Insulin-like Growth Factor 1 Inhibits Apoptosis Using the Phosphatidylinositol 3'-Kinase and Mitogen-activated Protein Kinase Pathways

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The role of insulin-like growth factor 1 (IGF-1) in preventing apoptosis was examined in differentiated PC12 cells. Induction of differentiation was achieved using nerve growth factor, and apoptosis was provoked by serum withdrawal. After 4–6 h of serum deprivation, apoptosis was initiated, concomitant with a 30% decrease in cell number and a 75% decrease in MTT activity. IGF-1 was capable of preventing apoptosis at concentrations as low as 10−8 M and as early as 4 h. The phosphatidylinositol 3′ (PI3′)-kinase inhibitors wortmannin (at concentrations of 10−8 M) and LY294002 (10−6 M) blocked the effect of IGF-1. The pp70 S6 kinase inhibitor rapamycin (10−8 M) was, however, less effective in blocking IGF-1 action. Moreover, stable transfection of a dominant-negative p85 (subunit of PI3′-kinase) construct in PC12 cells enhanced apoptosis provoked by serum deprivation. Interestingly, in the cells overexpressing the dominant-negative p85 protein, IGF-1 was still capable of inhibiting apoptosis, suggesting the existence of a second pathway involved in the IGF-1 effect. Blocking the mitogen-activated protein kinase pathway with the specific mitogen-activated protein kinase/extracellular-response kinase kinase inhibitor PD098059 (10−5 M) inhibited the IGF-1 effect. When wortmannin and PD098059 were given together, the effect was synergistic. The results presented here suggest that IGF-1 is capable of preventing apoptosis by activation of multiple signal transduction pathways.

Apoptosis or programmed cell death plays an important role in embryonic development, involution of organs, and tumorigenesis. During development of the nervous system, a large proportion of neurons die by this process; about 50% of embryonic postmitotic neurons ultimately die during the period when synapses are formed between neurons and their targets (1, 2). The survival of neurons is dependent on neurotrophins secreted by the target cells (3). In PC12 cells (rat pheochromocytoma cells) differentiated in the presence of nerve growth factor (NGF), 1 NGF itself, as well as other growth factors (including platelet-derived and epidermal growth factors, and insulin at high doses), protect cells maintained in serum-free media from apoptosis (4).

Insulin-like growth factor 1 (IGF-1) prevents apoptosis in a number of cell types. For example, IGF-1 at physiological concentrations was effective in inhibiting apoptosis in fibroblasts overexpressing c-myc (5, 6), in cells treated with the topoisomerase II inhibitor etoposide (7), in neuroblastoma cells under hyperoxic stress (8), and potassium-deprived cerebellar granule cells (9, 10). Most cell types require IGF-1 for growth in culture (11), and a decrease in IGF-1 receptor number induces apoptosis in tumor cells (12). IGF-1 can also act as a survival factor in the absence of other factors. Specifically, IGF-1 inhibits apoptosis of several interleukin-3-dependent cell lines when IL-3 is removed (13).

Insulin and the IGFs exert their biological effects by binding to their respective transmembrane receptors. Insulin and IGF-1 receptors are similar, heterotetrmeric proteins with intrinsic tyrosine kinase activity (14, 15). Both receptors are capable of binding insulin and IGF-1, but each receptor binds its own ligand with a 100-1000-fold higher affinity than that of the heterologous peptide. In addition, IGF-1 activity is also regulated by binding to specific IGF-binding proteins that do not bind insulin (16). IGF-1, the IGF-1 receptor, and the binding proteins are expressed in many tissues, creating an autocrine-paracrine hormonal system. One of the earliest steps in signal transduction by both insulin and IGF-1 is the extensive phosphorylation of IRS-1, a 185-kDa protein. Tyrosyl-phosphorylated IRS-1 then interacts with numerous SH2 domain-containing proteins, including PI3′-kinase and the guanine-nucleotide exchange factor Grb2/SOS. PI3′-kinase then initiates phospholipid turnover, and Grb2/SOS activation results in initiation of the MAP kinase signal transduction cascade by sequential phosphorylation and activation of proto-oncogenes Ras and Raf and the MAP/ERK kinases (MEK1 and MEK2).

