

Insulin-Like Growth Factor-1–Induced Phosphorylation of Transcription Factor FKHRL1 Is Mediated by Phosphatidylinositol 3-Kinase/Akt Kinase and Role of This Pathway in Insulin-Like Growth Factor-1–Induced Survival of Cultured Hippocampal Neurons

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ABSTRACT

Insulin-like growth factor-1 (IGF-1) is a trophic factor promoting cell survival by activating phosphatidylinositol 3-kinase (PI3K)/Akt kinase pathway. FKHRL1, a member of the Forkhead family of transcription factors possibly involved in cell cycle and apoptosis, is a downstream target of Akt in fibroblasts. However, very little information is available concerning neurons. We report herein that IGF-1 rapidly induced the phosphorylation of endogenous FKHRL1 in hippocampal neurons. The PI3K/Akt kinase pathway mediates this action, as evidenced by the use of different kinase inhibitors, the expression of constitutively active Akt, and *in vitro* kinase assay. IGF-1 blocked the nuclear translocation of FKHRL1 in hippocampal neurons and promoted survival in parallel to the phosphorylation of Akt and FKHRL1. Similarly, the expression of constitutively active Akt in

PC-12 cells increased the phosphorylation of FKHRL1 and promoted survival, whereas the expression of kinase dead Akt attenuated IGF-1–mediated survival of PC-12 cells. Moreover, the overexpression of wild-type FKHRL1 and its nonphosphorylated mutant induced apoptosis in cultured hippocampal neurons. Interestingly, IGF-1 and PI3-kinase inhibitors have no significant effect on the cell cycle inhibitor p27kip1 in hippocampal neurons. This finding suggests that in contrast to fibroblasts, FKHRL1 is unlikely to be involved in cell cycle in neurons. Taken together, these data reveal that endogenous FKHRL1 is a downstream substrate of PI3K/Akt in IGF-1 receptor signaling in hippocampal neurons and suggest that the phosphorylation of this transcription factor may play an important role in the neuronal survival properties of IGF-1.

Insulin-like growth factor-1 (IGF-1) is a polypeptide growth factor playing an important role in the normal development and maintenance of cellular integrity of the organism, including the central nervous system (Butler et al., 1998; Zheng et al., 2000a). IGF-1 and its receptors are expressed in different brain regions, including hippocampal formation, which play important roles in learning processes and are severely affected in Alzheimer's Disease (Doré et al., 1997; Zheng et al., 2000a). Recent studies have shown that

IGF-1 possesses trophic effects in the hippocampus and promotes cell survival of cultured hippocampal neurons against different insults (Doré et al., 1997; Matsuzaki et al., 1999; O'Kusky et al., 2000; Yamaguchi et al., 2001; Trejo et al., 2001).

The biological actions of IGF-1 are mostly mediated by type I IGF receptor. Binding of IGF-1 to this receptor activates its intrinsic receptor tyrosine kinase, which phosphorylates several intracellular substrates such as the insulin receptor substrate-1 and Shc (Myers et al., 1993; Sasaoka et al., 1994; LeRoith et al., 1995), leading to the activation of various signaling pathways, including the mitogen-activated protein (MAP) kinase (also called extracellular signal-regulated ki-

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ABBREVIATIONS: IGF-1, insulin-like growth factor-1; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; PI3, phosphatidylinositol 3; GSK3, glycogen synthase kinase 3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; LF2000, LipofectAMINE 2000; RIPA, radioimmunoprecipitation assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS+T, phosphate-buffered saline containing 0.3% Triton X-100; NDS, normal donkey serum; HA, hemagglutinin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; MEK, mitogen-activated protein kinase kinase; PD98059, 2(2'-amino-3'-methoxyphenyl); LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; IP, immunoprecipitated or immunoprecipitation; CMV, cytomegalovirus; Bim, Bcl-2 interacting mediator.

nase; ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt (Butler et al., 1998; Zheng et al., 2000a) pathways.

Akt is a serine/threonine kinase and a downstream target of PI3-kinase involved in cell survival induced by various growth factors (Dudek et al., 1997; Datta et al., 1999). The enzymatic phosphorylation of the Thr308 and Ser473 residues on Akt kinase activates this kinase (Alessi et al., 1996; Delcommenne et al., 1998; Kitamura et al., 1998; Balendran et al., 1999). Active Akt in turn phosphorylates and inhibits several proapoptotic proteins such as Bad (del Peso et al., 1997), caspase-9 (Cardone et al., 1998), and most recently the winged-helix family of transcription factors, FKHRL1 (Brunet et al., 1999; Zheng et al., 2000b), FKHR (Guo et al., 1999; Nakae et al., 1999; Tang et al., 1999), and AFX (Kops and Burgering, 1999), leading to cell survival (Datta et al., 1999).

FKHRL1 is a member of the Forkhead transcription factors possibly involved in cell survival and cell cycle (Brunet et al., 1999; Kops and Burgering, 1999; Medema et al., 2000). Recent studies have suggested that FKHRL1 is a proapoptotic protein and a downstream target of Akt in several cell types, including neuronal cells (Brunet et al., 1999, 2001; Zheng et al., 2000b). However, no information is currently available on the effect of IGF-1 on the phosphorylation of endogenous FKHRL1 and its effect on the survival of hippocampal neurons. Accordingly, the major aim of the present study was to investigate whether IGF-1, acting via the PI3K/Akt kinase pathway, was able to induce the phosphorylation of FKHRL1 in hippocampal neurons and the possible role of this event on cell survival.

Experimental Procedures

Materials. Human recombinant IGF-1 was obtained from Genentech (South San Francisco, CA). Wortmannin, leupeptin, and aprotinin were from Sigma-Aldrich (St. Louis, MO). U0126 was obtained from Promega (Madison, WI). Anti-FKHRL1-Ser-253, anti-FKHRL1-Thr-32, anti-FKHRL1, and anti-Akt α antibodies and purified active Akt were from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Akt, anti-phospho-ERK, anti-phospho-GSK3 α/β antibodies, and GSK3 α fusion protein were from New England Biolabs (Beverly, MA). Anti-ERKs and all secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmids of CMV6, and CMV6-M179A-Akt-HA were kindly provided by Drs. Sandeep S. R. Datta and M.E. Greenberg (Harvard Medical School, Boston, MA), and GAP-Akt was a generous gift from Dr. B. M. Burgering (Utrecht University, Utrecht, The Netherlands). β -Gal staining kit containing CMVLacZ control vector and pcDNA3.1 were from Invitrogen (Carlsbad, CA). G418, trypsin, B27, N2, LipofectAMINE 2000, and other cell culture reagents were purchased from Invitrogen, whereas all other reagents were from Sigma-Aldrich or Fisher Scientific (Nepean, ON, Canada).

PC-12 Cell Culture. PC-12 cells were kindly provided by Dr. Gordon Guroff (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD). Cells were maintained in 75-cm² flasks in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (FBS), 5% horse serum, 100 μ g of streptomycin/ml, and 100 U of penicillin/ml and incubated at 37°C with 5% CO₂ humidified atmosphere. Cultured mediums were replaced twice a week with fresh medium as described above. Stock culture was routinely subcultured at 1:5 ratio at a weekly interval.

