Type 1 Insulin-like Growth Factor Receptor (IGF-IR) Signaling Inhibits Apoptosis Signal-regulating Kinase 1 (ASK1)*

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Veronica Galvan‡, Anna Logvinova‡, Sabina Sperandio‡, Hidenori Ichijo§, and Dale E. Bredesen¶‡

From the Buck Institute for Age Research, Novato, California 94945-1400 and the Laboratory of Cell Signaling, Graduate School, Tokyo Medical and Dental University, 1-5-45 Bunkyo-ku, Tokyo 113-8549, Japan

The type 1 insulin-like growth factor receptor (IGF-IR) is a receptor-tyrosine kinase that plays a critical role in signaling cell survival and proliferation. IGF-IR binding to its ligand, insulin-like growth factor (IGF-I) activates phosphoinositide 3-kinase (PI3K), promotes cell proliferation by activating the mitogen-activated protein kinase (MAPK) cascade, and blocks apoptosis by inducing the phosphorylation and inhibition of proapoptotic proteins such as BAD. Apoptosis signal-regulating kinase 1 (ASK1) is a MAP kinase kinase kinase (MAPKKK) that is required for c-Jun N-terminal kinase (JNK) and p38 activation in response to Fas and tumor necrosis factor (TNFα) receptor stimulation, and for oxidative stress- and TNFα-induced apoptosis. The results presented here indicate that ASK1 forms a complex with the IGF-IR and becomes phosphorylated on tyrosine residue(s) in a manner dependent on IGF-IR activity. IGF-IR signaling inhibited ASK1 irrespective of TNFα-induced ASK1 activation and resulted in decreased ASK1-dependent JNK1 stimulation. Signaling through IGF-IR rescued cells from ASK1-induced apoptotic cell death in a manner independent of PI3K activity. These results indicate that IGF-IR signaling suppresses the ASK1-mediated stimulation of JNK/p38 and the induction of programmed cell death. The simultaneous activation of MAP kinases and the inhibition of the stress-activated arm of the cascade by IGF-IR may constitute a potent proliferative signaling system and is possibly a mechanism by which IGF-I can stimulate growth and inhibit cell death in a wide variety of cell types and biological settings.

The type 1 insulin-like growth factor receptor (IGF-IR) is a receptor-tyrosine kinase that plays a critical role in signaling cell survival and proliferation. Cells lacking this receptor cannot be transformed by most oncogenes, with the exception of v-Src (1). In addition, IGF-I can stimulate proliferation of a variety of cell types in culture in the absence of other growth factors. Gene knockout experiments in mice (2–4) and flies (5) have demonstrated that the IGF-I axis is required for normal growth at the organismal level as well. In mammalian cells, the assembly of a signaling complex at the cytoplasmic domain of IGF-IR results in the activation of phosphoinositide 3-kinase (PI3K) and its target, Akt/PI3K (6) and promotes cell proliferation, and in some situations differentiation, by engaging the mitogen-activated protein kinase (MAPK) cascade (7, 8) and the Ras pathway (9). The inactivation of the pro-apoptotic Bcl-2 family member BAD through phosphorylation by Akt/PI3K (10) is thought to underlie the strong anti-apoptotic activity of IGF-I and II. Activation of IGF-IR by its ligand also initiates metabolic cascades that result in the stimulation of protein synthesis, glucose intake, glycogen synthesis, and lipid storage.

The MAPK signaling cascade is conserved in evolution and controls transcriptional responses to mitogenic or stress stimuli by the sequential activation of protein kinases. There are at least 6 independent MAPK signaling units described in mammals (11). The pathways that culminate in the activation of the extracellular signal-regulated kinases (ERKs) and stress-activated protein kinases (SAPK/JNK and p38) have been characterized in some detail. Apoptosis signal-regulating kinase (ASK1) is a MAPKKK that activates the JNK/p38 pathway in response to proinflammatory cytokines such as TNFα and FasL (12–14). As for other MAPKKK, deletion of the N-terminal domain of ASK1 results in its constitutive activation, suggesting that the N terminus contains a regulatory domain (15). Consistent with this observation, Daxx (12), TRAF2 (14), and reduced thioredoxin (15) have been shown to interact with sequences in the ASK1 N-terminal domain.

