Antiapoptotic Signaling by the Insulin Receptor in Chinese Hamster Ovary Cells

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ABSTRACT: We have sought to determine whether insulin can promote cell survival and protect Chinese hamster ovary (CHO) cells from apoptosis induced by serum starvation. Low concentrations of insulin were antiapoptotic for cells overexpressing wild-type insulin receptors but not in cells transfected with kinase-defective insulin receptor mutants that lacked a functional ATP binding site. However, treatment with orthovanadate (50 μM), a widely used tyrosine phosphatase inhibitor, led a dramatic reduction in internucleosomal DNA fragmentation in both cell lines. Cells transfected with truncated receptor mutants in either the juxtamembrane or C-terminal domain were as responsive as cells overexpressing wild-type receptors in mediating insulin antiapoptotic protection. The mechanisms underlying insulin antiapoptotic protection were investigated using a variety of pharmacological tools known to inhibit distinct signaling pathways. The phosphatidylinositol-3′ kinase inhibitors wortmannin and LY294002 had only a modest influence whereas blocking protein farnesylation with manumycin severely disrupted the antiapoptotic capacity of the insulin receptor. Of interest, cells gained antiapoptotic potential following inhibition of extracellular signal-regulated kinase activation with the pharmacological agent PD98059. Insulin induced MKK3/MKK6 phosphorylation and activation of p38 MAP kinase whose activity was inhibited with SB203580. However, the inhibition of p38 MAP kinase had no effect on the protection offered by insulin. We conclude that the antiapoptotic function of the insulin receptor requires intact receptor kinase activity and implicates a farnesylation-dependent pathway. Increase in cellular phosphotyrosine content, however, triggers antiapoptotic signal that may converge downstream of the insulin receptor.

Following insulin binding, the insulin receptors undergo activation of their intrinsic tyrosine kinase function and subsequent tyrosine phosphorylation of cellular proteins including insulin receptor substrate (IRS)[1]-1, IRS-2, and a family of "src and collagen homology" (Shc) adaptor proteins (*1*). Tyrosine phosphorylation of IRS-1 and Shc proteins results in activation of several kinases that include phosphatidylinositol-3′ kinase (PI-3′ kinase) and p21ras by GTP loading with the subsequent activation of Raf-1, mitogen-activated protein (MAP) kinase kinase (also referred to as MEK1), and the extracellular signal-regulated kinases, ERK1 or ERK2, two members of the MAP kinase superfamily (*2*). Insulin regulates the activity of a number of cytosolic enzymes and nuclear transcription factors (*3*). Upon insulin stimulation, the nuclear factor kB (NF-kB) is activated through a Raf-1 kinase-dependent pathway (*4, 5*), which allows its translocation to the nucleus and binding to DNA sequences that regulate gene transcription (*5*). Moreover, Chen et al. (*6*) reported that insulin promoted rapid tyrosine phosphorylation of cytoplasmic Stat5 transcription factor thereby enhancing its ability to bind DNA. Both NF-kB and Stat5 have been shown to be involved in various cellular functions including the control of apoptosis (*7*−*9*).

Apoptosis, also referred to as programmed cell death, plays a major role in normal turnover of cells. It is characterized by changes within the nucleus including chromatin condensation and internucleosomal DNA cleavage (*10*). DNA degradation occurs well before the loss of plasma membrane integrity (*10*). The process of apoptosis has been the subject of intense study over the past few years since the identification of gene products involved in prevention or induction of apoptosis (*11*). Insulin has been shown to rescue cells from apoptotic death in a number of cell types (*12*−*17*); however, the concentrations of insulin used in these studies were supraphysiologic and could have exerted protection by activating the type I insulin-like growth factor (IGF-1) receptor. Activation of the IGF-1 receptor prevents cells from entering a death program following exposure to apoptotic stimuli, including serum withdrawal, UV irradiation, and chemotherapeutic agents (*18*−*20*). PI-3′ kinase has been implicated in the transduction of IGF-1 survival signals whereas Ras signaling through the activation of ERK pathway has been demonstrated to exert antiapoptotic func-

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[1] Abbreviations: IRS, insulin receptor substrate; PI-3′ kinase, phosphatidylinositol-3′ kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; ERK, extracellular signal-regulated kinase; IGF-1, insulin-like growth factor-1; CHO, Chinese hamster ovary cells; HIRc, CHO cells overexpressing the wild-type human insulin receptor; SFM, serum-free medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PVDF, poly(vinylidene difluoride); HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NMP, nuclear matrix protein.

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tion in some cell lines and not in others (*18*−*21*). Although intrinsic receptor properties and cellular context may explain how activation of the IGF-1 receptor elicits survival function, the question remains whether similar signaling pathways are being used by insulin to provide antiapoptotic protection, and whether insulin's ability to delay apoptosis is mediated by activation of the insulin receptor. In this study, we have sought to determine if low doses of insulin promote cell survival with a concomitant delay in apoptosis following serum deprivation of Chinese hamster ovary (CHO) cells transfected with the human insulin receptors.

In recent years, the identification of a number of functional domains in the insulin receptor has provided the impetus for assessing their importance in the receptor's regulation of glucose metabolism and mitogenesis. Using stably transfected cells overexpressing mutated forms of the insulin receptors, it has been shown that mutations within the juxtamembrane region, ATP binding site, and tyrosine cluster in the kinase core cause a severe impairment for both insulin-dependent metabolic and mitogenic signaling (for a review, see ref *22*). Moreover, some studies provided evidence that receptors with a deletion in the carboxyl-terminal tail failed to display normal metabolic responses while retaining full mitogenic potential in response to insulin (*22*). In this present work, we investigated the importance of various insulin receptor cytoplasmic domains in the antiapoptotic capacity of insulin. Furthermore, the signal transduction pathways involved in this action of insulin has been investigated.

MATERIALS AND METHODS

Cell Culture. CHO cell lines used in this study include the CHO cell lines stably transfected with a plasmid containing neomycin resistance gene driven by SV40 promoter without (neo) or with a plasmid encoding either the wild-type minus exon 11 variant of the human insulin receptor (HIRc), a truncated receptor lacking 69 amino acids at the carboxyl-terminus (ΔCT), a receptor with a deletion of 12 amino acids from its juxtamembrane domain (Δ960), and a mutant receptor with a Lys→Ala substitution at position 1018 in the ATP binding domain (K1018A). CHO/HIRc and CHO/Δ960 cells were gifts from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA), CHO/ΔCT cells were a gift from Dr. Jeremy M. Tavaré (University of Bristol, Bristol, U.K.), and CHO/K1018A cells were from Dr. Richard A. Roth (Stanford University, Stanford, CA). Cells were grown on tissue culture plates in Ham's F-12 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

Cell Treatment. Confluent cells in serum-free medium were incubated with 0.1% bovine serum albumin (BSA, RIA grade, Sigma Chemical, St. Louis, MO) in the absence or presence of a range of concentrations of insulin (Calbiochem, La Jolla, CA), recombinant IGF-1 (Upstate Biotechnology Inc. (UBI), Saranac Lake, NY) or 10% FBS. Eighteen hours later, floating cells were collected by centrifugation, harvested, and combined with the cells remaining attached to the plate. In some experiments, the PI-3′ kinase inhibitors wortmannin (Sigma) (50 nM) or LY294002 (Calbiochem) (3 μM) were added 30 min prior to the addition of insulin. In other experiments, cells in serum-free medium were preincubated either with the MEK1 inhibitor PD98059 (Calbiochem) at 100 μM, the Ras farnesylation inhibitor manumycin (Calbiochem) at 20 μM, p38 MAP kinase inhibitor SB203580 (Calbiochem) at 10 μM, or sodium orthovanadate (Sigma) at 50 μM for 30 min prior to the addition of insulin where indicated.