Hippocampal Neuronal Cultures. Hippocampal cultured neurons were prepared from fetuses (embryonic day 19) obtained from pregnant Sprague-Dawley rats (Charles River Canada, Montreal, PQ, Canada) and cultured in serum-free condition as described pre-

viously with minor modifications (Doré et al., 1997, nearly pure neuronal population are obtained under this condition). Animal care was according to protocols and guidelines approved by McGill University Animal Care Committee and the Canadian Council for Animal Care. Hippocampi were dissected in Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (HBSS) supplemented with 15 mM HEPES, 10 U/ml penicillin, and 10 μ g/ml streptomycin. Tissues were collected and washed four to five times with HBSS and then submitted to an enzymatic digestion at 37°C with 0.25% trypsin in HBSS media for 10 min. After the reaction was stopped by the addition of FBS (final concentration 10% for FBS), tissue was rinsed with HBSS four to five times to remove FBS. The cellular suspension was then obtained by repeating aspirations through a Pasteur pipette. After a centrifugation at 800g for 10 min, the medium were removed and cells were resuspended in a chemically defined serum-free medium, Neurobasal, supplemented with 2% B27 (or 10% FBS for mixed culture), 20 μ M L-glutamine, 15 mM HEPES, 10 U/ml penicillin, and 10 μ g/ml streptomycin. Neurons were plated at density of 5 to 8 \times 10⁵ cells/ml in cultured plates (coated with 10 μ g/ml poly-D-lysine) under serum-free conditions and grown at 37°C with 5% CO₂ humidified atmosphere. On the day after the plating, the medium was replaced with fresh culture medium. Medium was changed again with either the same medium as described above or Neurobasal supplemented with 1% N2 after 4 to 5 days. Experimental treatments were performed on the 7th day after plating.

Subcloning and Transfection. pcDNA-FKHRL1-WT (wild type) and pcDNA-FKHRL1-TM (a mutant of FKHRL1 in which the Akt phosphorylated sites Thr32, Ser253, and Ser315 are mutated for Ala) were constructed by subcloning the Hind III-XbaI fragments from PECE HA-FKHRL1-WT and PECE HA-FKHRL1-TM plasmids (Brunet et al., 1999; kindly provided by Drs. J. Zieg and M. E. Greenberg, Harvard Medical School). Transfection of PC-12 cells and hippocampal neurons with Akt was performed as indicated by Invitrogen with LipofectAMINE 2000 (LF2000) as described previously with minor modification (Zheng et al., 2000b). Briefly, cells were plated in 24-well plates at 2.5 \times 10⁵ cells/well in 0.5 ml of DMEM containing 5% FBS and 5% horse serum without antibiotics. On the following day, 0.8 μ g of DNA in 50 μ l of Opti-MEM reduced serum medium was mixed with 2 μ l of LF2000 prediluted in 50 μ l of Opti-MEM reduced serum medium for each well. After incubation at room temperature for 20 min to allow DNA-LF2000 complexes to form, 100 μ l of mixture was added to each well, which contained 0.2 ml of medium from the previous day. Then, the plate was shaken gently and cells were incubated at 37°C in a 5% CO₂ incubator for 24 to 48 h before the assay was performed. For cultured hippocampal neurons, transfection was performed on 3rd day after plating in serum-free medium. Transfection efficiency was determined by cotransfection with a CMVLacZ control vector at a 1:10 (control vector/DNA) ratio followed by in situ staining with a β -Gal staining kit as suggested by Invitrogen. The expression of Akt was confirmed by Western blot using an anti-Akt antibody.

Treatments. Before each experiment, cells were detached using 5 mM EDTA in HBSS and seeded in 12- or six-well plates (coated with poly-D-lysine, 10 μ g/ml) at a density of 4 to 8 \times 10⁵ cells/well in 2% serum medium for 24 h. Culture medium was replaced with DMEM (for primary cultures, medium was replaced with Neurobasal) 2 to 3 h before the desired reagents were added. To study the effect of different stimuli on the phosphorylation of various signaling proteins, cells were treated with 10 to 100 nM IGF-1. Alternatively, cells were pretreated with wortmannin (0.25–2 μ M, 20 min), LY294002 (12.5–100 μ M, 20 min), rapamycin (50 nM, 20 min), PD98059 (50 μ M, 40 min), and U0126 (20 μ M, 30 min) followed by stimulation with 10 to 100 nM IGF-1 or other stimuli.

Western Blotting. Western blotting was performed as described previously with some modifications (Zheng et al., 2000b,c). Briefly, treated cells from different experimental conditions were rinsed twice with ice-cold HBSS and lysed in either RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630,

0.1% SDS, 50 mM NaF, 1 mM NaVO₃, 5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 50 µg/ml aprotinin) or sample buffer [62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 1% glycerol, 50 mM dithiothreitol, and 0.1% (w/v) bromphenol blue]. Samples with equal amounts of protein were then separated by 4 to 20% polyacrylamide gel electrophoresis, and the resolved proteins were electrotransferred to Hybond-C nitrocellulose. The respective phosphorylation of Akt, MAP kinase, and FKHRL1 was determined by using anti-phospho-Akt, anti-phospho-ERK, or a mixture of anti-phospho-FKHRL1-Thr32 and phospho-FKHRL1-Ser253 antibodies, respectively. To establish the phosphorylation of FKHRL1 at Thr32 or Ser253 residues, anti-phospho-FKHRL1-Thr32 or anti-phospho-FKHRL1-Ser253 was used, respectively. Blots were stripped and reprobed with antibodies for the above-mentioned proteins to ensure that equal amounts of these proteins are present. In some experiments, two parallel running gels loaded with identical samples were used. One of them was to evaluate the phosphorylation of FKHRL1, whereas the other was to determine FKHRL1 levels in cell extracts. Quantification of the blots was performed by using an MCID image analyzer (Imaging Research, St. Catharines, ON, Canada).

Akt in Vitro Kinase Assay. The Akt kinase assay was performed as described previously (Zheng et al., 2000c) with some modifications. Briefly, cells were treated with 100 nM IGF-1 with or without 0.5 µM wortmannin, and Akt was separated by immunoprecipitation using anti-Akt antibody as mentioned above. The immunoprecipitates were then washed four times with RIPA buffer and once with kinase buffer [25 mM Tris-HCl pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, and 10 mM MgCl₂]. Subsequently, an in vitro kinase reaction was carried out in 40 µl of kinase buffer containing precipitated Akt, 200 µM ATP, and 0.2 µg of GSKα fusion protein as substrate. After 30-min incubation at 34°C, the reaction was stopped by addition of 10 µl of 5× reduced SDS sample buffer. Akt activity was then determined by Western blots by measuring the level of phosphorylation of GSK3α fusion protein with anti-phospho-GSK3 α/β antibody.

Phosphorylation of FKHRL1 by Active Akt in Vitro. PC-12 cells and hippocampal neurons were pretreated with 0.5 µM wortmannin and then FKHRL1 was separated as described above by immunoprecipitation using anti-FKHRL1 antibody. The immunoprecipitates were washed four times with RIPA buffer and once with kinase buffer as described above. Next, an in vitro kinase reaction was carried out in 40 µl of kinase buffer containing precipitated FKHRL1 with or without 0.2 µg of purified active Aktα and 200 µM ATP. In the positive control tubes, precipitated FKHRL1 was replaced by 0.2 µg of GSKα fusion protein as substrate. After a 30-min incubation at 34°C, the reaction was stopped by addition of 10 µl of 5× reduced SDS sample buffer. Phosphorylation of FKHRL1 at Thr32 and Ser253 was determined by Western blots using anti-phospho-FKHRL1-Thr32 and anti-FKHRL1-Ser253, respectively. Akt activity was determined by Western blot by measuring the phosphorylation of GSK3α fusion protein with anti-phospho-GSK3 α/β antibody.