ASK1 selectively activates the SEK1-JNK1 and M KK3/ M KK6-p38 pathways (16) and is required for the sustained activation of p38 in response to TNFα and oxidative stress (17). The biological outcome of JNK/p38 activation is largely dependent on cell type and cellular context (18), and in some situations leads to the activation of programmed cell death (19–21). TNFα-induced JNK activation can be blocked by IGF-II in neuronal cell lines (22). Also, IGF-I can suppress apoptosis downstream of TNF-R activation (23) and block JNK activation by amyloid-β (24).

Withdrawal of growth factors present in serum (15) or withdrawal of nerve growth factor (25) has been shown to lead to a severalfold increase in ASK1 activity. Also, ASK1-induced death in cultured cells requires either the deletion of its N-terminal domain (19) or low serum conditions (16). We hypothesized that a pathway activated by trophic factor(s) could thus negatively regulate ASK1 activity. Given that IGF-I is a potent activator of cell proliferation, and that in many cases it can replace serum to stimulate growth of cells in culture, we sought...
to investigate whether IGF-I signaling modulates ASK1 activity. The results presented here indicate that IGF-IR signaling inhibits ASK1 and relieves ASK1-induced apoptotic cell death. We found that ASK1 formed a complex with IGF-IR both in the presence and in the absence of exogenously added IGF-I and became phosphorylated on tyrosine residue(s) on its regulatory N-terminal domain in a manner dependent on IGF-IR kinase activity.

IGF-IR signaling inhibited ASK1 activation in the presence of TNF-α and attenuated ASK1-dependent JNK1 stimulation. We propose that one of the mechanisms underlying the cytoprotective activity of IGF-1 signaling may involve the down-regulation of the stress-activated arm of the MAP kinase cascade through the phosphorylation and inhibition of ASK1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—Unless indicated otherwise, human embryonic kidney 293 and L929 cells were grown in 60-, 100-, or 150-mm dishes at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin/streptomycin (Cellgro). 293 cells grown in 60- or 100-mm dishes were transiently transfected with 3-5 or 6-8 μg of the indicated plasmid constructs, respectively, using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions and maintained in serum-free medium for the duration of the experiment. Lysates were collected 13–15 h after transfection. 8–10 h post-transfection, cultures of 293 cells were treated with 100 ng/ml of recombinant TNF-α (Sigma) for 20 min. In all transfection experiments, the amount of plasmid DNA transfected was equalized by the addition of pCDNA3.1 vector DNA. For the analysis of endogenous proteins, 8–10 150-mm dishes of L929 cells were incubated in serum-free media for 2 h and then either left untreated or treated with 200 nM wortmannin (Sigma) 20 min prior and during treatment with 100 ng/ml purified IGF-I (Sigma) for 10 additional minutes.

DNA Constructs and Antibodies—Hemagglutinin-tagged wild-type and kinase-mutant human ASK1 (ASK1-HA and ASK1KD-HA) and the glutathione S-transferase fusion of MKK6KD were described previously (16). The ASK1Y574A-HA construct was generated by site-directed mutagenesis using primers CACAACAAATCTACACCTTGGTCTTGGTGCTCAAAACTAGAAGTTGAGG and CCTCAACTTCATTGTTTGGAGT and processed in a SGI Octane R12 computer running Biplane’s Advanced Imaging Software suite. Briefly, deconvolution was done using a maximum likelihood estimation with Hillary software and then processed by the Imaaris imaging interface (Biplane AG).

Assessment of Apoptotic Cell Death—Percentages of apoptotic cells in cultures were determined by standard propidium iodide (PI) staining protocols and flow cytometry (27) 60 h after transfection. IGF-I-expressing cells were treated with 100 ng/ml IGF-I and 200 nM wortmannin (Sigma) after transfection as indicated and maintained in their presence for the duration of the experiment. Fresh IGF-I was added to the cultures every 8 h.