DNA Laddering. Cells were lysed in lysis solution (Puregene, Gentra Systems, Inc., Minneapolis, MN) and incubated overnight at room temperature prior to the addition of 20 μg/mL RNase A for 1 h at 37 °C. The samples were deproteinized, followed by DNA precipitation with 2-propanol. The concentration and purity of DNA were determined spectrophotometrically by measuring UV adsorbance ratio at 260 over 280 nm. Equal amounts of DNA from each sample (1 μg) were 3′-OH-labeled with 5 U Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA) and 0.5 μCi [α-^{32}P]dCTP (∼3000 Ci/mmol, Amersham Corp., Arlington Heights, IL) in the presence of 10 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$. After 10 min incubation at room temperature, the reaction was terminated by the addition of 10 mM EDTA. The samples were electrophoresed on 6% polyacrylamide gel. Following electrophoresis, the gel was fixed in a solution composed of 15% MeOH and 5% AcOH, dried, and analyzed by autoradiography using Kodak BioMax film and intensifying screens. In addition, the radioactivity associated with the DNA fragments was quantitated by electronic autoradiography with a Packard InstantImager (Meriden, CT).

PI-3′ Kinase Activity. PI-3′ kinase activity was measured as described previously (*23*) with some minor modifications. Serum-deprived cells grown in 35-mm dishes were preincubated in the absence or presence of wortmannin (50 nM) or LY294002 (3 μM) for 30 min prior to the addition of 0.1% BSA with or without insulin (10 nM). Eighteen hours later, cells were lysed, and lysates were immunoprecipitated with a polyclonal anti-p85 antibody (UBI). L-α-Phosphatidylinositol (Sigma) was used as substrate in the kinase assay.

ERK Immune Complex Assay. ERK1/ERK2 activity was measured as described (*23*) with some minor modifications. Briefly, cells grown in 35-mm dishes were switched to serum-free medium containing 0.1% BSA and then preincubated in the absence or presence of manumycin (10 μM) or PD98059 (50 μM) for 1 h prior to the addition of insulin (10 nM). Five minutes later, cells were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM NaF, 10 mM sodium pyrophosphate, 0.15 mM Pefabloc SC (Boehringer Mannheim), 1 mM sodium orthovanadate, 20 μg/mL aprotinin, and 10 μg/mL leupeptin. The lysates were centrifuged at 14000*g* for 20 min at 4 °C, and equal amounts of protein from each clarified lysate were immunoprecipitated with a polyclonal anti-rat ERK1/ERK2 antibody (ERK1-CT, UBI) and protein G-Plus/protein A-agarose (Oncogene Science, Manhasset, NY) for 16 h. After a series of washes, the immune pellets were incubated with 10 μg/mL myelin basic protein (UBI) and 20 μM [γ-^{32}P]ATP for 15 min at 22 °C. The reaction was stopped by addition of Laemmli sample buffer (*24*), and the samples were analyzed by one-dimensional 4−20% (Novex, San Diego, CA) SDS−polyacrylamide gel electrophoresis (PAGE) under reducing conditions and autoradiography.

p38 MAP Kinase Activity. p38 MAP kinase activity was measured as recently described (*25*) with some minor modifications. Confluent cells grown in 35-mm dishes were switched to serum-free medium containing 0.1% BSA and then treated with various factors, as described in the figure legends, followed by lysis at 4 °C for 15 min in 0.5 mL of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 1 mM orthovanadate, 0.15 mM Pefabloc SC, 10 μg/mL aprotinin, 5 μg/mL leupeptin, 10 nM okadaic acid, 10 nM tautomycin, and 10 nM cypermethrin. Equal amounts of protein from clarified lysates were immunoprecipitated with a rabbit polyclonal anti-p38 MAP kinase antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 90 min at 4 °C after which protein G-Plus/protein A-agarose was added for another 90 min. The immune complexes were washed twice in lysis buffer, then two times in lysis buffer containing 0.5 M NaCl, followed by two additional washes with kinase assay buffer (20 mM HEPES (pH 7.4), 1 mM EGTA, 25 mM β-glycerophosphate, 20 mM $MgCl_2$, and 0.2 mM orthovanadate). The kinase reaction was performed by resuspending the pellet in 20 μL of kinase assay buffer containing 0.1 mg/mL recombinant GST-ATF2 fusion protein (generous gift from Dr. Roger J. Davis, University of Massassuchetts, Worcester, MA), 25 μg/mL cAMP-dependent protein kinase inhibitor peptide (UBI), 20 μM [γ-^{32}P]-ATP (10 cpm/fmol), and 1 mM dithiothreitol for 15 min at 22 °C. The reaction was stopped by addition of Laemmli sample buffer, and the samples were analyzed by one-dimensional 4−12% SDS−PAGE under reducing conditions and autoradiography.

Immunoprecipitation and Western Blot Analysis. Cells grown to confluency in 35-mm dishes were fed with serum-free Ham's F-12 medium containing 0.1% BSA for 3−4 h. After appropriate treatments, the fluid was removed, and the dishes were rapidly immersed in liquid nitrogen. The cells were scraped and lysed into a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM sodium orthovanadate, 100 mM NaF, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% NaN_3, 0.15 mM Pefabloc SC, 1 mM benzamidine, 8 μg/mL aprotinin, and 2 μg/mL leupeptin. For immunoprecipitation, the clarified lysates were incubated with a monoclonal anti-insulin receptor antibody (clone 29B4, Oncogene Sciences) in the presence of protein G-Plus/protein A-agarose beads. After being washed, the samples were denatured in Laemmli sample buffer containing 5% 2-mercaptoethanol. Proteins were separated by one-dimensional 4−12% SDS−PAGE under reducing conditions, along with prestained protein markers, and electrotransferred onto poly(vinylidene difluoride) (PVDF) membrane. Blots were probed either with polyclonal antibodies specific for insulin receptor α-subunit (sc-710, Santa Cruz) or a monoclonal anti-phosphotyrosine antibody conjugated with horseradish peroxidase (sc-508, Santa Cruz). The signal was detected by Western immunoblotting using the ECL chemiluminescence detection system (Amersham).

Alternatively, confluent cells were lysed directly in Laemmli sample buffer containing 5% 2-mercaptoethanol, and equal amounts of protein from each sample were subjected to SDS−PAGE and electrotransferred. The blots were probed with polyclonal antibodies specific for either IRS-1 (UBI), p38 (Santa Cruz), MKK3 (New England BioLabs) or phospho-MKK3/MKK6 (New England BioLabs) proteins. When appropriate, membranes were also probed with a monoclonal anti-phosphotyrosine antibody conjugated with horseradish peroxidase (Santa Cruz).

MTT Assay. This measure of mitochondrial function was performed as described previously (*20*) with cells seeded on 24-well plates. Following treatments, the medium was removed from the wells, and 200 μL of MTT reagent (Sigma) at a concentration of 1 mg/mL in RPMI-1640 medium without phenol red was added to each well. After 1 h incubation at 37 °C, the cells were lysed by addition of 1 volume of 2-propanol and shaking for 20 min. Absorbance of converted dye was measured at a wavelength of 570−690 nm.

Determination of Nuclear Matrix Protein. Cells grown in 35-mm dishes were incubated in serum-free medium containing 0.1% BSA in the absence or presence of 10 nM insulin. Forty-eight hours later, the culture supernatant was collected, centrifuged at 10000*g* for 15 min at 4 °C, and transferred into a new tube followed by lyophilization. The dried material was resuspended in water and used for NMP determination, which was conducted according to the manufacturer's protocol (Calbiochem).