While the effectiveness of IGF-1 on inhibition of apoptosis is well established, the signaling pathways leading to apoptosis and the mechanisms of action by which IGF-1 and other agents prevent apoptosis are largely unknown. The PI3′-kinase inhibitor wortmannin is known to block the protective action of NGF and platelet-derived growth factor in serum-deprived PC12 cells (4); this finding suggests an important role of the PI3′-kinase pathway in apoptosis prevention by growth factors. PI3′-kinase is an important component of intracellular signal

IRS-1, insulin receptor substrate 1; SH2, Src homology region 2; ERK, extracellular-response kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP/ERK kinase; pp70S6K, pp70 S6 kinase; DMEM, Dulbecco’s modified Eagle’s medium; SFM, serum-free DMEM; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
transduction processes linked directly or indirectly to diverse receptor types. PI3'-kinase is an heterodimer composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (17). The p85 subunit, through its SH2 domains, mediates the association of p110 with activated protein tyrosine kinase receptor

In the present work, the involvement of PI3'-kinase in mediating IGF-1 prevention of apoptosis in PC12 cells was further explored using the PI3'-kinase inhibitors wortmannin and LY294002, and PC12 cells were stably transfected with either a wild-type p85 (Wp85) construct or a dominant-negative p85 construct (Δp85) (19). The Δp85 protein lacks the inter-SH2 region required for binding to p110, thereby blocking activation of p110 (20). Rapamycin, which inhibits pp70 S6 kinase (pp70S6K) activation, was also used (21). In addition, the involvement of the MAP kinase pathway in the prevention of apoptosis by IGF-1 was tested using PD098059, a specific inhibitor of MEK and the MAP kinase cascade (22, 23).

Experimental Procedures

Materials—Recombinant IGF-1 and mouse 2.5S NGF were purchased from NeuroBiotechnology (Lake Placid, NY), buffer salts were from Sigma, and LY294002 was from DuPont NEN.

Cell Culture and Experimental Design—PC12 cells (a gift from Dr. G. Guroff, NICHD, NIH, Bethesda, MD) were typically maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% horse serum and 5% fetal bovine serum, in a humidified atmosphere of 95% air and 5% CO2 at 37°C. For each experiment, cells were grown in 100-mm culture dishes in DMEM until they reached 60–70% confluence. Cell differentiation was then allowed to proceed as described previously (24) by changing cells to low serum (2% horse serum and 1% fetal bovine serum) medium supplemented with 100 ng/ml 2.5S NGF. After 6–7 days, the cells were washed twice with phosphate-buffered saline (PBS), and the medium was changed overnight to serum-containing DMEM without NGF. The next day, the cells were incubated in the absence or presence of IGF-1 (concentrations ranging from 10−3 to 10−5 M) in serum-free DMEM. At various intervals as stated in the figure legends, the cells remaining attached to the plate were harvested and combined with those cells suspended in the medium, which were collected by centrifugation.

In some experiments, the PI3'-kinase inhibitors wortmannin (10−8−10−5 M) or LY240023 (10−8−10−5 M) were added 6 h prior to harvesting the cells to the serum-free DMEM, either in the absence or presence of IGF-1 (10−8 M). For cells growing in either absence or presence of IGF-1, rapamycin (10−8−10−7 M) was added 6 or 24 h prior to collecting the cells. In other experiments, the MEK inhibitor PD098059 (10−6−10−4 M) was added to cells in serum-free medium as described (22, 23). Cells were preincubated for 30 min with PD098059 prior to the addition of IGF-1.

Insulin and IGF-1 Binding—Insulin and IGF-1 receptor number in PC12 cells were determined by the Scatchard method as described previously (25) using tracer amounts of Tyr-A14 monoiodinated insulin (2000 Ci/mmol specific activity, Amersham LifeScience, Inc., Arlington Heights, IL) or des(1–31)-IGF-1 (44 μCi/μg, GroFep, Adelaide, Australia).