RESULTS

IGF-I and the Activated IGF-I Inhibit ASK1 Activation—Given that maximal activation of ASK1 in cultured cells is achieved when cultures are maintained in the absence of serum, we hypothesized that a pathway activated by trophic factor(s) could negatively regulate ASK1 activity. IGF-I is a potent activator of cell proliferation and in many cases it can replace serum to stimulate growth of cells in culture. We therefore asked whether IGF-I would be sufficient to decrease ASK1 activity in serum-deprived L929 cells. The activity of ASK1 was reduced 40% in L929 cells grown in 10% serum in comparison to L929 cells maintained in the absence of serum (Fig. 1, panels A and B). IGF-I treatment of serum-starved L929 cells decreased the activity of ASK1 to levels identical to those observed in cultures treated with 10% serum, implying that the inhibition of ASK1 activity by serum may be attributed to the effect of IGF-I. Recently, Chao and co-workers (28) showed that ASK1 can be inhibited by Akt, a serine/threonine kinase that is activated by PI3K and has a crucial role in the signal transduction pathway initiated by IGF-I. To determine the contri-
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Fig. 1. IGF-I inhibits ASK1 activity. A, ASK1 was immunoprecipitated from L929 cells treated as indicated. Complexes were resolved in gels and immunoblotted (upper panel) or used in kinase assays with GST-MKK6KD as a substrate in the presence of [γ-32P]ATP. The products of the reaction were resolved in gels and detected by phosphorimeter analysis (lower panel). IP, immunoprecipitation; WB, Western blot; KA, kinase assay. B, the activity of ASK1 in L929 cells treated as indicated was determined by phosphorimeter analysis and densitometry as described under “Experimental Procedures.” The fold activation of ASK1 was determined in reference to its activity in cells maintained in the presence of 10% serum, which was equal to 1. The average of two independent experiments is shown. C, the activity of ectopically expressed ASK1 is decreased in the presence of kinase-competent IGF-IR. ASK1-HA was transiently expressed in the presence of full-length IGF-IR (IGF-IR) or a kinase-inactive IGF-IR (IGF-IRIC) or kinase-mutant (IGF-IRKM) myristylated form of the intracellular domain of the receptor. ASK1-HA was immunoprecipitated and used in kinase assays in the presence of [γ-32P]ATP and myelin basic protein (MBP) as a substrate. The products of the reaction were resolved in gels and detected by phosphorimeter analysis of 32P incorporation in ASK1 (upper panel) and MBP (middle panel). Immunoprecipitated HA-ASK1 was analyzed by immunoblot (lower panel). D, the histogram shows the mean ± S.E. for densitometric ratios of 32P-labeled protein determined by phosphorimeter analysis. Values of 32P-labeled protein were normalized to ASK1 protein levels by immunoblotting as described in C. p values were calculated by Student’s paired t test.

bution of Akt to the decrease in ASK1 activity induced by IGF-I, we incubated serum-starved, IGF-I-stimulated cells in the presence of wortmannin, a potent PI3K inhibitor. Inhibition of PI3K signaling did not have a significant effect on the decrease in ASK1 activity resulting from IGF-I stimulation of serum-starved cells (Fig. 1, panel B). In some experiments, we observed a moderate decrease in ASK1 activity in the presence of wortmannin (Fig. 1, panel A). This could be caused by a general effect of wortmannin on protein synthesis, even at the relatively short incubation times used in these experiments. Given that Akt activation was effectively inhibited by wortmannin in IGF-I-stimulated L929 cells (not shown), we concluded that the inhibition of ASK1 by IGF-I occurred, at least partially, upstream or independently of PI3K.