Cell Viability Using the MTT Assay. PC-12 cells (10,000–20,000 cells/well) in serum-free medium DMEM or DMEM supplemented with 1% FBS was added to 96-well plates and incubated at 37°C with 5% CO₂ for 1 h. For primary cultured neurons, cells were deprived of N2 supplements to induce cell death at day 7 by replacing medium with Neurobasal without N2. Cells were treated with different reagents (1% FBS, 100 nM IGF-1, 25 µM LY294002, 25 µM PD89059, and 20 nM rapamycin) for different times as indicated in individual figure legend and were grown for 1 or 2 days for PC-12 cells and 4 to 5 days for cultured neurons. After replacement of the medium with 0.5 mg/ml MTT in DMEM, cells were returned into the incubator for a 3-h period. Cells and MTT formazan crystals were then solubilized by trituration in a solution of isopropanol/HCl (0.1 N), and the survival profile of these cells was quantified by spectrophotometrically measuring the plate at 570 nm. Assays were repeated at least three to six times in quadruplicate. To

evaluate the effect of Akt on the survival of PC-12 cells, PC-12 cells transfected with CMV/GAPAkt/M179A-Akt, and survival of transfected PC-12 cells was determined at day 2 as parental PC-12 cells.

Detection of Apoptotic Nuclei by Hoechst 33342 Staining. After various treatments, hippocampal cultured neurons were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min with nonspecific binding blocked using 5% normal goat serum (Vector Laboratories, Burlingame, CA) in 0.01 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBS+T). Cells were washed twice with PBS and incubated with 10 µg/ml Hoechst 33342 in PBS for 10 min at room temperature. Chromatin staining pattern was then analyzed for individual cells by fluorescence microscopy.

Immunofluorescence Staining of HA-Akt and Subcellular Localization of FKHRL1. Immunofluorescence in primary cultured neurons was performed as described previously with minor modifications (Ma et al., 2000). After various treatments, hippocampal cultured neurons were fixed in 4% paraformaldehyde in 0.1 M PB for 20 min with nonspecific labeling blocked using 5% normal donkey serum (NDS; Vector Laboratories) in 0.01 M PBS containing PBS+T. Cells were washed twice with PBS+T and incubated with anti-FKHRL1 antibody (1:100)/anti-HA (1:100) diluted in PBS+T containing 5% NDS at 4°C for 48 h. After the removal of the primary antibodies and rinsing (2 times) with PBS+T, cells were stained with corresponding secondary antibodies conjugated with fluorescein for anti-FKHRL1 (fluorescein isothiocyanate, 1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) or Texas-Red for anti-HA-Akt (1:100; Jackson ImmunoResearch Laboratories) diluted in PBS+T containing 5% NDS for 1 h. Cells stained with FKHRL1 or HA-Akt were evaluated by fluorescence microscopy, whereas the subcellular localization of FKHRL1 was visualized by confocal microscopy (PCM 2000; Nikon, Tokyo, Japan).

Double Labeling of HA-Texas-Red for FKHRL1 and TUNEL Staining. Cells transfected with FKHRL1 stained with HA-Texas-Red as described above and apoptotic profile were determined by TUNEL staining using In Situ Cell Death Detection kit (Roche Applied Science, Mannheim, Germany) as suggested by the manufacturer. HA-FKHRL1 positive (red) and apoptosis (green) TUNEL positive cells were visualized by fluorescence microscopy as described above.

Statistical Analysis. Data are expressed as mean ± S.E.M. A one-way analysis of variance with Student-Newman-Keuls test was used to establish statistical significance set at $p < 0.05$.

Results

IGF-1 Time and Concentration Dependently Induced the Phosphorylation of Akt and FKHRL1 in Cultured Hippocampal Neurons. To establish the effect of IGF-1 on endogenous FKHRL1 in neurons, rat cultured hippocampal neurons were treated with 100 nM IGF-1 for different periods of time or for 10 min with various concentrations of IGF-1, and the phosphorylation of Akt and FKHRL1 was investigated. Figure 1, A and B, shows that IGF-1 rapidly induced the phosphorylation of Akt and FKHRL1 in cultured hippocampal neurons. The phosphorylation of Akt and FKHRL1 was already evident at 2.5 min, peaked, and remained stable for at least 40 min. The phosphorylation of Akt and FKHRL1 was observed at a minimal concentration of 1 to 3 nM IGF-1 and reached maximal levels at about 10 to 30 nM IGF-1 (Fig. 1, C and D).

IGF-1-Induced Phosphorylation of Akt and FKHRL1 Is Mediated by the PI3-Kinase Pathway. Hippocampal neurons were then pretreated with different kinase inhibitors and stimulated with 100 nM IGF-1 for 10 min. IGF-1 induced a 2- to 4-fold increase in the phosphorylation of FKHRL1 and Akt (Fig. 2, lane 5 versus 1). Pretreatment with

the PI3-kinase inhibitor wortmannin (0.5 μ M) blocked IGF-1-induced phosphorylation of FKHRL1 and Akt (Fig. 2, lane 6 versus 5). In contrast, the MEK inhibitor PD98059 (50 μ M), an upstream blocker of MAP kinase, did not affect IGF-1-induced phosphorylation of Akt and FKHRL1 (Fig. 2, lane 7 versus 5). Similarly, the p70 S6 kinase inhibitor rapamycin (50 nM) failed to alter IGF-1-induced phosphorylation of FKHRL1 and Akt (Fig. 2, lane 8 versus 5). These data suggest that the PI3-kinase is located upstream of Akt and FKHRL1. Consistent with this hypothesis, an *in vitro* kinase assay using GSK3 α fusion protein as substrate showed that IGF-1-induced activation of Akt is blocked by the PI3-kinase inhibitor wortmannin (Fig. 2B). Figure 3 demonstrates that the inhibitory effects of wortmannin and LY294002 against IGF-1-induced Akt and FKHRL1 phosphorylation are concentration-dependent. Interestingly, IGF-1 and the PI3-kinase inhibitors failed to have any significant effect on the expression of the cell cycle inhibitor p27kip (Fig. 2A), suggesting that this protein is unlikely to be a downstream target of FKHRL1 in our model.

To investigate the phosphorylation of FKHRL1 in greater details, the effect of IGF-1 on the phosphorylation of the Thr32 and Ser253 residues was studied. Figure 4 shows that in hippocampal neurons, the phosphorylation of FKHRL1 at these two sites is induced by 100 nM IGF-1, and in a PI3-kinase-dependent manner as demonstrated by the inhibitory action of wortmannin (lane 6 versus 5) but not by PD98059 (lane 7 versus 5) or rapamycin (lane 8 versus 5).