Statistical Analysis. Data are presented as the mean ± the standard error of the mean (mean ± SEM). Comparison between groups were made by analysis of variance coupled to Fisher's PLSD post-hoc test using STATVIEW II software for Apple Macinstosh computers (Abascus Concepts, Berkeley, CA).

RESULTS

Insulin Receptor Activation Confers Protection against Apoptosis. To examine the involvement of insulin as a survival factor, CHO/HIRc cells were incubated in serum-free medium (SFM) in the presence of a range of concentrations of insulin for 18 h. Apoptosis was then detected by DNA laddering. The protective effect of insulin was detected at concentrations as low as 1 nM, with increasing protection at 10 nM (Figure 1A, lanes 6−9). In contrast, no reduction in internucleosomal DNA fragmentation was evident in CHO/neo cells treated with 2.5 or 10 nM insulin (lanes 3 and 4 vs lane 1). However, the addition of a supraphysiological concentration of insulin (100 nM) conferred antiapoptotic protection in these cells (data not shown), possibly as a result of IGF-I receptor activation. Indeed, addition of 10 nM IGF-1 inhibited apoptosis very effectively in CHO/neo cells, while having reduced effectiveness in CHO/HIRc cells. This finding is in agreement with the work of Maggi et al. (*26*), who showed that overexpression of the insulin receptor makes CHO cells resistant to the action of IGF-1. Several lines of evidence suggest that insulin receptor tyrosine kinase activity may be involved in most of the actions of insulin. To assess the requirement for kinase function in the antiapoptotic protection, CHO/K1018A cells that overexpress tyrosine kinase-deficient receptor mutants, in which the critical ATP binding lysine residue within the kinase active site has been substituted (*27*), were incubated in SFM in the presence or absence of insulin. Despite having the same affinity for insulin as the wild-type insulin receptor, this mutant receptor has been reported to be completely

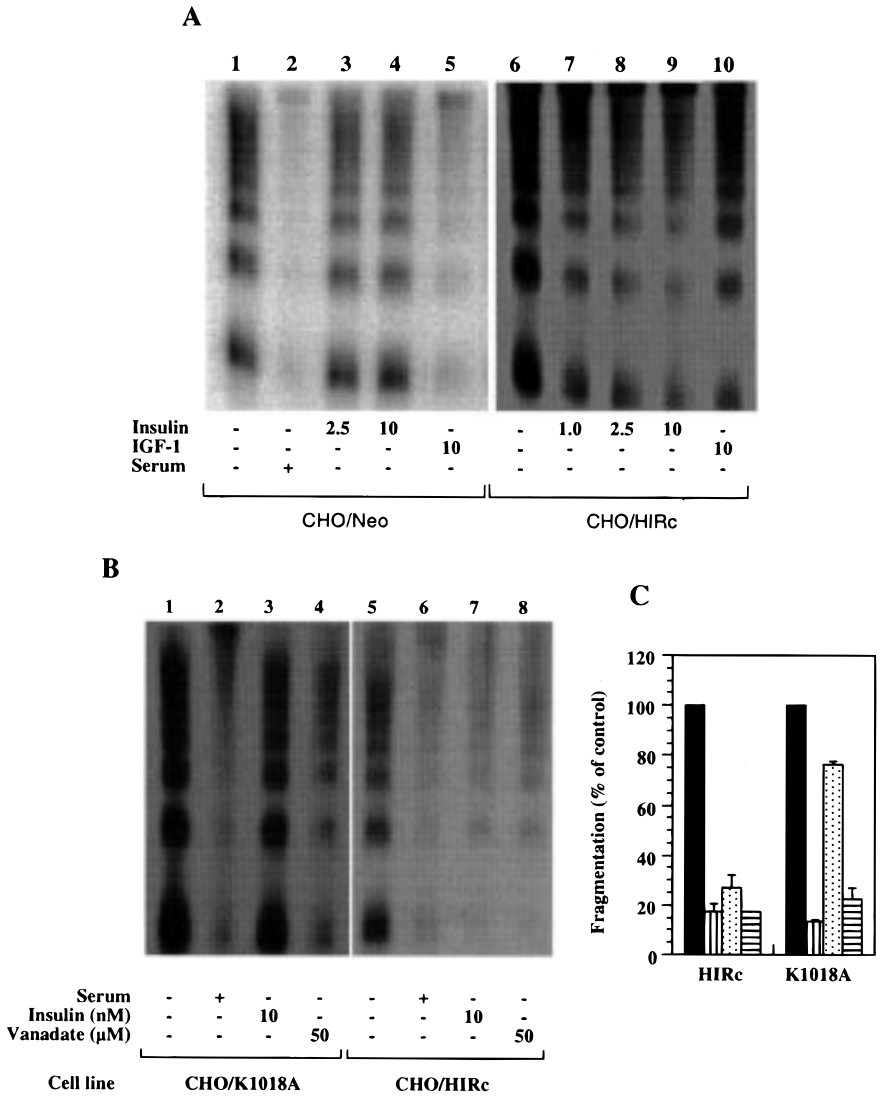

FIGURE 1: Protection from apoptosis induced by serum withdrawal in cells overexpressing insulin receptors. (A) Effect of insulin or IGF-1. Confluent CHO/neo (lanes 1−5) and CHO/HIRc (lanes 6−10) cells were shifted to SFM and maintained for 18 h in the absence (−) or presence of the indicated concentrations of insulin or IGF-1 (in mM), or 10% FBS (+). Both floating and attached cells were harvested, lysed, and the genomic DNA radiolabeled as described under Materials and Methods. Results are representative of three separate experiments. (B) Survival function of insulin or orthovanadate in CHO cells overexpressing kinase-dead insulin receptor mutants. Confluent CHO/K1018A (lanes 1−4) and CHO/HIRc (lanes 5−8) cells were shifted to SFM and maintained for 18 h in the absence (−) or presence of insulin (10 nM), orthovanadate (50 μM), or 10% FBS. DNA extraction, labeling, and separation on polyacrylamide gels were performed as described. (C) The data represent the means ± SEM of three independent experiments, where the relative level of fragmentation in SFM-treated cells was arbitrarily set at 100%. Closed bars, SFM; vertical hatched bars, 10% FBS; dotted bars, insulin; horizontal hatched bars, orthovanadate.

inactive for both the metabolic and the mitogenic functions of insulin (*27*). CHO cells expressing K1018A mutant exhibited negligible insulin-mediated protection from serum withdrawal (Figure 1B, lane 3 vs lane 1). However, marked reduction in DNA laddering was observed following the incubation with orthovanadate (50 μM) both in CHO/HIRc and in CHO/K1018A cells. The results of the densitometric scans are shown in Figure 1C, expressed relative to the amount of DNA fragmentation present in their respective control SFM-treated cells.

The ability of insulin or orthovanadate to maintain cell survival was studied in CHO/HIRc cells using MTT assay. Both insulin and orthovanadate were able to prevent a reduction in MTT activity induced by a 24-h growth factor withdrawal (Figure 2). However, as compared with insulin, orthovanadate exhibited a reduction in MTT activity after a 48-h treatment, approaching the levels seen in control SFM-treated cells. These results indicate that overexpression of the wild-type insulin receptors and their activation by insulin has conferred enhanced cell survival and protection against SFM-mediated apoptosis. Furthermore, it appears that tyrosine phosphorylation events downstream of the insulin receptors may also participate in mediating protection against apoptosis. For subsequent experiments, we chose to use insulin at a concentration of 10 nM so to detect effects of agents that might enhance or inhibit the antiapoptotic actions of insulin.

