixed and nuclei were stained with the DNA dye bisbenzimide.

* This research was supported by National Institutes of Health Grant RO1 CA18689. 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.
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The abbreviations used are: PI, phosphatidylinositol; GSK-3, glycogen synthase kinase-3; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; NGF, nerve growth factor.
RESULTS AND DISCUSSION

Inhibition of GSK-3 Activity Is Mediated by the PI 3-Kinase/Akt Signaling Pathway during Cell Survival—Previous studies have demonstrated that GSK-3 is inhibited as a result of growth factor stimulation and activation of the PI 3-kinase/Akt signaling pathway in several cell types (15, 25–27). We therefore sought to determine whether stimulation of cells with growth factors known to signal cell survival by suppression of apoptosis similarly resulted in inhibition of GSK-3 activity. Treatment of PC12 cells with NGF, which signals cell survival via the PI 3-kinase pathway (2), resulted in a 30–40% inhibition of GSK-3 activity (Fig. 1), which is similar to that reported in other cell systems (15, 25–27). This inhibition of GSK-3 was prevented by preincubation with the PI 3-kinase inhibitors LY294002 (28) and wortmannin (29, 30) (Fig. 1), indicating that NGF inhibition of GSK-3 was mediated by the PI 3-kinase pathway.

Induction of Apoptosis by Overexpression of GSK-3—Because GSK-3 was inhibited by PI 3-kinase signaling in NGF-stimulated PC12 cells, we investigated whether overexpression of active GSK-3 induced apoptosis. Both PC12 cells and Rat-1 fibroblasts, which are also dependent upon PI 3-kinase signaling for survival (3), were used as recipients in transient trans-
Infection assays. Cells were transfected with a construct expressing GFP and cotransfected with plasmids expressing either wild-type GSK-3β or catalytically inactive R85 GSK-3β cDNAs (21) from the EF1α promoter (22). Transfected cells were identified by fluorescence microscopy to detect GFP expression, and apoptotic cells were scored by nuclear morphology after staining with Hoechst dye.

Fluorescence micrographs of a representative experiment are shown in Fig. 2, and data from several experiments with both PC12 and Rat-1 cells, presented as in Fig. 3.

We next examined whether apoptosis induced by GSK-3β could be blocked by known inhibitors of apoptotic cell death (Fig. 4). Cotransfection with a dominant-negative mutant of p53 (V143A) (23) protected cells from apoptosis induced by GSK-3, consistent with the involvement of p53 in apoptosis induced by inhibition of PI 3-kinase in both PC12 and Rat-1 cells (31). Induction of apoptosis by GSK-3β was also blocked by a peptide inhibitor of CPP32-like caspases, which are effectors of cell death activated by diverse apoptotic stimuli (32), including PI 3-kinase inhibition (4, 31). Finally, apoptosis induced by GSK-3β was inhibited by cotransfection with plasmids expressing either Bcl-2 or Bcl-xL, which protect cells from apoptosis induced by a variety of agents, including inhibitors of PI 3-kinase (4). Apoptosis induced by GSK-3β thus required caspase activity and was modulated by p53 and Bcl-2 family members, consistent with a role for GSK-3β as a downstream element in the PI 3-kinase signaling pathway.

Dominant-negative GSK-3 Inhibits Apoptosis Resulting from Inhibition of PI 3-Kinase—We next sought to determine whether GSK-3β activity was required for apoptosis induced by inhibition of PI 3-kinase. PC12 and Rat-1 cells were transfected with the catalytically inactive R85 GSK-3β mutant, which acts as a dominant-negative inhibitor of GSK-3 activity (21), and then treated with the specific PI 3-kinase inhibitor LY294002 (Fig. 5). Treatment with LY294002 induced apoptosis of approximately 60% of control cells that had been transfected with the pEF1α vector alone. Transfection with the R85 GSK-3β mutant significantly inhibited apoptosis induced by LY294002 to levels of 30–40%, approaching the background level of 20% apoptosis observed in control cells that were not treated with the PI 3-kinase inhibitor. The activity of the R85 GSK-3β mutant in inhibiting apoptosis in these experiments was similar to that of dominant-negative p53.

Similar results were obtained when cells were transfected with a plasmid expressing dominant-negative PI 3-kinase (24) rather than being treated with LY294002 (Fig. 6). Expression of dominant-negative PI 3-kinase induced apoptosis in approximately 60% of transfected cells, and this was significantly
inhibited by cotransfection with dominant-negative R85 GSK-3. Thus, GSK-3 activity was necessary for apoptosis resulting from PI 3-kinase inhibition.

Taken together, these results indicate that GSK-3 is a key target of PI 3-kinase signaling leading to prevention of apoptosis. Overexpression of active GSK-3 is sufficient to induce apoptosis, whereas expression of dominant-negative GSK-3 effectively protects cells from apoptosis resulting from inhibition of PI 3-kinase. Although other targets of the Akt protein kinase, including the Bcl-2 family member Bad (33, 34), may also be important in the regulation of cell survival, these findings implicate GSK-3 as a central element in the PI 3-kinase/Akt survival pathway, with phosphorylation of one or more targets of GSK-3 presumably serving to activate apoptotic cell death. Identification of the GSK-3 targets that regulate apoptosis thus poses a critical next step to understanding the signaling pathways by which growth factors control cell survival.

Acknowledgments—We are grateful to I. Dominguez for GSK-3 plasmids, to C. E. Rudd for the dominant-negative PI 3-kinase plasmid, and to P. Erhardt for kind help and advice.

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