Activated Akt Directly Phosphorylated FKHRL1 on Thr32 and Ser253 Residues, whereas the Expression of Constitutively Active Akt (GAP-Akt) Increased the Phosphorylation of FKHRL1 in Hippocampal Neurons. Our data indicated that the phosphorylation of FKHRL1 induced by IGF-1 is mediated by the PI3K/Akt kinase pathway. However, it is not known whether Akt can directly phosphorylate FKHRL1 in hippocampal cultured neurons. Therefore, an *in vitro* kinase assay with purified recombinant activated Akt was performed. The incubation of purified GSK3 α fusion protein (used as a positive control) with active

Akt increased the phosphorylation of GSK3 α (Fig. 5, A and B, lane 2 versus 1). These results established the functionality of our Akt kinase assay. Figure 5, A and B, shows that in parallel assays with immunoprecipitated (IP) FKHRL1 from PC-12 cells, significant increases in the phosphorylation of the Thr32 and Ser253 residues are observed from reaction with the purified enzyme, whereas a control reaction had no effect (Fig. 5, A and B, lane 4 versus 3). Similar results were obtained with IP-FKHRL1 from hippocampal neurons (Fig. 5, A and B, lane 6 versus 5). Furthermore, the expression of constitutively active Akt (GAP-Akt) increased the phosphorylation of Akt and FKHRL1 in hippocampal neurons and PC-12 cells (Fig. 5C). These results indicate that the activation of Akt is sufficient to induce the phosphorylation of FKHRL1 in these two preparations and further support a role for Akt kinase in the phosphorylation of FKHRL1 induced by IGF-1.

IGF-1 Inhibits the Nuclear Translocation of FKHRL1 in Hippocampal Neurons. A recent study in fibroblasts suggested that the dephosphorylation of FKHRL1 promoted the translocation of this transcription factor in the nucleus, whereas its phosphorylation by Akt maintained its cytoplasmic localization in an inactive form (Brunet et al., 1999). To establish whether this is true in neurons, cultured hippocam-

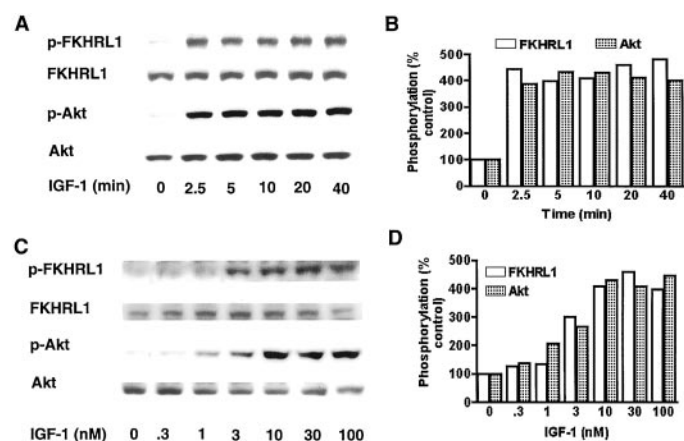


Fig. 1. IGF-1 induced time- (A) and concentration (C)-dependent phosphorylation of FKHRL1 and Akt in cultured hippocampal neurons. Hippocampal culture was treated with 100 nM IGF-1 (A) for various times or with different concentrations of IGF-1 for 10 min (C) and then the phosphorylation of FKHRL1 and Akt was determined as described under *Experimental Procedures*. Blots represent prototypical examples of experiments replicated at least three times. Quantitative data (OD, optical density) for A and C are shown in B and D, respectively.

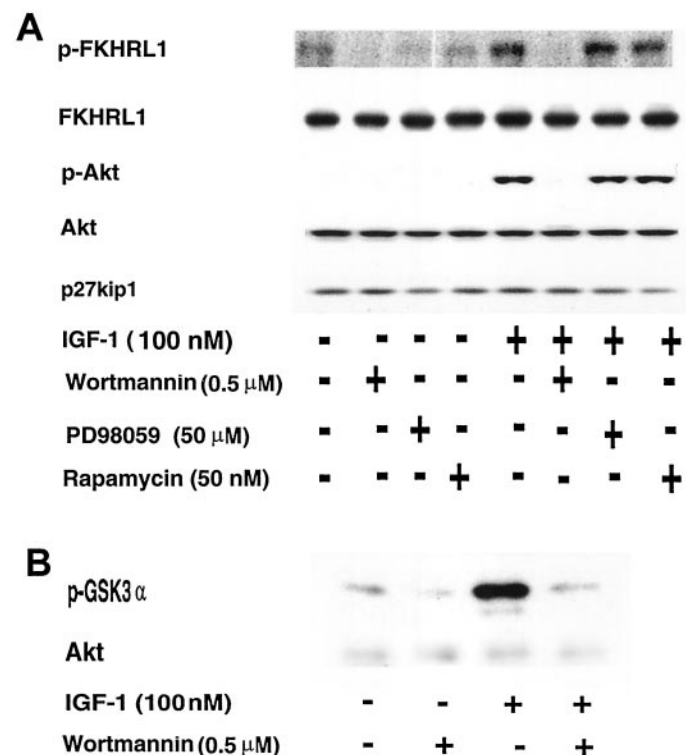


Fig. 2. IGF-1 induced the phosphorylation of FKHRL1 in cultured hippocampal neurons via a PI3-kinase pathway. A, after treatment with different kinase inhibitors, hippocampal neurons were exposed to 100 nM IGF-1 and then the phosphorylations of FKHRL1 and Akt were determined by Western blot using anti-phospho-FKHRL1/Akt antibodies. The PI3-kinase inhibitor wortmannin inhibited IGF-1-induced phosphorylation of FKHRL1 and Akt in hippocampal cultured neurons, whereas an MEK kinase inhibitor, PD98059, and a p70S6 pathway inhibitor, rapamycin, did not have any significant effect. B, activity of Akt separated from hippocampal neurons treated with IGF-1 or wortmannin was determined by *in vitro* kinase assay as described under *Experimental Procedures*. IGF-1 stimulated activation of Akt and this is blocked by the PI3-kinase inhibitor wortmannin. Blots represent prototypical example of experiments replicated at least three times.

pal neurons were treated either with the PI3K inhibitor LY294002 to block the phosphorylation of FKHRL1 by endogenous Akt or with IGF-1 (100 nM) to increase its phosphorylation, and the subcellular localization of FKHRL1 was visualized by immunofluorescence using an anti-FKHRL1 antibody. Figure 6A shows that FKHRL1 is distributed into various cellular compartments under control conditions, whereas a decrease in its phosphorylation induced by LY294002 led to a clearly more nuclear localization. In contrast, IGF-1-induced increases in the phosphorylation of FKHRL1 caused the retention of this protein into the cytoplasm (Fig. 6A).

IGF-1 Protects PC-12 Cells and Hippocampal Cultured Neurons from Serum/N2 Deprivation. It is well documented that serum deprivation induces cell death (Maroto and Perez-Polo, 1997). To establish further the role of Akt and FKHRL1 in cell survival, PC-12 cells and cultured hippocampal neurons were serum/N2-deprived. Figure 6, B and C, shows that a 4- to 5-day N2 deprivation resulted in 40 to 60% neuronal losses in cultured hippocampal neurons, whereas a 48- to 72-h serum deprivation caused 50 to 70%

death in PC-12 cells. IGF-1 significantly increased the survival of PC-12 cells and hippocampal neurons with the maximal effect observed at 10 to 30 nM in PC-12 cells and 30 to 100 nM in hippocampal neurons.

IGF-1 Promotes the Survival of Hippocampal Neurons by the PI3 Kinase Pathway. Hippocampal cultured neurons pretreated with different kinase inhibitors were stimulated with IGF-1 and then the survival was determined. Figure 6D reveals that IGF-1 promoted hippocampal neuron survival and that this effect is inhibited by the PI3-kinase inhibitor LY294002 but not by the MEK inhibitor PD89059 or the S6p70 pathway inhibitor rapamycin.