Antiapoptotic Properties of CHO Cells Overexpressing Truncated Insulin Receptor Mutants. We looked first at the ability of an insulin receptor mutant with a 12-amino acid deletion in the juxtamembrane region (Δ960) to protect cells from SFM-induced apoptosis. This mutation prevents the insulin receptor to interact with and phosphorylate IRS-1/2, a prequisite step for subsequent activation of PI-3′ kinase

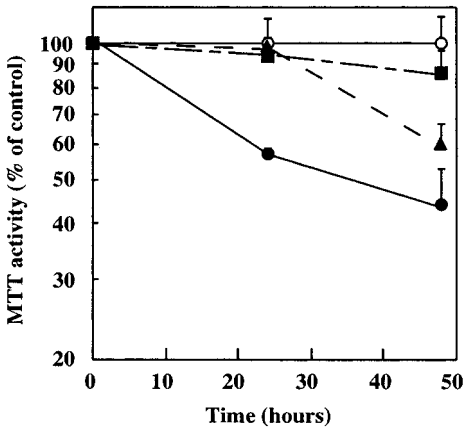

FIGURE 2: Loss of mitochondrial function in CHO/HIRc cells maintained in the absence of trophic factor. Confluent CHO/HIRc cells were maintained for 24 and 48 h in SFM in the absence (●) or presence of 10 nM insulin (■), 50 μM orthovanadate (▲), or 10% FBS (○). At the time points indicated, plates were washed and MTT activity was assayed as described under Materials and Methods. Results are the means ± range of two experiments performed in triplicate.

(*28*, *29*). Cells expressing the Δ960 receptor mutants showed typical internucleosomal DNA fragmentation in the absence of serum. The addition of 10 nM insulin led to an antiapoptotic protection comparable to that of CHO/HIRc cells (results not shown).

Next, a 69 amino acid C-terminal truncation mutant of the insulin receptor (ΔCT) was analyzed for its ability to protect cells from apoptosis. This mutant displays normal binding affinity for insulin but exhibits enhanced insulin-mediated increase in MAP kinase activity (*30*). The tyrosine phosphorylation state of immunoprecipitated ΔCT receptor was assessed by immunoblot analysis with an antiphosphotyrosine antibody. In the absence of insulin stimulation, the level of insulin receptor autophosphorylation was undetectable as measured by densitometric analysis (Figure 3A). Treatment of cells with insulin for 5 min resulted in the phosphorylation of the ΔCT receptor although to a lesser extent than the wild-type receptor. This is likely due to the deletion of the carboxyl-terminal tail of the receptor that contains two tyrosine autophosphorylation sites (22). Reprobing the blot with an antibody against the insulin receptor α-subunit revealed similar amounts of receptor proteins in each lane (Figure 3A). Insulin-stimulated tyrosine phosphorylation of the endogenous substrate IRS-1 was also examined by immunoblotting of cell lysates, and the results indicated a similar response between CHO/HIRc and ΔCT cells (Figure 3B).

SFM-treated CHO cells expressing ΔCT receptor demonstrated protection both under basal and insulin conditions (Figure 3C). It has been previously shown that soluble nuclear matrix proteins (NMP) are released in culture supernatant from dead and dying cells (*31*). Using an enzyme immunoassay for quantitative determination of the amount of NMP released, we found that insulin's antiapoptotic protection was associated with the lowering in NMP level in both cell lines (Figure 3D). The level of NMP released was greatly reduced in CHO/ΔCT cells maintained in the absence of insulin when compared to cells expressing wild-type insulin receptors (Figure 3D).

Effect of PI-3′ Kinase Inhibition on Insulin-Mediated Delay in Apoptosis. Activation of the insulin receptor increases PI-3′ kinase activity, whose function has been associated in the antiapoptotic signaling in various cell types (*18*, *32*). To evaluate the importance of this kinase in the protective effect of insulin, CHO/HIRc cells were switched to SFM containing or not wortmannin (50 nM) or LY294002 (3 μM) for 30 min prior to a 18-h incubation in the presence of insulin. As shown in Figure 4A, the ability of insulin at exerting antiapoptotic protection was not altered by wortmannin or LY294002, two chemically unrelated inhibitors of PI-3′ kinase (*33*, *34*). In the absence of ligand, neither wortmannin nor LY294002 did enhance the degree of apoptosis detected in SFM-treated cells (Figure 4A, lanes 4 and 6 vs lane 1). Additional experiments were performed to demonstrate that the dose of inhibitors used can block insulin stimulation of PI-3′ kinase in these cells. An immunoprecipitation/kinase assay of PI-3′ kinase has been carried out in the presence of phosphatidylinositol as the substrate. As anticipated, the stimulatory effect of insulin was markedly attenuated by the presence of 50 nM wortmannin (Figure 4B). Similarly to wortmannin, 3 μM LY294002 blocked the effect of insulin on PI-3′ kinase (data not shown). Thus, antiapoptotic signaling by the insulin receptor remained intact despite PI-3′ kinase inhibition, indicating that insulin-mediated survival functions does not require PI-3′ kinase activity in these cells.

Farnesylation Reaction Is Involved in the Antiapoptotic Function of Insulin. The role of protein farnesylation in the anti-apoptotic function of insulin was studied in CHO/HIRc cells in the presence of insulin plus manumycin, an inhibitor of Ras farnesylation (*35*). Attachment of Ras protein to the plasma membrane is required for effective Ras signaling and is initiated by the enzyme farnesyl protein transferase whose activity has been shown to be increased by insulin (*36*). Cells pretreated with 20 μM manumycin showed a marked attenuation in insulin's ability to confer antiapoptotic protection, whereas the degree of apoptosis detected in unstimulated cells remained unaffected (Figure 5A, compare lanes 3 and 1 vs lanes 5 and 4). Ras activation is required for initiation of the downstream events leading to ERK activation. Indeed, preincubation with 10 μM manumycin greatly reduced insulin-stimulated ERK activity in CHO/HIRc cells (Figure 5B). Thus, the possibility exists that the antiapoptotic effect of insulin involves an activation of the Ras/Raf/ERK pathway that has been reported to inhibit apoptotic death in some cell lines but not in others (*20*, *37*).

Effect of ERK1/ERK2 Inhibition on Insulin-Mediated Delay in Apoptosis. A specific inhibitor of MAP kinase kinase (also referred to as MEK1), PD98059 (*38*), was used to assess the role of ERK in insulin-mediated protection against apoptosis. Interestingly, incubation with PD98059 (100 μM) alone reduced the degree of apoptosis detected in SFM-treated CHO/HIRc cells (Figure 6A, lane 4 vs lane 1), resulting from the inhibition of constitutive ERK activity. Moreover, the protective effect of insulin was clearly enhanced by PD98059 (Figure 6A, lane 5 vs lane 3). Under these experimental conditions, the dose of PD98059 used can block insulin-stimulated ERK activity that was measured in an immunoprecipitation/kinase assay with MBP as the substrate (Figure 6B). These data support the conclusion that the ERK pathway plays a role in inducing apoptosis.