DNA Laddering—Genomic DNA was prepared from PC12 cells using the TACS Apoptotic DNA laddering kit. The concentration and purity of DNA were determined by measuring UV absorbance at 260 and 280 nm. Equal amounts of DNA from each sample (1 μg) were 3'-end-labeled for 10 min at room temperature using the Klenow fragment of DNA Pol I and the radiolabeled nucleotide (0.5 μCi [32P]dCTP, 6000 Ci/mmol). The reaction was stopped by adding 6× DNA loading buffer, and samples were then electrophoresed through a 1% Trew Gel and a 5× Tris acetate/EDTA buffer. Following electrophoresis, the gel was fixed in 10% acetic acid for several hours, dried on Whatman 3 MM paper (Whatman Int., Maidstone, United Kingdom) in a gel dryer at 70°C, and analyzed by autoradiography using Kodak X-OMat AR film and Cronex Lightning Plus enhancing screens.

Cell Transfection and Screening—PC12 cells were grown in 60-mm plates until 60–70% confluent. Cells were co-transfected with 15 μg of plasmid DNA (SRa-Wp85 or SRa-Δp85, the kind gift of Professor Masato Kasuga, Kobe University, Japan) (19) and 1 μg pMC1-neo (Clontech, Palo Alto, CA), using Lipofectin reagent (Life Technologies, Inc.). Transfections were performed for 12 h, and the medium was then changed to DMEM for an additional 24 h. After 24 h of recovery, the cell mixtures were serially diluted and plated onto 150-mm dishes. Stably transfected cells were selected using 500 μg/ml geneticin (Life Technologies, Inc.). Independent colonies were picked by using cloning cylinders (Specialty Media Inc., Lavallette, NJ). Individual clones overexpressing bovine Wp85 or Δp85 were selected by Western blotting using a polyclonal anti-p85 antibody (Transduction Laboratories). Phosphatidylinositol-4-monophosphate (Sigma) was used as substrate in the kinase assay.

Measurement of MAPK/ERK Activity—MAPK/ERK activity was assayed as described (27) with some minor modifications. Briefly, cells maintained overnight in serum-free medium were stimulated with IGF-1 (10−7 M) for 12 min at 37°C. The cells were then rapidly washed in ice-cold 1× PBS and solubilized in ice-cold lysis buffer (50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM EDTA, 1 mM diithiothreitol, 100 mM sodium orthovanadate, 10 mM benzimidamide, and 2 μg/ml pepstatin A). The lysates were cleared by centrifugation, and equal amounts of protein from each sample (5 μg) were incubated for 15 min at 30°C in the presence of 400 μg/ml specific MAP kinase substrate peptide (myelin basic protein 95–98, Upstate Biotechnology Inc.) and 100 μM (γ32P)ATP. The reaction was stopped by spotting samples onto Whatman P81 paper squares following by extensive washing in 150 mM phosphoric acid. The papers were air dried and counted in a β-counter with scintillation liquid.

MTT Assay and Cell Number—A modification of the MTT assay that measures mitochondrial function was used (28). Differentiated PC12 cells were plated on 24-well plates (50,000–80,000 cells/well) and maintained overnight in complete medium. Cells were then changed to serum-free medium the next day, and the effects of different inhibitors at the indicated periods of time, the medium was aspirated from the wells, and 200 μl of MTT reagent (1 mg/ml) were added to each well. The cells were then incubated for 1 h at 37°C and lysed by addition of 200 μl of isoaamyl alcohol and shaking for 20 min. A 200-μl aliquot of each sample was then translated to 96-well plates and read in an enzyme-linked immunosorbent assay reader at 570–690 nm.

In parallel, direct counting of the cells remaining attached to the plate was performed. After treatment, medium was aspirated, and plates were washed twice with 1× PBS. Cells still attached to the plate were trypsinized and counted in a Neubauer chamber.