Next, we transiently expressed a human ASK1 complementary DNA (cDNA) tagged with a hemagglutinin epitope (ASK1-HA) in the presence of a kinase-competent, full-length human IGF-IR cDNA (IGF-IR), a kinase-competent, myristylated intracellular domain of the IGF-IR β-chain (IGF-IRβC), or a kinase-inactive form of the myristylated IGF-IR intracellular domain (IGF-IRβM) in 293 cells. As reported previously (3), ectopically expressed ASK1-HA showed high constitutive activity in in vitro kinase assays (Fig. 1, panel C). We observed variability in ASK1-HA protein levels when it was expressed in cells in the presence of IGF-IR; therefore, to determine whether ASK1-HA activity was affected by IGF-IR signaling independent of variations in ASK1-HA protein levels, we determined the ratio between ASK1-HA kinase activity by radionuclide labeling and phosphorimeter analysis and the densitometric values of ASK1-HA protein by immunoblot in cells expressing ASK1-HA in the presence of different forms of IGF-IR. ASK1 specific activity was decreased in the presence of full-length IGF-IR (Fig. 1, panel D). IGF-IRβM, which showed high constitutive kinase activity (Fig. 2, panel D and data not shown), further inhibited both ASK1 autophosphorylation and the phosphorylation of myelin basic protein by ASK1 (Fig. 1, panels C and D). This effect was partially abolished by mutation of the ATP binding site in the IGF-IR kinase domain. Mutation of lysine 1003 to arginine in the ATP binding site of the receptor has been shown to abolish its kinase activity (29). The partial (rather than complete) restoration of ASK1 kinase activity observed in the presence of the kinase-inactive form of the IGF-IR intracellular domain suggests that a kinase-independent mechanism for ASK1 inhibition by IGF-IR may exist.
IGF-IR may become active in the absence of exogenously added stimulation. To determine whether transiently expressed IGF-IR may signal in the absence of exogenous IGF-I, we assayed IGF-IR complexes immunoprecipitated from 293 cells in in vitro kinase assays and found that these complexes were able to transphosphorylate in vitro (data not shown). These observations suggest that IGF-IR may form kinase-active complexes when transiently expressed in 293 cells in the absence of ligand binding. Even though survival of 293 cell cultures requires the addition of serum or IGF-I to the media, we cannot rule out the possibility that suboptimal amounts of IGF-I may be produced by 293 cells maintained in serum-free conditions for the duration of the transient transfection experiments described in these studies, —12 h. We concluded that ASK1 is phosphorylated on a tyrosine residue in a manner dependent on IGF-IR activity and independent of exogenously added IGF-I.

To determine whether ASK1 is a direct substrate for IGF-IR, we immunoprecipitated ectopically expressed IGF-IR, the intracellular domain of the IGF-IRβ chain and a kinase-mutant form of ASK1-HA from 293 cells and performed in vitro kinase assays in the presence of 32P-labeled γ-ATP. A kinase-mutant form of ASK1-HA (ASK1-KM-HA), which possesses no detectable kinase activity (Fig. 2C, right panel), was labeled with 32P when IGF-IR or the kinase-competent intracellular domain of the receptor’s β-chain were present in the reaction (Fig. 2C, left panel), suggesting that IGF-IR can phosphorylate ASK1 in vitro. Kinase-active ASK1-HA was included in the experiments as a control for the kinase reaction and to indicate the position of migration of 32P-labeled ASK1-HA protein bands in gels.

ASK1 Is Found in a Complex with IGF-IR—Because the detection of a complex formed by IGF-IR and ASK1 would provide additional support for the notion that ASK1 is a substrate of the IGF-IR, we performed reciprocal immunoprecipitation experiments using lysates from L929 cells maintained in the presence of 10% serum or deprived of serum with and without IGF-I stimulation. We found that the β-chain of IGF-IR was present in complexes immunoprecipitated with an anti-ASK1 antibody in extracts from L929 cells maintained both in the presence and in the absence of serum (Fig. 3, panel A). A relative increase in the amount of IGF-IR β-chain present in complexes immunoprecipitated with anti-ASK1 was observed in lysates from serum-starved L929 cells.