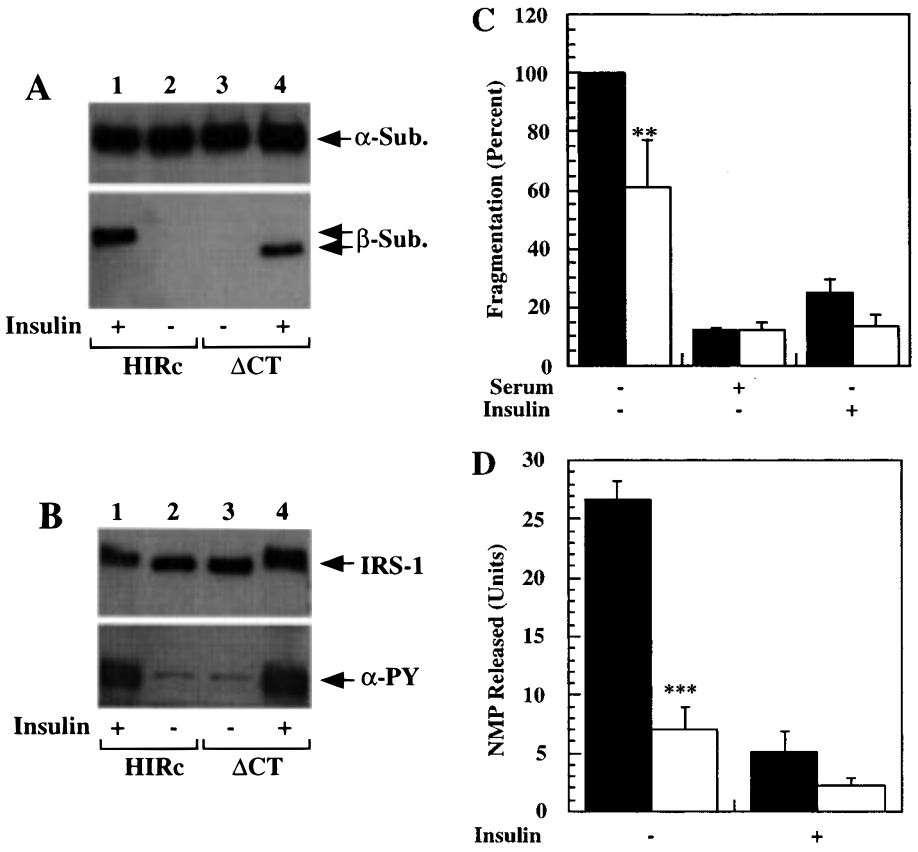

FIGURE 3: Antiapoptotic properties of CHO cells expressing insulin receptor mutants with a C-terminal truncation. (A) Tyrosine phosphorylation of the insulin receptor in intact cells. Serum-starved CHO/HIRc and ΔCT cells were incubated in the absence (−) or presence (+) of 10 nM insulin for 5 min at 37 °C. Insulin receptors were immunoprecipitated from the cell lysates with monoclonal anti-insulin receptor antibody and protein G-plus/protein A-agarose. Immunoblot analysis was performed using polyclonal antibodies specific for phosphotyrosine (lower panel) or insulin receptor α-subunit (upper panel), followed by enhanced chemiluminescence detection. The positions of the α-subunit (α-Sub.) and *β*-subunit (*β*-Sub.) of the insulin receptor are shown. (B) In vivo substrate phosphorylation. Lysates (30 *μ*g) from cells were subjected to SDS−polyacrylamide gel electrophoresis and immunoblotted using anti-IRS-1 (upper panel) or antiphosphotyrosine (lower panel) antibodies. The position of tyrosine phosphorylated IRS-1 is indicated in the lower panel. (C) CHO/HIRc (closed bars) and ΔCT (open bars) cells were incubated in SFM for 24 h in the absence (−) or presence (+) of 10 nM insulin or 10% FBS prior to cell harvesting. DNA extraction, labeling, and separation on polyacrylamide gels were performed as described in the legend to Figure 1. Results are means ± SD of three separate experiments. **, $P < 0.01$. (D) Reduced NMP release in ΔCT cells. Cells were maintained in SFM in the absence (−) or presence (+) of 10 nM insulin for 24 h. The amount of NMP released in culture supernatant was assayed as described in Materials and Methods. Results are the means ± SD of four independent determinations. ***, $P < 0.001$.

Furthermore, it appears that insulin's ability to protect cells requires a Ras-dependent, but ERK-independent, stimulatory event.

Role of p38 MAP Kinase Activity in Insulin-Mediated Delay in Apoptosis. Recent reports indicate an important role for p38 MAP kinase, a member of the MAP kinase superfamily, in neuronal cell apoptosis (*25*, *37*). Therefore, we were interested in determining whether this MAP kinase plays a role in antiapoptotic signaling by the insulin receptor in CHO/HIRc cells. MKK3 and MKK6, the upstream regulators of p38 MAP kinase, are dual specificity kinases whose activities depend on serine and threonine phosphorylation (*39*−*41*). Since no information is currently available regarding MKK3/MKK6 activation by insulin, we performed an immunoblot analysis of their phosphorylation status using a phosphospecific anti-MKK3/MKK6 antibody. An increase in MKK3/MKK6 phosphorylation was evident after a 16-h incubation of SFM-treated CHO/HIRc cells with either 10% serum or insulin (Figure 7, upper panel). The addition of 50 *μ*M orthovanadate had an insulin-like effect and, when combined with insulin, did not lead to a greater response than with insulin alone (lane 5 vs lane 3). Neither of these agents augmented cellular expression of MKK3 protein (Figure 7, lower panel), which indicates that increased MKK3/MKK6 phosphorylation was not accompanied by alteration in MKK3 protein levels. On the basis of these results, it is expected that insulin will stimulate p38 MAP kinase activity.

Indeed, when SFM-treated CHO/HIRc cells were incubated with 10 nM insulin for 16 h, there was a 4.3-fold increase in p38 MAP kinase activity (Figure 8A,B). This phosphotransferase activity was measured in an immunoprecipitation-based kinase assay that uses recombinant GST-ATF2 fusion protein as the substrate (*39*). Exposure of the cells to 10% serum or orthovanadate (50 *μ*M) for 16 h resulted in a comparable increase in p38 MAP kinase activity. As a control, 1-h treatment with arsenite (0.4 mM), a known activator of p38 MAP kinase (*42*), led to a 10-fold increase in its activity (Figure 8A, lanes 5 and 6). SB203580, a highly specific inhibitor of the p38 MAP kinase (*43*), markedly attenuated p38 MAP kinase activation by arsenite. Orthovanadate but not insulin stimulated p38 MAP kinase activity severalfold over basal in CHO/K1018A cells (data not shown). Additional experiments were performed whereby