Results

IGF-1 Inhibition of Apoptosis in PC12 Cells—Differentiation of PC12 cells was induced by incubating the cells with NGF-containing medium for 6–7 days. Apoptosis in these differentiated cells was induced by removal of NGF and incubation of the cells in serum-free DMEM (SFM). Within the first 4–6 h, apoptosis was detected as early as 4 h and at concentrations as low as 10−9 M, with maximal effects seen at 10−8 M (Fig. 1B). At the same time, 6 h of serum deprivation provoked a 29 ± 10% decrease in cell number (Fig. 2A) as well as a decrease in MTT activity (Fig. 2B). IGF-1, on the other hand, was capable of inhibiting both effects. The effect of insulin on inhibition of apoptosis was detected by increased DNA laddering (Fig. 1A). After 24 h of recovery, the cell mixtures were serially diluted and plated onto 150-mm dishes. Stably transfected cells were selected using 500 μg/ml geneticin (Life Technologies, Inc.). Independent colonies were picked by using cloning cylinders (Specialty Media Inc., Lavallette, NJ). Individual clones overexpressing bovine Wp85 or Δp85 were selected by Western blotting using a polyclonal anti-p85 antibody (Transduction Laboratories). Phosphatidylinositol-4-monophosphate (Sigma) was used as substrate in the kinase assay.

The Effect of Wortmannin, LY294002, and Rapamycin on
**Apoptosis**—To determine whether the effect of IGF-1 on inhibition of apoptosis involved the PI3-kinase pathway, we used the specific PI3-kinase inhibitors wortmannin, a fungal protein, and LY294002 (29), a synthetic inhibitor of PI3-kinase. The cells were incubated with IGF-1 (10^{-8} M) and varying concentrations of wortmannin (10^{-9}-10^{-7} M). Wortmannin at 10^{-7} M inhibited the action of IGF-1. This inhibition was maximal at 10^{-7} M wortmannin (Fig. 3). The presence of DNA laddering as a consequence of the incubation with wortmannin was detected at both 6 h (Fig. 3) and 24 h (data not shown), being maximal at 6 h. This is not unexpected, due to the instability of the inhibitor at the physiological temperature of the medium, as previously reported (4, 30). To confirm that the PI3-kinase pathway was involved, we used LY294002, a reversible PI3-kinase inhibitor. Similarly to wortmannin, concentrations of 10^{-6} and 10^{-5} M LY294002 blocked the effect of IGF-1 on inhibition of apoptosis as measured by increased DNA laddering (data not shown), decrease in cell number (Fig. 2A).

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![Image of DNA laddering and cell enumeration](http://www.jbc.org/)

**Fig. 1.** IGF-1 inhibits apoptosis in PC12 cells. **A**, time-course of induction of apoptosis and inhibition by IGF-1. PC12 cells differentiated in the presence of NGF as described under "Experimental Procedures" were maintained in SFM in the absence (-) or presence (+) of 10^{-8} M IGF-1. Cells were collected after the indicated time periods, and DNA was extracted and radiolabeled. **B**, dose-response of IGF-1 effect. Differentiated PC12 cells were maintained in SFM without or with IGF-1 at the indicated concentrations. After a 6-h incubation, DNA was extracted and radiolabeled. **C**, dose-response of insulin effect. Differentiated PC12 cells were maintained for 6 h in SFM in the absence or presence of different insulin concentrations as indicated. IGF-1 at 10^{-8} M (lane 1) was used as a control. Results are representative of three separate experiments.
and mitochondrial activity (Fig. 2B).