Given that ASK1 is a cytoplasmic protein, we next asked whether the interaction of ASK1 with IGF-IR involved the cytoplasmic portion of the receptor’s β-chain, which encompasses its kinase domain. ASK1-HA was transiently expressed in 293 cells together with IGF-IR or with kinase-active and kinase-inactive forms of the IGF-IR β-chain intracellular domain and reciprocal immunoprecipitations were performed. Both a kinase-active and a kinase-inactive form of the IGF-IR β-chain, as well as the full-length receptor, were found in complexes immunoprecipitated with anti-IGF-IR antibodies (Fig. 3, panel B). Conversely, complexes immunoprecipitated with anti-IGF-IR antibodies contained ASK1-HA (Fig. 3, panel C). These results indicate that the interaction between ASK1 and the IGF-IR involves the cytoplasmic portion of the IGF-IR β-chain. Consistent with the constitutive nature of the interaction between the endogenous ASK1 and IGF-IR in L929 cells, the formation of IGF-IR/ASK1 complexes was not affected by mutation of the ATP-binding site in the IGF-IR kinase domain.

It has been shown that the mechanism of ASK1 activation involves, in part, stimulus-dependent homo-oligomerization (30). Thus, it is possible that the interaction with IGF-IR blocks ASK1 homo-oligomerization and activation, and may explain why kinase-mutant forms of IGF-IR partially retained the ability to inhibit ASK1 (Fig. 1, panels C and D).

**Fig. 2. ASK1 is phosphorylated on Tyr in the presence of kinase-active IGF-IR.** A, ASK1-HA was transiently expressed in 293 cells in the presence of kinase-competent or kinase-mutant forms of IGF-IR and immunoprecipitated using anti-HA antibodies. Complexes were separated on 3–8% Tris acetate gels and immunoblotted with anti-HA antibodies. B, lysates from 293 cells transiently expressing the indicated proteins were subject to immunoprecipitation using anti-ASK1 antibodies, resolved in gels and immunoblotted with antiphospho-Tyr antibodies (upper panel) or anti-ASK1 antibodies (lower panel). C, the indicated IGF-IR forms and either ASK1-HA or ASK1-KM-HA were separately immunoprecipitated from lysates of 293 cells and used alone or combined in in vitro kinase assay reactions in the presence of [γ-32P]ATP as indicated. The [32P]-labeled products were separated in gradient gels and detected by phosphorimager analysis (left panel). The activity of ASK1-HA or ASK1-KM-HA immunoprecipitated from 293 cells was determined in in vitro kinase assays by incorporation of [γ-32P]ATP (right panel).
We then sought to determine the subcellular localization of the interaction between ASK1 and IGF-IR. The two proteins were found in structures adjacent to the nucleus of 293 cells ectopically expressing ASK1 and the IGF-IR precursor (Fig. 3D, upper panels). The presence of ASK1/IGF-IR complexes in perinuclear structures may reflect the accumulation of immature forms of IGF-IR in the endoplasmic reticulum (ER) compartment and their interaction with ASK1, which is associated with components of the unfolded protein response machinery that assembles at the ER membrane (31). In agreement with the observation that the intracellular domain of IGF-IR mediates its interaction with ASK1, we found that IGF-IRcA co-localized with ASK1 in a compartment juxtaposed to the cytoplasmic face of the plasma membrane in 293 cells in a manner independent of its kinase activity (Fig. 3D, middle and lower panels).

The N-terminal Domain of ASK1 Is Tyr-phosphorylated—It has been shown that the N-terminal domain of ASK1 contains regulatory domains that affect the activity of the kinase through phosphorylation (28) and protein-protein interactions (14, 15). To determine whether phosphorylation by the IGF-IR occurs at the N-terminal regulatory domain of ASK1 we transiently expressed a mutant ASK1-HA construct in which the N-terminal 648 amino acids of the kinase had been deleted (ASK1/H-9004 N-HA) (15) together with IGF-IR in 293 cells maintained in the absence of serum. While full-length ASK1-HA became phosphotyrosine immunoreactive in the presence of IGF-IR, no detectable phosphorylated tyrosine residues were found in ASK1/H-9004 N-HA, irrespective of IGF-I stimulation (Fig. 4, panel A). Therefore, the Tyr residue(s) that are phosphorylated in ASK1 by IGF-IR map to the N-terminal 648 amino acids of the kinase. Both the IGF-IR and the intracellular portion of its cA-chain were detected in complexes immunoprecipitated with ASK1/H-9004 N-HA, suggesting that the N-terminal domain of ASK1 is not required for its interaction with IGF-IR (data not shown).