In accordance with these findings, the PI3-kinase inhibitor LY294002 concentration dependently decreased the phosphorylation of endogenous Akt and FKHRL1 and the viability of hippocampal neurons (Fig. 7, A and B).

Expression of Constitutively Active Akt (GAP-Akt) Promotes the Survival of PC-12 Cells, whereas the Expression of Kinase Dead (179M-Akt) Akt Attenuates IGF-1-Mediated Survival in These Cells. To further establish the role of Akt in the survival of IGF-1-treated neuronal cells, PC-12 cells were transiently transfected with CMV control plasmid, constitutively active Akt (GAP-Akt), and kinase dead Akt, and the effect of these kinases on the survival of PC-12 cells was determined. Figure 8A shows that serum deprivation of PC-12 cells for 48 h induced about 50% cell death, whereas the expression of constitutively active-Akt significantly protected the cells. Moreover, 10 nM IGF-1 protected PC-12 cells from serum deprivation, an effect

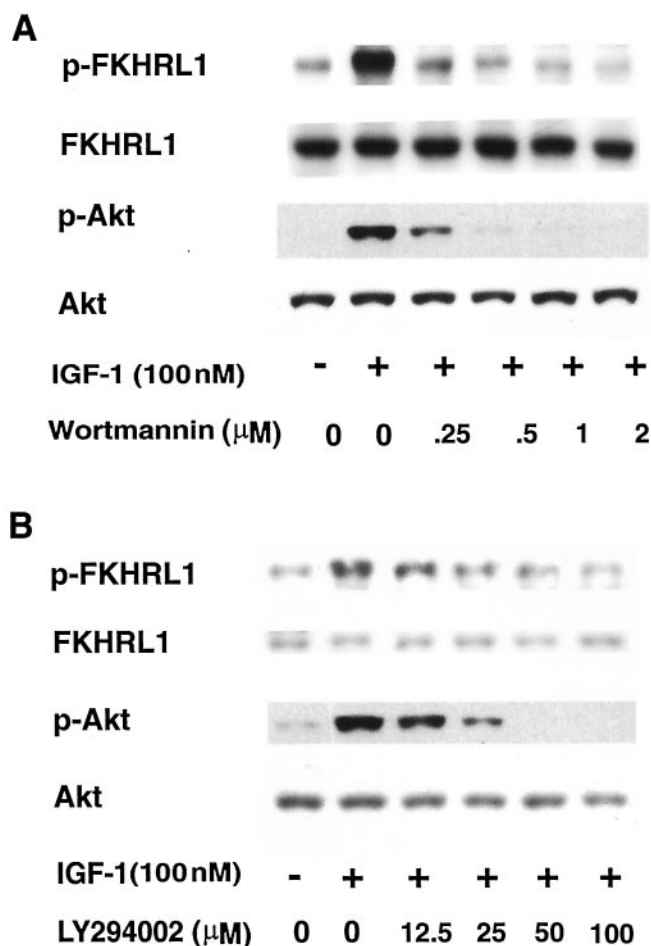


Fig. 3. IGF-1-induced phosphorylation of Akt and FKHRL1 is concentration dependently inhibited by the PI3K inhibitors wortmannin (A) and LY294002 (B). Cultured hippocampal neurons pretreated with different concentrations of wortmannin or LY294002 were stimulated with 100 nM IGF-1, and the phosphorylation of Akt and FKHRL1 was determined as described under *Experimental Procedures*. Wortmannin and LY294002 block the IGF-1-induced phosphorylation of Akt and FKHRL1 in a concentration-dependent manner. Blots represent prototypical example of experiments replicated at least three times.

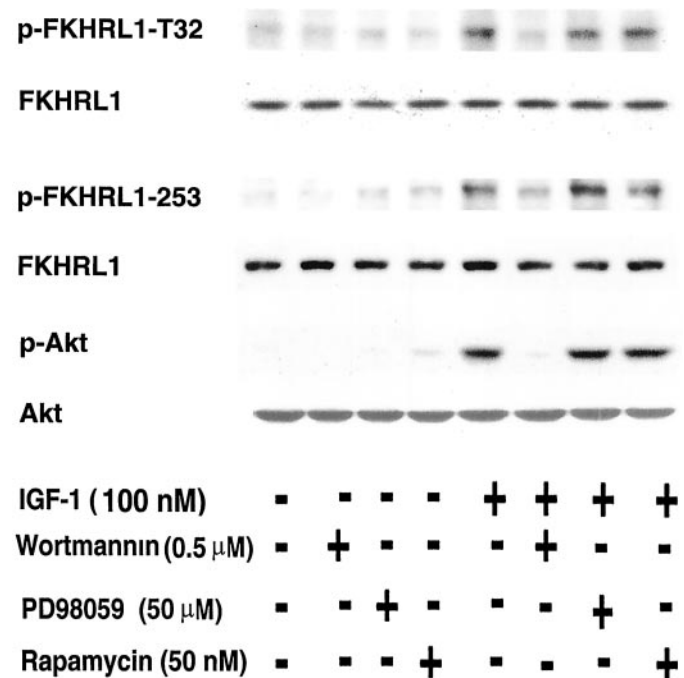


Fig. 4. PI3K/Akt kinase pathway mediates IGF-1-induced phosphorylation of Thr32 and Ser253 residues of FKHRL1 in hippocampal neurons. Hippocampal neurons pretreated with different pathway inhibitors were stimulated with 100 nM IGF-1 and then the phosphorylation of FKHRL1 at Thr32 or Ser253 residues was determined by Western blots using anti-phospho-FKHRL1-Thr32 or anti-phospho-FKHRL1-Ser253 antibodies, respectively. Only the PI3K/Akt inhibitor wortmannin blocked IGF-1-induced phosphorylation of residues Thr32 and Ser253. Blots represent prototypical examples of experiments replicated at least three to five times.

blocked by the expression of kinase dead Akt (Fig. 8A). In accordance of these findings, overexpression of HA-Akt protected hippocampal neurons from apoptosis induced by N2 deprivation (Fig. 8B).

Overexpression of FKHRL1 Induced Apoptosis of Cultured Hippocampal Neurons. To examine the effect of FKHRL1 on apoptosis, hippocampal neurons transfected with FKHRL1 or its nonphosphorylated mutant were treated with IGF-1, and the apoptotic profiles were determined by TUNEL and Hoechst staining as described under *Experimental Procedures*. Consistent with previous reports (Brunet et al., 1999, 2001; Shin et al., 2001), Fig. 8C showed that although the overexpression of both wild-type and mutant FKHRL1 caused apoptosis in some hippocampal neurons, IGF-1 only attenuated the apoptotic effect of wild-type FKHRL1.