Interestingly, both wortmannin and LY294002 in the absence of IGF-1 enhanced the degree of apoptosis detected (Fig. 3, lanes 6–8), possibly resulting from the inhibition of basal PI3'-kinase activity. However, the level of apoptosis seen with the inhibitor alone is greater than when the inhibitor and IGF-1 are combined (Fig. 3, lane 3 versus 7 and lane 4 versus 8). These results support the conclusion that the PI3'-kinase pathway plays a role in mediating apoptosis. Furthermore, the IGF-1 effect on inhibition of apoptosis is, at least in part, via PI3'-kinase activation.

pp70S6K is known to play a role in the signaling cascade initiated by PI3'-kinase (21). Stimulation of pp70S6K can also be blocked by wortmannin (31, 32) or LY294002 (21). The inhibitor rapamycin is known to block the activation of pp70S6K at some point downstream of PI3'-kinase although its specific target remains unknown (31, 32). In our system, $10^{-7}$ M rapamycin completely blocked IGF-1-stimulated phosphorylation of pp70S6K as detected by a shift of the band in a 15% SDS-polyacrylamide electrophoresis gel blotting with anti-pp70S6K antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (data not shown). However, incubation of PC12 cells with $10^{-8}$ M or $10^{-7}$ M rapamycin resulted only in partial inhibition of the IGF-1 effect on protecting the cells from apoptosis. This effect was detected as a faint laddering (data not shown) concomitant with a loss of cells. The effect of $10^{-8}$ M rapamycin was significant only after a 24-h incubation (Fig. 2A). Incubation with $10^{-7}$ M rapamycin, on the other hand, resulted in a significant, although moderate, decrease in cell number after 6 h (87 ± 4% of cells survived), which was more prominent after 24 h (73 ± 9% of cells survived). Rapamycin alone at $10^{-8}$ or $10^{-7}$ M did not significantly increase the degree of apoptosis in cells maintained in SFM without IGF-1.

Overexpression of p85—To further establish that the PI3'-kinase pathway is involved in apoptosis, we stably transfected PC12 cells with vectors containing either a dominant-negative p85 construct (Dp85) or a wild-type p85 construct (Wp85) as a control. Multiple clones of each cell type were obtained, and Western blot analyses established enhanced expression of the proteins (Fig. 4A). Overexpression of the wild-type p85 construct (Wp85) did not significantly affect the IGF-1 effect on apoptosis (data not shown). Conversely, expression of Δp85 resulted in enhanced apoptosis (Fig. 4B and C), concomitant with a partial decrease in IGF-1-stimulated PI3'-kinase activity (Fig. 4D). The addition of LY294002 yielded an even greater effect on induction of apoptosis (Fig. 4B, lane 6 versus 3). Similar results were obtained using wortmannin (data not shown).
shown). After 16 h of incubation in SFM, the difference in the degree of apoptosis between the parental cells and the different Δp85 clones studied was maintained or increased (data not shown). Interestingly, in the cells overexpressing the dominant negative p85 protein, IGF-1 was still capable of inhibiting apoptosis although its sensitivity was decreased compared with its effect on parental cells (Fig. 4C). The IC₅₀ of the IGF-1 effect was 10⁻⁸ M in two Δp85 clones studied as compared with 10⁻⁹ M in the parental cells, suggesting that inhibition of PI3'-kinase activity resulted in a decreased effectiveness of IGF-1 on inhib-
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bition of apoptosis. On the other hand, the IGF-1 effect was not completely eliminated by inhibiting the PI3’-kinase pathway. This could be explained in part by the fact that the level of expression of the dominant-negative p85 is insufficient to totally block the PI3’-kinase pathway (Fig. 4D). Alternatively, other signaling pathways may be involved in the IGF-1 effect.