IGF-IR Inhibits ASK1 Downstream of TNF-R and Blocks JNK1 Activation—ASK1 is activated by TNF-R (13, 14) and by cellular stress (11, 20), and it activates JNK and the p38 MAP kinase pathways (16). The possibility that transiently overex-
The specific activity of ASK1 was calculated as the ratio between phosphorylated and total ASK1. The complexes were resolved in gels and immunoblotted with anti-phospho-Tyr (upper panel) or anti-HA antibodies (lower panels).

IGF-IR Inhibits ASK1 irrespective of TNFα stimulation and decreases ASK1 signaling to JNK1 in 293 cells. The activity of ASK1-HA immunoprecipitated from lysates of 293 cells transiently expressing the indicated proteins was determined in vitro kinase assays using [γ-32P]ATP (upper panel). ASK1-HA complexes were resolved in gels and immunoblotted as indicated (lower panel). The specific activity of ASK1 (Sp activity) was calculated as the ratio between 32P incorporation and the densitometric values of ASK1 protein by phosphorimager analysis of kinase assays and densitometry as described under “Experimental Procedures” and multiplied by 100.

Fig. 5. IGF-IR inhibits ASK1 irrespective of TNFα stimulation and decreases ASK1 signaling to JNK1 in 293 cells. A, the activity of ASK1-HA immunoprecipitated from lysates of 293 cells transiently expressing the indicated proteins was determined in vitro kinase assays using [γ-32P]ATP (upper panel). ASK1-HA complexes were resolved in gels and immunoblotted as indicated (lower panel). The specific activity of ASK1 (Sp activity) was calculated as the ratio between 32P incorporation and the densitometric values of ASK1 protein by phosphorimager analysis of kinase assays and densitometry as described under “Experimental Procedures” and multiplied by 100.

Fig. 6. IGF-IR suppresses ASK1-induced apoptosis. Percentages of apoptotic cells present in cultures of 293 cells expressing the indicated proteins were determined by PI staining and flow cytometry as described under “Experimental Procedures.” p values were determined by Student’s t test.

**DISCUSSION**

The response of cells to changes in their physical and chemical environment is key to the control of many aspects of cellular function. Environmental changes happen in ranges of intensities and duration and in a combinatorial fashion in most biological settings. It is therefore likely that most signaling pathways have evolved in the context of their interconnection in an entire network, and thus the investigation of how indi-
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Signal through IGF-IR is required for growth of most cell types, and is sufficient to support survival of a variety of cells in culture in the absence of other growth factors. IGF-I was shown to suppress apoptosis through the activation of the PI3K/Akt axis, which results in the phosphorylation and inhibition of BAD, a pro-apoptotic member of the Bcl-2 family (10). IGF-I was also shown to suppress apoptosis downstream of TNF-R activation (23) and to block the activation of JNK by the toxic fragment derived from the amyloid precursor protein, amyloid β (24). The mechanisms by which IGF-I signaling blocks the stress-activated MAP kinase cascade, however, have not yet been elucidated.

In the present work we have addressed the modulation of one of the apical stress-activated MAP kinases, ASK1, by the IGF-IR signaling pathway. Our findings indicate that ASK1 is inhibited by IGFIIR and becomes phosphorylated on Tyr residue(s) on its regulatory N-terminal domain in a manner dependent on IGF-IR activity. IGF-IR and ASK1 formed a complex in cells irrespective of the functionality of either kinase, and irrespective of IGF-IR ligand stimulation. This interaction involved the C-terminal domain of ASK1 and the intracellular domain of the IGF-IR β-chain. We found that ASK1 was present in a complex with the IGF-IR in a compartment juxtaposed to the nucleus in 293 cells overexpressing both kinases. Ectopically expressed ASK1 also colocalized with kinase-active and kinase-inactive myristylated forms of the intracellular domain of the IGF-IR at the plasma membrane of 293 cells. Consistent with our observation that endogenous IGF-IR and ASK1 were found in complexes immunoprecipitated from L929 cells irrespective of IGF-I or serum stimulation, the two kinases colocalized in perinuclear structures reminiscent of ER in serum-stimulated or serum-deprived L929 cells (data not shown).