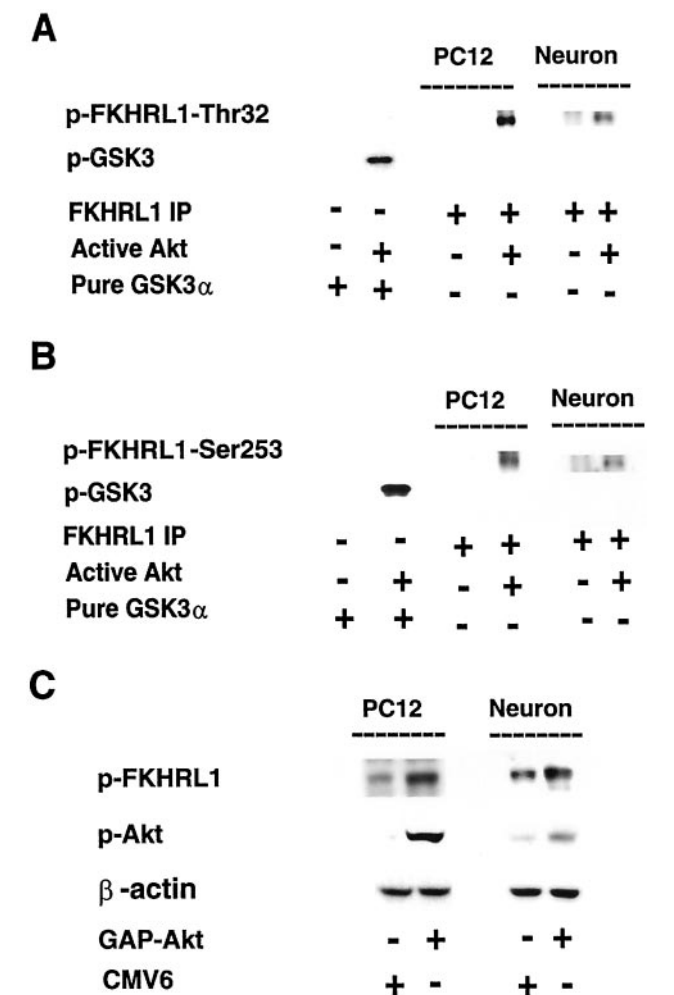


Fig. 5. Akt kinase directly phosphorylates Thr32 and Ser253 residues of FKHRL1 isolated from PC-12 cells and hippocampal neurons, whereas expression of constitutive active Akt increases the phosphorylation of FKHRL1 in these cells. A and B, FKHRL1 from PC-12 cells or hippocampal neurons was separated by IP and used in *in vitro* kinase assay with active Akt. The phosphorylation of FKHRL1 at the Thr32 (A) and Ser253 (B) residues was determined as described under *Experimental Procedures*. C, PC-12 cells and hippocampal cultured neurons were transfected with Gap-Akt, and the phosphorylation of FKHRL1 and Akt was determined. Active Akt phosphorylated FKHRL1 at Thr32 and Ser253, whereas constitutively active Akt increases the phosphorylation of FKHRL1 in PC-12 cells and hippocampal neurons. Blots represent prototypical example of experiments replicated at least three times.

Discussion

In the present study, we have shown that IGF-1 can induce, via the PI3-kinase pathway, the phosphorylation of both Akt (leading to its activation) and FKHRL1 (leading to its inactivation) in hippocampal neurons. Moreover, our data

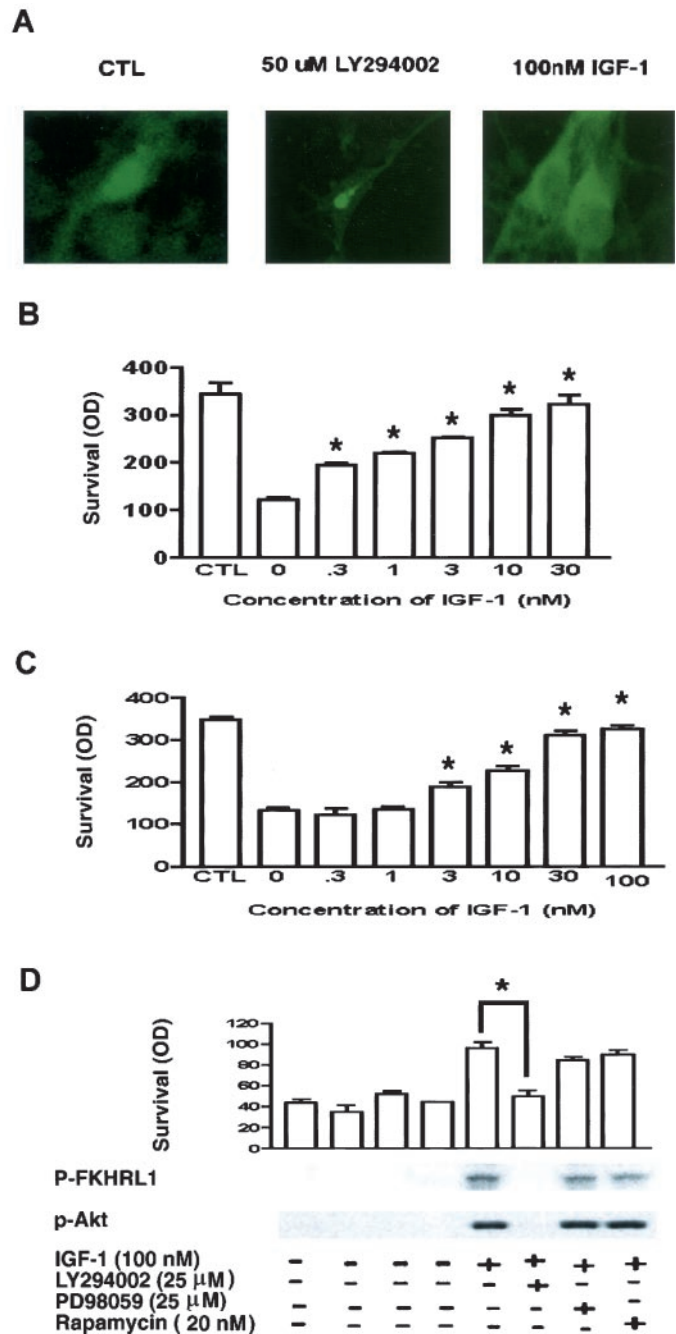


Fig. 6. IGF-1 blocks the nuclear translocation of FKHRL1 in hippocampal neurons and protects these neurons from N2 deprivation. Cultured hippocampal neurons were treated with IGF-1 or different reagents, and the subcellular localization and survival were determined as described under *Experimental Procedures*. A, IGF-1 blocked the nuclear localization of FKHRL1 in hippocampal neurons. B, IGF-1 concentration dependently protected PC-12 cells from serum deprivation. C, IGF-1 protected hippocampal neurons from N2 deprivation in a concentration-dependent manner. D, IGF-1-mediated survival of hippocampal neurons is mediated by the PI3-kinase pathway based on LY294002-inhibiting effect of IGF-1. Data represent assays from at least three independent experiments (in quadruplicate for MTT assay). *, $p < 0.05$.

suggest that these events play an important role in IGF-1–induced survival of hippocampal neurons and PC-12 cells.

FKHRL1 Is a Component of IGF-1 Receptor Signaling in Cultured Hippocampal Neurons. FKHRL1 is a member of the family of Forkhead transcription factors characterized by the presence of a highly conserved Forkhead domain with a winged-helix motif and DNA binding activity (Kops and Burgering, 1999; Zheng et al., 2000b). Recent studies have shown that FKHRL1 is a proapoptotic protein and a downstream target of PI3K/Akt in IGF-1 signaling in fibroblasts and PC-12 cells (Brunet et al., 1999; Zheng et al., 2000b). The phosphorylation of this transcription factor on its Thr32 and Ser253 residues by Akt inhibits its proapoptotic properties and leads to cell survival (Brunet et al., 1999, 2001; Dijkers et al., 2000; Shin et al., 2001). However, no information was available on the possible role of this transcription factor in IGF-1–mediated effects in hippocampal neurons. The present study shows that IGF-1 time and concentration dependently induced the phosphorylation of endogenous FKHRL1 in hippocampal neurons, demonstrating

that this transcription factor is indeed a target and a component of IGF-1 receptor signaling in neurons.