The Role of the MAP Kinase Pathways—Since the MAP kinase pathway mediates many of the known effects of IGF-1, we next blocked this pathway to determine its role in IGF-1 prevention of apoptosis. For this purpose, we used a specific MEK inhibitor, PD098059 (22, 23), that inhibits MAPK activity in a dose-dependent fashion (Fig. 5A). When incubated with IGF-1, PD098059 at concentrations of $10^{-5}$ and $10^{-4}$ M inhibited the IGF-1 effect on apoptosis prevention (Fig. 5B, lanes 3–4 versus lane 1). This effect was detectable at 10 h but was maximal at 25 or 30 h (Fig. 5C). PD098059 alone (10$^{-5}$–10$^{-4}$ M) also increased apoptosis (Fig. 5B, lanes 6–7 versus lane 5). These results suggest that the MAPK pathway is also involved in apoptosis and mediates the IGF-1 prevention of apoptosis. In agreement with the DNA laddering experiments, a decrease in cell number was found at 24 h but not at 6 h (Fig. 2A) although a decrease in MTT activity was already detected at 6 h, being significant at 12 h (Fig. 2B).

Since both wortmannin and PD098059 were effective in inhibiting the IGF-1 action, we next determined whether their effects were additive or synergistic. As can be seen in Fig. 6, the effect of addition of both inhibitors is synergistic. For example, the degree of apoptosis in lane 8 is greater than the degree of apoptosis predicted by the addition of lanes 2 and 4. Moreover, the extensive DNA laddering obtained by incubation of the cells with both inhibitors was easily visible in electrophoretic gels after staining the DNA with ethidium bromide. In contrast, the laddering provoked after the incubation with either one or the other inhibitor could not be visualized that way, needing radioactive labeling (data not shown). Macroscopically, incubation with both inhibitors simultaneously resulted in 70–80% of the cells detaching from the plate, in contrast to about 30% when either of the inhibitors was used separately (data not shown).

DISCUSSION

During embryonic development, rapid cellular proliferation is necessary for organ growth. However, appropriate function of each organ also depends on an orderly removal of certain cells. This is achieved by the process of apoptosis, also known as programmed cell death. Apoptosis is detected initially as internucleosomal fragmentation of genomic DNA, followed by chromatin condensation, nuclear disintegration, and cellular fragmentation (33, 34). In addition, apoptosis may play an important role in regulating tumorigenesis. For example, the tumor suppressor gene product, p53, may regulate tumorigen-

FIG. 5. PD098059 blocks MAPK activity and induces apoptosis in PC12 cells. A, PD098059 blocks MAP kinase activity in PC12 cells. PC12 cells were maintained in SFM for 12 h and preincubated with PD098059 (PD) for 18 h or with wortmannin (WT) for 6 h at the indicated concentrations. The cells were then stimulated with $10^{-7}$ M IGF-1 for 12 min and lysed. Lysates were cleared by centrifugation and analyzed for MAPK activity as described under “Experimental Procedures.” Results are presented as radioactivity incorporated into the substrate as a percentage of the basal values (those in absence of IGF-1 stimulation). Results shown are means ± S.E. of three experiments done in triplicate. *, p < 0.05. B, specific MEK inhibitor PD098059 induces apoptosis in PC12 cells. Differentiated PC12 cells were maintained in SFM without (-) or with (+) IGF-1 $10^{-8}$ M in the presence of the indicated concentrations of PD098059 for 24 h prior to collection and labeling of the DNA. The results presented are representative of two separate experiments. C, time-course of apoptosis initiation by PD098059. PC12 cells were maintained for the indicated periods of time in SFM in the absence (-) or presence (+) of $10^{-8}$ M IGF-1, with or without $10^{-4}$ M PD098059. Results are representative of three separate experiments.
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- **Fig. 6.** Wortmannin and PD098059 have synergistic effects on apoptosis induction in PC12 cells. Differentiated PC12 cells were maintained for 18 h in 10^{-8} M IGF-1 in the absence (−) or presence (+) of different concentrations of PD098059. Wortmannin was then added at various final concentrations, and incubation was allowed to proceed for 6 h. DNA was then extracted and analyzed. The results presented are representative of two separate experiments.

| Treatment | 10^{-8} M | 10^{-7} M | 10^{-6} M | 10^{-5} M | 10^{-4} M | 10^{-3} M | 10^{-2} M | 10^{-1} M |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| IGF-1     | −         | +         | +         | +         | +         | +         | +         | +         |
| PD098059  | 10^{-5} M | 10^{-6} M | 10^{-7} M | 10^{-8} M | 10^{-9} M | 10^{-10} M | 10^{-11} M | 10^{-12} M |
| Wortmannin| −         | −         | 10^{-8} M | 10^{-7} M | 10^{-6} M | 10^{-5} M | 10^{-4} M | 10^{-3} M |