Our observation that transiently expressed IGF-IR may be active in the absence of ligand binding allowed us to examine signaling by the receptor in the absence of IGF-I stimulation of the endogenous IGF-IR, which is sufficient to inhibit ASK1 activation and ASK1-induced cell death. Our results demonstrate that ASK1-dependent JNK activation and cell death (which occur only in the absence of IGF-I) were inhibited in a manner dependent on the presence of IGF-IR and independent of PI3K function.

In this experimental system, TNFα stimulation did not affect IGF-IR inhibition of ASK1. Whether IGF-IR blocked ASK1 activation or whether the TNFα-stimulated ASK1 activity was inhibited by IGF-IR could not be deduced from our data. On the other hand, the activation of JNK1 and the induction of programmed cell death downstream of ASK1 activation were inhibited by IGF-IR even when PI3K activity was blocked by wortmannin.

The observation that ASK1 and IGF-IR were present in a complex in L929 cells irrespective of IGF-I stimulation and that this interaction was independent of either IGF-IR or ASK1 kinase activity in 293 cells may indicate that ASK1 molecules bind the intracellular portion of IGF-IR heterodimers in a constitutive fashion. ASK1 has been shown to signal in the cytoplasmic compartment (16) and to be activated by recruitment to the membrane-associated signaling complexes such as TNF-R/TRAF2, Fas/Daxx (12, 14), and IRE1/TRAF2 (31). Formation of these complexes is thought to favor the homo-oligomerization and transphosphorylation of the kinase (12, 33). Our observation that a kinase-inactive form of IGF-IR partially retained the ability to inhibit ASK1 suggests that IGF-IR may also inhibit ASK1 activation by interfering with its homooligomerization through protein-protein interactions. It is possible that a membrane-associated pool of ASK1 molecules exists in equilibrium between active and inactive forms, which could be shifted in either direction by selective ligand binding of activators such as TNF-R or of inhibitors such as IGF-IR.

Our results indicate that ASK1 is phosphorylated on multiple Tyr residues at its N-terminal domain in a manner dependent on IGF-IR activity and suggest that ASK1 is a bona fide substrate of IGF-IR. The N-terminal domain of ASK1 contains 23 Tyr residue(s) of which 5 are predicted as candidate phosphorylation sites by NetPhos 2.0 (Tyk-95, Tyk-210, Tyk-390, Tyr-574, and Tyr-625). One of these five Tyr residues is phosphorylated in the presence of kinase-competent IGF-IR, but the additional phosphorylated Tyr residue(s) remain to be identified. The identification of all phosphorylated Tyr residues in the N-terminal domain of ASK1 is currently underway and will allow us to further our understanding of the physiological significance of the phosphorylation of ASK1 by the IGF-IR.

Kim et al. (28) recently showed that PKB/Akt, a downstream effector of the IGF-I signaling pathway, phosphorylates and inhibits ASK1. These observations, together with the results presented here, suggest that the IGF-I signaling pathway can antagonize the activation of ASK1 through two different kinases. It is possible that inhibition of ASK1 by IGF-IR and its effector PKB/Akt occurs in a stepwise fashion and that phosphorylation, and/or protein-protein interactions with both kinases is required for complete inhibition of ASK1. This hypothesis is supported by our observation that inhibition of PI3K activity by wortmannin only partially reversed IGF-I-dependent inhibition of ASK1. Alternatively, phosphorylation and/or protein-protein interactions by either IGF-IR or PKB/Akt may be sufficient to inhibit ASK1 activity to levels below those required for activation of JNK/p38. The observation that two different kinases in the same pathway inhibit ASK1 may indicate that the metabolic responses triggered by IGF-I stimulation require that the inhibition of the stress-activated arm of the MAPK cascade be ensured.

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Bredesen

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