Phosphorylation of FKHRL1 Induced by IGF-1 in Hippocampal Neurons Is Mediated by the PI3K/Akt

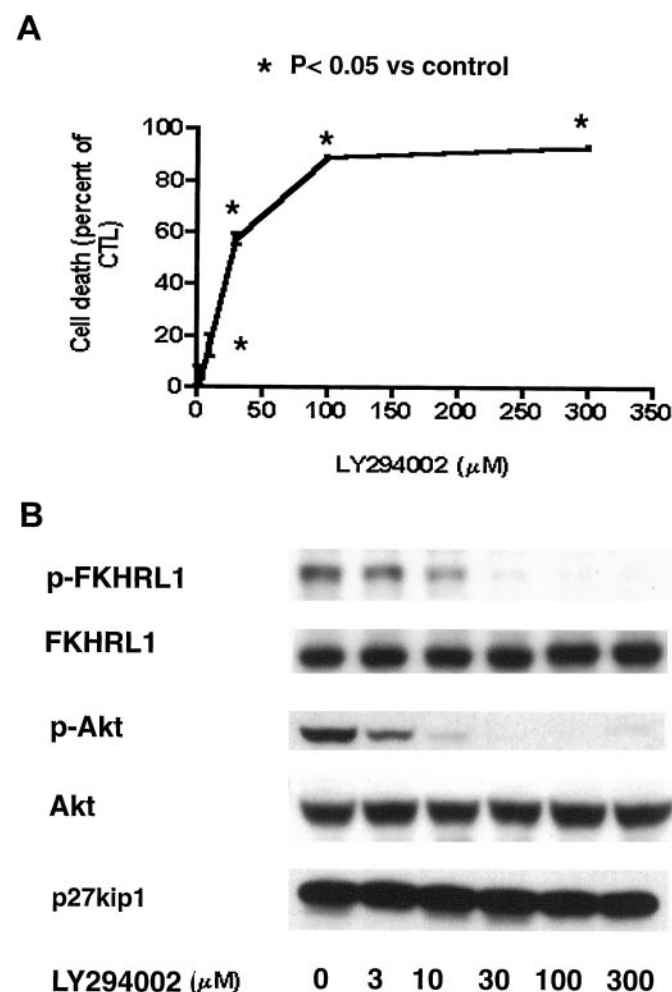


Fig. 7. PI3-kinase inhibitor LY294002 concentration dependently decreased the phosphorylation of FKHRL1 and Akt and induced cell death in hippocampal neurons. Cultured hippocampal neurons were treated with different concentrations of LY294002, and the phosphorylation of FKHRL1 and Akt, as well as survival of hippocampal neurons, were determined. Data represent assays from at least three independent experiments (in quadruplicate for MTT assay). *, $p < 0.05$.

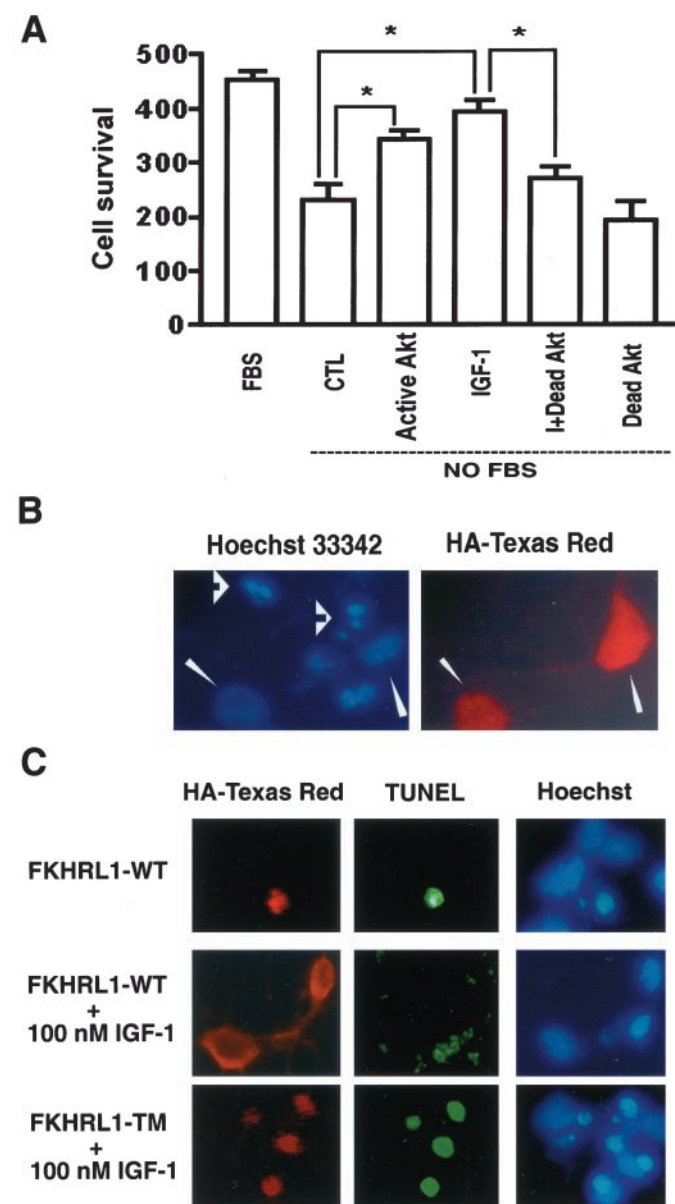


Fig. 8. Protective effect of Akt in the survival of PC-12 cells and hippocampal neurons. **A**, PC-12 cells transfected with CMV control plasmid, GAP-Akt, or kinase dead Akt were treated with or without IGF-1, and the survival of these cells was determined as described under *Experimental Procedures*. Constitutively active Akt promoted the survival of PC-12 cells, whereas kinase dead Akt attenuated cell survival of PC-12 cells promoted by IGF-1. **B**, hippocampal cultured neurons transfected with HA-Akt were N2-deprived for 48 to 72 h to induce apoptosis; apoptosis and HA-Akt positive cells were determined as described under *Experimental Procedures*. Expression of Akt protected hippocampal cultured neurons from apoptosis induced by N2 deprivation (small arrow points to HA-Akt positive cell; bigger arrows point to apoptotic cells). **C**, hippocampal neurons transfected with FKHRL1-WT and FKHRL1-TM were treated with or without IGF-1, and apoptosis and HA-FKHRL1 positive cells were determined as described under *Experimental Procedures*. The overexpression of both FKHRL1 and FKHRL1-TM induced apoptotic-like profile in cultured hippocampal neurons, whereas IGF-1 only attenuated the apoptotic effect of wild-type FKHRL1. Data represent assays from at least three independent experiments (in quadruplicate for MTT assay). *, $p < 0.05$.