**Results:**

- Wortmannin demonstrates a substantially greater degree of specificity for PI3' kinase compared with a number of other lipid kinases, especially when wortmannin is used at low concentrations (10^{-9} or 10^{-8} M) (39). PI3' kinase comprises an 85-kDa regulatory subunit and a 110-kDa catalytic subunit that phosphorylates phosphatidylinositol at d-3-hydroxy of the inositol ring. Wortmannin binds irreversibly to the catalytic subunit (p110), thereby inhibiting the signaling pathway following PI3' kinase activation. Wortmannin has previously been shown to completely inhibit PI3' kinase activity in PC12 cells at 10^{-7} M, with a half-maximal dose (IC_{50}) of approximately 3 × 10^{-9} M (4, 30). Due to the instability of the inhibitor in the medium, the peak inhibitory effect of wortmannin takes place after 3–4 h of incubation in the cells (30). In the present study, wortmannin at low concentrations enhanced apoptosis and inhibited the effect of IGF-1 when added to PC12 cells, suggesting that PI3'-kinase is involved in the regulation of apoptosis. Support for this hypothesis comes from the use of the synthetic PI3'-kinase inhibitor LY294002 (29), which gave essentially the same results as wortmannin. In both cases, detection of characteristic DNA laddering coincided with a 20–30% decrease in the number of cells attached to the plate and with an even more marked decrease in mitochondrial activity as measured by MTT assay. The acceleration of apoptosis by addition of wortmannin to cells in the absence of growth factor has been observed previously (40) and can be attributed to the inhibition of the basal PI3'-kinase activity.

Since wortmannin (and LY294002) may not be absolutely specific for PI3'-kinase, we chose to further modulate the PI3'-kinase system by stably transfecting PC12 cells with a dominant-negative p85 or a wild-type p85 construct. Overexpression of these proteins has enabled us to confirm the role of PI3'-kinase in apoptosis. Overexpression of wild-type p85 did not significantly affect IGF-1 effects on apoptosis following NGF withdrawal. The dominant-negative p85 subunit, on the other hand, can bind phosphorylated IRS-1 but, because it lacks the inter-SH2 domain, cannot bind the p110 subunit. Therefore, PI3'-kinase activation by IGF-1 is effectively inhibited by blocking access of functional p85 to phosphorylated IRS-1 (19). Overexpression of Δp85 thus resulted in a partial decrease of PI3'-kinase activity and a consequent increase in the degree of apoptosis observed in serum-free medium and an increased sensitivity to the presence of wortmannin or LY294002. Thus, our results support the findings of Yao and Cooper (4) who similarly demonstrated the importance of PI3'-kinase in mediating the NGF modulation of apoptosis in PC12 cells. The involvement of the PI3'-kinase pathway on prevention of apoptosis by IGF-1, but not by IL-3, was also recently observed in hemopoietic progenitor cells (41). The effect of rapamycin, an inhibitor of p70S6K (21), further supports the role of the PI3'-kinase pathway in mediating the IGF-1 effect on apoptosis.

However, we have noted that overexpression of Δp85 did not result in a complete inhibition of the IGF-1 action, suggesting the possibility that IGF-1 may inhibit apoptosis via other signal transduction pathways. We chose, therefore, to study the role of the MAP kinase pathway using a synthetic inhibitor of MEK. The MAP kinase pathway is responsible for mediating numerous effects of both insulin and IGF-1. Both insulin and IGF-1 receptors activate the MAP kinase cascade through a p21ras-dependent signal transduction pathway. Activated Ras interacts with the serine-threonine kinase Raf and localizes it to the membrane, thereby initiating Raf activation. Activated Raf then initiates the kinase cascade by phosphorylating and activating MEK, which in turn phosphorylates and activates the MAPK/ERK.