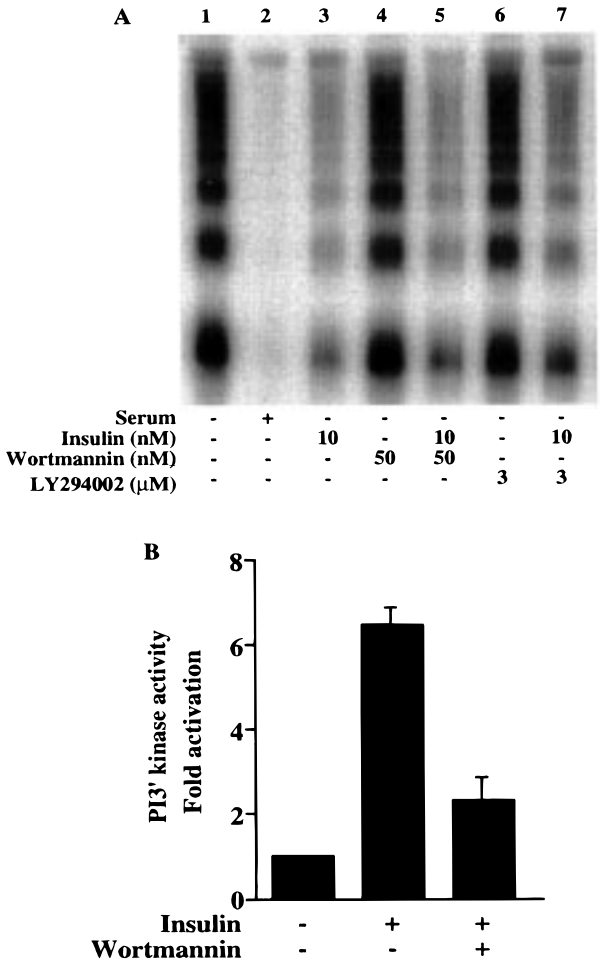

FIGURE 4: PI-3′ kinase is not required for the antiapoptotic function of insulin. (A) The protective effect of insulin is resistant to PI-3′ kinase inhibition. CHO/HIRc cells maintained in SFM were treated with wortmannin or LY294002 at the indicated concentrations for 30 min followed by a 18-h incubation in the absence or presence of insulin (10 nM). The collection and labeling of the DNA were performed as in the legend to Figure 1. The results presented are representative of three separate experiments. (B) Pharmacological inhibition of PI-3′ kinase activity. CHO/HIRc cells maintained in SFM were treated with wortmannin (50 nM) for 30 min prior to the addition (+) or not (−) of insulin (10 nM) for 18 h. The cells were lysed, and clarified lysates were immunoprecipitated with anti-p85 antibody. The immune pellets were analyzed for PI-3′ kinase activity as described in Materials and Methods. The level of radioactivity incorporated into phosphatidylinositol in the absence of inhibitor is arbitrarily set at 1.0. Results presented are means ± range of two experiments.

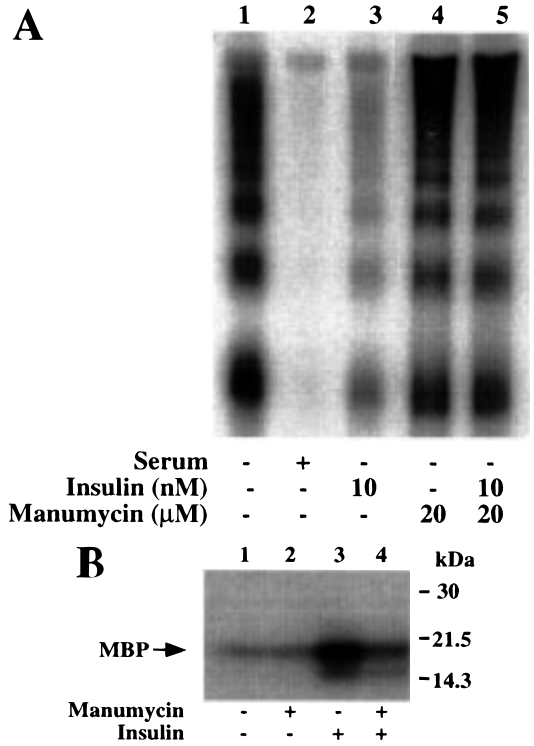

FIGURE 5: Manumycin blocks the protective effect of insulin. (A) CHO/HIRc cells maintained in SFM were treated with manumycin (20 μM) for 30 min followed by a 18-h incubation in the absence (−) or presence (+) of insulin (10 nM). The collection and labeling of the DNA were performed as in the legend to Figure 4. The results presented are representative of three separate experiments. (B) Manumycin inhibits activation of the ERK pathway. Cells maintained in SFM were treated with 10 μM manumycin for 1 h prior to the addition (+) or not (−) of insulin. Five minutes later, the cells were lysed, and ERK1/ERK2 activity was determined in an immunoprecipitation-based kinase assay using [γ-^{32}P]ATP and myelin basic protein as the substrate. A representative autoradiogram is shown.

p38 protein expression was studied in total cell protein extracts (50 μg) that were fractionated by SDS−PAGE and transferred to PVDF membrane. P38 was detected by immunoblotting using a polyclonal anti-p38 antibody. The results from a representative Western blot indicated that incubation with insulin, 10% serum, or vanadate for 16 h had no influence on p38 protein expression in these cells (Figure 8C).

In contrast with earlier reports (*25*, *37*), we found that SB203580 pretreatment was unable to prevent the antiapoptotic function of the insulin receptor in SFM-treated CHO/HIRc cells (data not shown), which indicates that p38 MAP kinase does not have a significant role in regulating the protective capacity of the insulin receptor in these cells.

DISCUSSION

In the present study, we examined the ability of insulin to exert antiapoptotic function in CHO cells and assessed the importance of various insulin receptor cytoplasmic domains in this function. Toward this end, CHO cells expressing large numbers of wild-type insulin receptors or various insulin receptor mutants were used. These mutant receptors, which included the kinase-defective K1018A, the signaling impaired Δ960 and the C-terminally truncated ΔCT, have been previously characterized for their metabolic and mitogenic properties in various cellular backgrounds (*22*). Of significance, these models are generally acceptable to assess intrinsic signaling properties of the insulin receptor. For example, insulin has been recently demonstrated to promote the expression of a DNA repair gene both in CHO/HIRc cells and fully differentiated 3T3-L1 adipocytes (*44*), a cell model known for its high responsiveness to insulin. This finding and others support the notion that the use of CHO cells with overexpressed receptors are valid experimental models for investigating the antiapoptotic function of the insulin receptor.

Insulin is a potent survival factor in primary neuronal cultures (*25*). Our data indicate that insulin inhibits also apoptosis in a concentration-dependent manner in CHO cells that overexpress wild-type insulin receptors (HIRc). Insulin

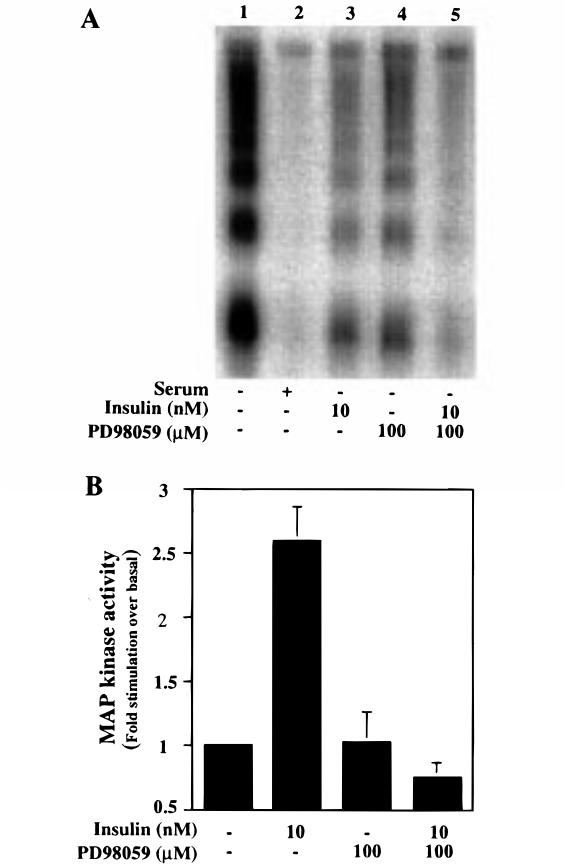

FIGURE 6: Inhibition of ERK1/ERK2 activity enhances insulin antiapoptotic signaling. (A) Reduction in apoptosis by PD98059 in CHO/HIRc cells. Cells maintained in SFM were treated with PD98059 (100 μM) for 30 min followed by a 18-h incubation in the absence (−) or presence (+) of insulin (10 nM). The collection and labeling of the DNA were performed. The results presented are representative of four separate experiments. (B) Pharmacological inhibition of ERK1/ERK2 activity. Cells maintained in SFM were treated with 100 μM PD98059 for 1 h prior to the addition (+) or not (−) of insulin. Five minutes later, the cells were lysed, and ERK1/ERK2 activity was determined. The level of radioactivity incorporated into the substrate in the absence of ligand stimulation is arbitrarily set at 1.0. Results presented are means ± range of two independent experiments.