Kinase Pathway. We have most recently shown that IGF-1-induced phosphorylation of FKHRL1 is mediated by the PI3K/Akt kinase pathway in PC-12 cells (Zheng et al., 2000b). In accordance with these results, IGF-1-induced phosphorylation of FKHRL1 in hippocampal neurons was inhibited by two PI3-kinase inhibitors, wortmannin (Yao and Cooper, 1995) and LY294002 (Vlahos et al., 1994), but not by the MAP kinase pathway inhibitor PD98059 (Alessi et al., 1995) or by rapamycin, an S6p70 kinase pathway inhibitor (Chung et al., 1992), suggesting a role for PI3K/Akt kinase. This finding is consistent with an early report showing that FKHRL1 was a downstream target of Akt in fibroblasts (Brunet et al., 1999) and supported by the fact that purified active Akt kinase can directly phosphorylate FKHRL1 extracted from PC-12 cells (Zheng et al., 2000b) and hippocampal neurons (this study). Moreover, the transient expression of constitutively active Akt in PC-12 cells and hippocampal neurons increased the phosphorylation of FKHRL1, whereas kinase dead Akt inhibited IGF-1-induced phosphorylation of FKHRL1 in PC-12 cells, indicating that the activation of Akt is sufficient to stimulate the phosphorylation of FKHRL1 in these cells. Taken together, these data demonstrate that FKHRL1 is a downstream target and a substrate of Akt in IGF-1 receptor signaling in hippocampal neurons. Interestingly, IGF-1 and the PI3K inhibitors have little or no significant effect on the cell cycle inhibitor p27kip1, although IGF-1 induced the phosphorylation of FKHRL1, whereas the PI3-kinase inhibitors blocked the phosphorylation of this protein. These results may indicate that p27kip1 is not the target of FKHRL1 in neurons, in contrast to other cell types such as A14 cells and Jurkat cells (Medema et al., 2000). These findings demonstrate further differences in Akt/FKHRL1 signaling in various cell types and are consistent with the notion that primary cultured neurons have more limited potency to proliferate than other cell types.

PI3-Kinase/Akt/FKHRL1 Pathway Plays a Role in IGF-1-Induced Survival of Hippocampal Neurons and PC-12 Cells. The functional relevance of the phosphorylation of FKHRL1 induced by IGF-1 in hippocampal neurons was studied next. It is known that IGF-1 can protect various cell types, including hippocampal neurons against apoptosis and cell death by activating the PI3K/Akt pathway (Yao and Cooper, 1995; Dudek et al., 1997; Datta et al., 1999; Matsuzaki et al., 1999; Kaplan and Miller, 2000; O'Kusky et al., 2000; Trejo et al., 2001; Yamaguchi et al., 2001). However, the mechanism(s) by which PI3K/Akt mediated the neuroprotective effects of IGF-1 is far from clear. Three proapoptotic proteins, Bad, caspase-9, and the Forkhead transcription factors, including FKHRL1, have been recently recognized as targets of Akt in other cells (del Peso et al., 1997; Cardone et al., 1998; Brunet et al., 1999, 2001). The phosphorylation of these proteins by Akt inhibits their proapoptotic properties, leading to cell survival. However, the role of Bad is probably limited in the central nervous system because neurons from Bad knockout mice did not show alterations in neuronal apoptosis (Kaplan and Miller, 2000). Similarly, caspase-9 is an unlikely target of Akt in neurons because an Akt phosphorylation site is not conserved in non-human pro-caspase-9, and Akt apparently failed to increase the phosphorylation of caspase-9 in neurons (Fujita et al., 1999; Kaplan and Miller, 2000). Hence, FKHRL1 is a prime candidate in IGF-1/PI3K/Akt-mediated neuronal survival.

The role of FKHRL1 in apoptosis was first implicated by Brunet and colleagues who found that the expression of unphosphorylated mutant FKHRL1 in cerebellar granule neurons, CCL39 fibroblasts, and Jurkat cells induced apoptosis (Brunet et al., 1999, 2001). Subsequently, the expression of unphosphorylated FKHRL1 mutant in mouse pro-B cell line Ba/F3 or the expression of wild type and its unphosphorylated FKHRL1 in epithelial cells was also found to induce death (Dijkers et al., 2000; Shin et al., 2001). These findings are consistent with the above-mentioned hypothesis that FKHRL1 is probably involved in IGF-1/PI3K/Akt-mediated neuronal survival. Indeed, we observed that IGF-1-induced phosphorylation of FKHRL1 in hippocampal neurons blocked its nuclear translocation and promoted survival, whereas the inhibition of its phosphorylation by PI3-kinase inhibitors caused the nuclear retention of this transcription factor, leading to death. Moreover, PI3-kinase inhibitors blocked both IGF-1-induced phosphorylation of FKHRL1 and the survival of hippocampal neurons and PC-12 cells. Interestingly, the expression of constitutively active Akt (GAP-Akt) increased the phosphorylation of FKHRL1 and promoted survival, whereas the expression of kinase dead Akt, which had been shown to attenuate IGF-1-mediated phosphorylation of FKHRL1 (Zheng et al., 2000b), blocked the neuroprotective action of IGF-1. Finally, the expression of HA-Akt in hippocampal neurons protected these cells from apoptosis induced by N2 deprivation, whereas the overexpression of FKHRL1 induced apoptosis of these neurons. Taken together, these data strongly suggest that IGF-1-induced survival of hippocampal neurons and PC-12 cells is mediated by the PI3K/Akt/FKHRL1 pathway.

Phosphorylation of FKHRL1 Inhibits the Transcription of Death Genes Such as Fas Ligand or the Bcl Family Member Bcl-2 Interacting Mediator (Bim), Leading to Survival of Hippocampal Neurons. The detailed mechanism(s) by which IGF-1-induced phosphorylation of FKHRL1 is involved in cell survival is not fully understood. Like other Forkhead transcription factors, FKHRL1 contains a core domain of 100 amino acids that mediates its interaction with DNA consensus sequence (Lai et al., 1993; Brunet et al., 1999). When Akt is inactive, FKHRL1 is not phosphorylated and is mostly localized to the nucleus where it binds to DNA consensus sites of Fas ligand gene to induce transcription, leading to apoptosis (Brunet et al., 1999). However, upon treating fibroblasts with IGF-1, it induces the activation of Akt kinase and the phosphorylation of FKHRL1 at its Thr32 and Ser253 residues, promoting the association of FKHRL1 with the 14-3-3 protein and its retention in the cytoplasm, leading to the inhibition of the Fas ligand gene transcription and to cell survival (Brunet et al., 1999). Hence, if this sequence of events is applicable herein, the neuroprotective effect of IGF-1 in PC-12 cells and hippocampal neurons is likely to be related to its action on Akt kinase and the phosphorylation of death-inducing factors, including the Forkhead transcription factors.

A recent report suggested that unphosphorylated FKHRL1 was able to induce apoptosis of mouse pro-B Ba F3 cells by inducing the expression of the Bim of cell death (Dijkers et al., 2000). Bim is a Bcl-2 family member, capable of interaction with anti-apoptotic Bcl-2 members such as Bcl-2 and Bcl-xL and causes apoptosis in various cell types (Dijkers et al., 2000; O'Reilly et al., 2000). Because Bim is also expressed

in neuronal cells, it is possible that the phosphorylation of FKHL1 induced by IGF-1 could block the expression of Bim, leading to cell survival (O'Reilly et al., 2000). Further experiments will be required to investigate this interesting possibility.

In summary, the present study demonstrates that IGF-1 is capable of stimulating the phosphorylation of the Forkhead transcription factor FKHL1 via the PI3K/Akt kinase pathway in hippocampal neurons. The phosphorylation of FKHL1 by IGF-1 may be an additional new mechanism by which IGF-1 promotes cell survival in the brain.

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