PD098059 is a small molecular weight inhibitor of MEK activity, as measured by MAP kinase activity (22, 23). PD098059 is a specific noncompetitive inhibitor of MEK, with respect to ATP binding, and does not inhibit several other kinases tested (22). PD098059 inhibits MAP kinase activity in
PC12 cells with an IC\textsubscript{50} of approximately 10^{-6} M (22, 23). In our study, incubation of PC12 cells with concentrations of PD908059 capable of significantly inhibiting MAP kinase activity resulted in increased apoptosis when given alone. PD908059 could also block the protective effect of IGF-1. After 6 h of incubation with PD908059, neither a decrease in cell number nor DNA laddering could be found although a decrease in the metabolic activity in the cells treated with the inhibitor could be already detected by this time. This decrease in mitochondrial activity precedes by some hours the initiation of apoptosis as detected by loss of cell number and the appearance of DNA laddering. This loss of metabolic activity preceding apoptosis triggering has been previously found in sympathetic neurons deprived of growth factor (42). Thus, our results demonstrate that the MAP kinase pathway is also involved in IGF-1 prevention of apoptosis. A different conclusion was reached by Yagi and Cooper (4) using PC12 cells. However, their studies involved the use of the expression of the dominant inhibitory mutant Ras N17, which interferes with normal Ras function and Raf activation. Since Raf and MEK activation may be achieved by pathways other than Ras (31, 32, 43), we suggest that inhibition of the pathway downstream of Raf, as in the present study, is important to establish the role of the MAP kinase pathway. Moreover, in PC12 cells, it has been described that the expression of an activated Raf mutant did not activate the MAP kinases although it resulted in gene expression similar to that induced by NGF (44). This result suggests that the MAP kinases may be activated by other pathways in addition to the Ras/Raf pathway in this cell type. Further confirmation for our conclusion that the MAP kinase pathway is involved in IGF-1 inhibition of apoptosis comes from a recent study (38) that demonstrates a role for the MAP kinases in apoptosis prevention by NGF in PC12 cells.

Since the PI3'-kinase and MAP kinase pathways converge at some point before MEK activation (31, 32), it is possible that the wortmannin inhibition of PI3'-kinase in our cells affects MAP kinase activation. Furthermore, recent studies have also shown that following certain stimuli, MEK may be activated by a PI3'-kinase-dependent pathway (32). Consistent with this are our findings that wortmannin is also capable of inhibiting IGF-1-stimulated MAP kinase activity to a certain extent. However, while the possibility that the wortmannin effect is entirely due to the inhibition of the MAP kinase pathway is consistent with our data, it is less likely because the wortmannin effect on IGF-1 inhibition of apoptosis occurs much earlier than the effect of PD908059 on IGF-1 action. Furthermore, rapamycin alone was also capable of inhibiting the effect of IGF-1 in preventing apoptosis. We therefore postulate that both pathways are involved separately in this process and that the synergistic effect we obtained by using the MEK inhibitor together with wortmannin suggests some convergence of the two pathways either at the level of MEK or at a more distal point. The convergence of the two pathways is supported by our findings that the effectiveness of wortmannin was greater than the effect of rapamycin.

While we have described in this study two separate IGF-1-stimulated pathways that inhibit apoptosis, it is also likely that IGF-1 inhibition of apoptosis involves other as yet unidentified pathways. The elucidation of these pathways should ultimately lead to a better understanding of cellular growth and apoptosis and enable investigators to design new and effective therapeutic agents.

Acknowledgments—We thank Professor Masato Kasuga (Kobe University, Japan) for the gifts of the wild-type and dominant-negative p85 constructs and Drs. V. Blakesley and E. Wertherme (National Institutes of Health, Bethesda, MD) for critical reading of the manuscript.
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J. Biol. Chem. 1997, 272:154-161.
doi: 10.1074/jbc.272.1.154

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