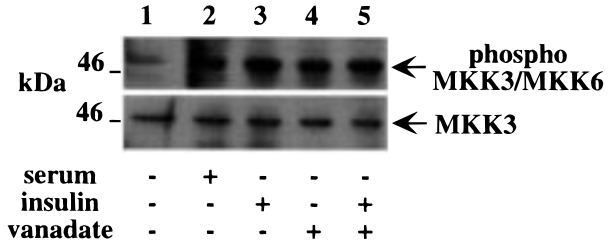

FIGURE 7: Effect of insulin and vanadate on MKK3/MKK6 phosphorylation. CHO/HIRc cells were shifted to SFM and maintained in this medium for 24 h in the absence (−) or presence (+) of 10% serum, insulin (10 nM), orthovanadate (50 μM), or both insulin and orthovanadate. Total cell lysates were run in denaturing polyacrylamide gel electrophoresis under reducing conditions. Proteins in the gel were electrotransferred to a PVDF membrane and analyzed by Western blotting using a polyclonal anti-PhosphoPlus MKK3/MKK6 antibody (upper panel) or anti-MKK3 antibody (lower panel). The results presented are representative of three separate experiments. Molecular mass markers are shown on the right in kilodaltons.

at concentrations as low as 1−10 nM was able to attenuate apoptosis in CHO/HIRc cells but not in parental CHO/neo cells, which express low levels of endogenous insulin receptors. The binding of insulin to its own receptor exhibits considerable high affinity ($K_d \sim 0.5-2$ nM); however, high concentrations of the hormone are known to activate IGF-1 receptors (*45*) whose antiapoptotic capacity has been reported in several studies (*18*−*21*). Indeed, in the study herein, we showed that the addition of IGF-1 at 10 nM could protect CHO/neo cells from apoptosis very efficiently but had a lower activity in CHO/HIRc cells. It is possible that this difference reflects the fact that the insulin receptor titrates out signaling molecules important for regulating apoptosis. The evidence that insulin is acting through its own receptors is supported by an earlier report indicating that the binding of ^{125}I-IGF-1 to CHO/HIRc cells was not affected by the presence of 10 nM insulin (*46*). These findings suggest that overexpression of the insulin receptors sensitizes cells to the protective capacity of insulin against apoptosis mediated by growth factor withdrawal.

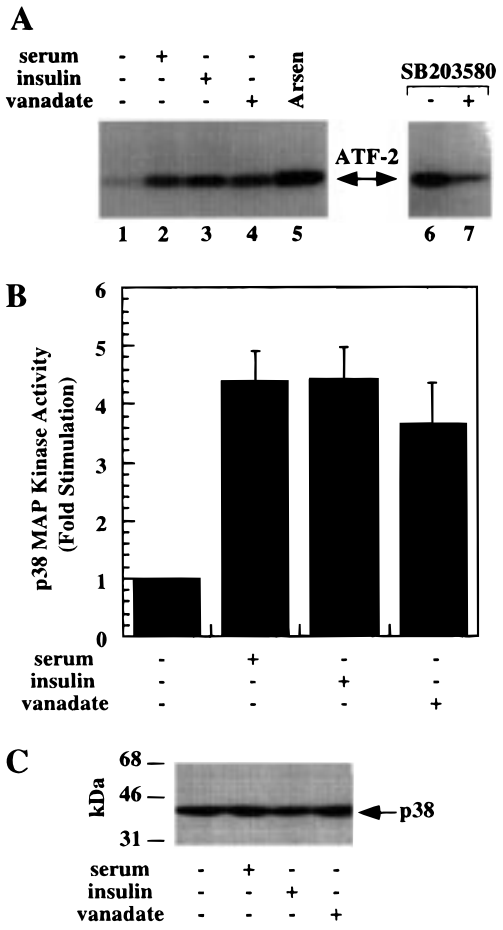

FIGURE 8: Effect of insulin and vanadate on p38 MAP kinase activity. (A) CHO/HIRc cells were shifted to SFM and maintained in this medium for 24 h in the absence (−) or presence (+) of 10% serum, insulin (10 nM), or orthovanadate (50 μM). As a control, SFM-treated cells were incubated with 400 μM arsenite (Arsen.) in the absence (lanes 5 and 6) or presence (lane 7) of 10 μM SB203580 for 1 h. The cells were lysed, and p38 MAP kinase activity was determined in an immunoprecipitation-based kinase assay using recombinant GST-ATF2 fusion protein as the substrate. (B) The level of radioactivity incorporated into the substrate in the absence of ligand stimulation is arbitrarily set at 1.0. Results presented are means ± SEM of four independent experiments. (C) Total cell extracts prepared from CHO/HIRc cells maintained with the indicated treatments for 24 h were separated by SDS−PAGE and immunoblotted with anti-p38 MAP kinase antibody.

Through experiments using the insulin receptor mutants, we have provided evidence that intact tyrosine kinase is important for triggering insulin's antiapoptotic signaling. Inactivation of the critical ATP-binding site in the K1018A receptor mutant severely abolished the ability of insulin to inhibit DNA fragmentation, in agreement with earlier reports indicating that this kinase-defective mutant was unable to transmit insulin signals to various biological end points (*22*). However, when CHO/K1018A cells were treated with orthovanadate, a potent inhibitor of tyrosine phosphatase, these cells displayed significant antiapoptotic resistance. This result indicates that a substantial protection against apoptotic death can be acquired through a receptor-independent increase in cellular phosphotyrosine content. We do not know whether inhibition of individual protein tyrosine phosphatases or dual-specificity Thr/Tyr phosphatases is responsible for the antiapoptotic activity of orthovanadate. Comparable results were obtained in hematopoietic cells (*47*), but not in HL-60 leukemic cells (*48*), nor in dentate gyrus cells in culture (*49*).

Recent reports have shown that the C-terminus of the IGF-1 receptor may encode a proapoptotic signal (*50*, *51*). Our studies indicate that truncation of 69 amino acids at the C-terminus of the insulin receptor did not alter insulin-mediated antiapoptotic protection but caused an apparent greater protection under basal conditions. Although this mutant receptor exhibits normal insulin-stimulated autophosphorylation, IRS-1 phosphorylation, and glucose utilization when expressed in CHO cells (*52*), it has been suggested that the C-terminal domain of the insulin receptor plays an important role in regulating mitogenic signaling through regulation of MAP kinases (*53*), possibly as a result of an impaired association of signaling mediators with the insulin receptor (*54*). In support of this view, it was found that the β-subunit of the truncated insulin receptors binds an activating peptide with greater capacity when compared to that of wild-type receptors (*55*).

In this study, the signal transduction pathway used by the activated insulin receptor to confer antiapoptotic protection was investigated. Various inhibitors of key steps throught to be required in insulin signaling have been widely used and have provided important insight into the understanding of the role of signaling molecules in the regulation of mitogenic and metabolic functions of insulin (*56*). We showed that the binding of insulin to its receptors led to the activation of PI-3′ kinase, whose inhibition by low doses of wortmannin and LY294002 failed to block the antiapoptotic capacity of insulin in CHO/HIRc cells. Insulin-stimulated PI-3′ kinase activity was still increased by 2-fold in the presence of wortmannin. One interpretation is that only a small increase in PI-3′ kinase activity is sufficient for the antiapoptotic response of insulin. However, our finding that the Δ960 mutant receptor which has an impaired activation of PI-3′ kinase by insulin (*28*) is able to inhibit apoptotis is in agreement with the mutagenesis data of Boehm et al. (*57*), who suggested that activation of IRS-1-associated PI-3′ kinase is not a critical element in insulin antiapoptotic signals. This result differs somewhat from the findings of Bertrand et al. (*7*), who showed that LY290042 partially inhibited the protective function of insulin. However, it is important to realize that these authors used a concentration of inhibitor that was 10-fold higher than the one used in our study and, therefore, could be the result of a nonspecific effect. Interestingly, a role for PI-3′ kinase in the antiapoptotic signaling of IGF-1 has been reported in several cell models (*18*−*21*). Indeed, expression of a dominant negative PI-3′ kinase mutant attenuated IGF-1 antiapoptotic signaling in PC12 cells (*19*, *20*). Moreover, wortmannin blocks the effect of IGF-1 in CHO cells (*58*). Thus, even though both hormones bind to receptors with intrinsic tyrosine kinase activity, IGF-1 activates a PI-3′ kinase-dependent antiapoptotic pathway, while insulin maintains survival of CHO/HIRc cells mainly via an alternative pathway.

We also examined the role of protein farnesylation in insulin antiapoptotic function by treatment of cells with manumycin. The inhibition of farnesyl transferase blocks activation of p21ras that is accompanied with marked inhibition of the stimulatory effect of insulin on Raf-1 activation (*59*) and ERK activity (*59*, *60*, and our data). Under these conditions, manumycin was able to block insulin-mediated inhibition of apoptosis in CHO/HIRc cells. However, the inhibitor used in these experiments may not be specific for Ras. Thus, the ability of manumycin to prevent the antiapoptotic effect of insulin could also be due to its action on other signaling molecules. Nevertheless, our data are consistent with the results of Lebowitz et al. (*61*), who observed that farnesyl transferase inhibitors induced apoptosis in Ras-transformed cells.

The ability of insulin to activate Raf-1 implies that Raf-1 kinase activity may play a role in the promotion of cell survival, as suggested by recent studies (*7*, *62*). However, Kaufman-Zeh et al. (*63*) reported that Ras activation through Raf-1 pathway induced apoptosis in fibroblasts. Raf-1 has been shown to transduce the signal from Ras to ERK (*64*, *65*) via phosphorylation and activation of MEK, an immediate upstream activating kinase for ERK. Thus, it raises the possibility of the involvement of MEK/ERK in the control of apoptotic death. Rather than promoting apoptosis, we observed that MEK inhibition by PD98059 offered a greater protection both in unstimulated and insulin-treated CHO/HIRc cells. Recall that a similar reduction in the extent of apoptosis was found following a 24-h serum deprivation of CHO/ΔCT cells maintained without PD98059. Such a result could be explained by the low level of MEK activity expressed in CHO/ΔCT cells. In agreement with this explanation, it was observed that MEK phosphorylation by insulin was attenuated in cells expressing C-terminal truncated insulin receptor mutants (*66*). Taken together, our finding indicates that the ERK1 and ERK2 isoforms of the MAPK family have intrinsic inhibitory activity that suppresses the survival signal in CHO/HIRc cells, in agreement with the conclusion of Sutherland et al. in hepatoma cells (*67*). However, there are conflicting data regarding the involvement of ERK in growth factor-mediated cell survival (*19*, *20*, *37*, *68*−*70*). In this regard, it is worth noting that the paradoxical ability of ERK to prevent apoptosis in some cell lines and not in others indicates that cell type and the nature of inducers of apoptosis (e.g., serum deprivation, chemotherapeutic agents, UV irradiation) may define the contribution of one signaling pathway versus another. Thus, our results provide evidence that protein farnesylation (of Ras or other signaling molecules) plays a major role in the antiapoptotic function of insulin in CHO/HIRc cells and

that ERK activation is not required for the action of this hormone on cell survival.

There are three types of mammalian MAP kinases that include ERKs, c-Jun N-terminal protein kinase (JNK)/stress-activated protein kinase (SAPK), and p38 MAP kinase. A recent report indicated that insulin stimulates JNK/SAPK activity by a Ras-dependent mechanism in Rat1/HIRc cells (*71*). However, the question of whether p38 MAP kinase activity is regulated by insulin gave rise to conflicting results. In one hand, insulin was found to have no effect on p38 MAP kinase activity in muscle (*72*) while causing a marked inhibition in PC12 cells and Rat1 fibroblasts (*70*). On the other hand, activation of p38 MAP kinase by insulin was recently reported in hepatoma cells (*73*). These controversial observations raise the possibility that the insulin's ability to modulate p38 MAP kinase may be related to the cell type studied. Indeed, we demonstrated here that insulin stimulated p38 MAP kinase activity, as assessed: (i) indirectly by showing an increased phosphorylation of MKK3/MKK6, immediate upstream activating kinases for p38 MAP kinase, and (ii) directly by reporting a 4-fold activation of p38 MAP kinase measured in an immunoprecipitation-based kinase assay from insulin-treated CHO/HIRc cells. Because cellular MKK3 and p38 protein levels remained unchanged under these conditions, one could argue for a role of insulin in p38 MAP kinase activation. Of interest, we established that the tyrosine phosphatase inhibitor orthovanadate mimicked insulin by increasing p38 MAP kinase activity. Such a result could be explained by the fact that p38 MAP kinase activation requires phosphorylation of critical tyrosine residues (*39*−*41*). Furthermore, vanadate has been found to increase p38 activity in CHO/K1018A cells. Our finding is in agreement with the result of D'Onofrio et al. (*74*), who observed that tyrosyl phosphorylation and activation of ERKs by orthovanadate was independent of insulin receptor autophosphorylation and activation in CHO/HIRc cells. Thus, under our experimental conditions, insulin and orthovanadate appeared to be capable of stimulating ERK and p38 MAP kinase activities and emphasizes that coordinated regulation of these complex signaling cascades may be important in determining cellular antiapoptotic capacity.

Recent studies indicate a correlation between changes in the activity of p38 MAP kinase and the degree of neuronal cell apoptosis induced by growth factor withdrawal (*25*, *37*). In contrast to these reports, Kulik et al. (*19*) failed to establish such correlation in IGF-1's antiapoptotic protection in Rat-1 fibroblasts. Our finding supports the notion that increases in p38 MAP kinase activity does not play an important role in the antiapoptotic response mediated by insulin. It was recently suggested that the antiapoptotic function of insulin was mediated, in part, through activation of the transcription factor nuclear factor κB (NF-κB) (*7*). This argues for a significant part of the entire process to be due to secondary changes in expression of antiapoptotic genes. The involvement of p38 MAP kinase in insulin-induced NF-κB activation appears unlikely due to the presence of distinct signaling cascades involved in their activation (*69*).

In conclusion, we have demonstrated that the antiapoptotic action of insulin is dependent upon intact insulin receptor kinase activity but does not need the receptor juxtamembrane nor the C-terminal domain. We also showed that treatment with orthovanadate mimicked the ability of insulin to exert antiapoptotic function while triggering activation of the p38 MAP kinase cascade. Whereas the survival signal does not require PI-3′ kinase, ERK, nor p38 MAP kinase activation, protein farnesylation plays an important role in insulin's ability to protect CHO cells against apoptotic death induced by serum starvation.

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