Journal of Cellular Biochemistry 82:610–618 (2001)

Regulation of the Akt/Glycogen Synthase Kinase-3 Axis by Insulin-Like Growth Factor-II Via Activation of the Human Insulin Receptor Isoform-A

Pierluigi Scalia,[1,3]* Emma Heart,[1] Lucio Comai,[2] Riccardo Vigneri,[3] and Chin K. Sung[1]

[1]Department of Physiology and Biophysics, University of Southern California, Keck School of Medicine, Los Angeles, California 90033

[2]Department of Molecular Biology and Immunology, University of Southern California, Keck School of Medicine, Los Angeles, California 90033

[3]Istituto di Medicina Interna, Malattie Endocrine e del Metabolismo, University of Catania, Ospedale Garibaldi, 95123 Catania, Italy

Abstract Insulin-like growth factor II (IGF-II) plays a key role in mitogenesis during development and tumorigenesis and is believed to exert its mitogenic functions mainly through the IGF-I receptor. Recently, we identified the insulin receptor isoform A (IR^A) as an additional high affinity receptor for IGF-II in both fetal and cancer cells. Here we investigated the mitogenic signaling of IGF-II via the Akt/Glycogen synthase kinase 3 (Gsk3) axis employing R-IR^A cells that are IGF-I receptor null mouse embryonic fibroblasts expressing the human IR^A. IGF-II induced activation of the proto-oncogenic serine kinase Akt, reaching maximal at 5–10 min. IGF-II also caused the rapid and sustained deactivation of glycogen synthase kinase 3-beta (Gsk3β), reaching maximal at 1–3 min, shortly preceding, therefore, maximal activation of Akt. Under our conditions, IGF-II and insulin induced 70–80% inhibition of Gsk3βactivity. In these cells IGF-II also deactivated Gsk3α although less effectively than Gsk3β. In parallel experiments, we found that IGF-II induced transient activation of extracellular-signal-regulated kinases (Erk) reaching maximal at 5–10 min and decreasing thereafter. Time courses and potencies of regulation of both mitogenic pathways (Akt/Gsk3β and Erk) by IGF-II via IR^A were similar to those of insulin. Furthermore, IGF-II like insulin effectively stimulated cell cycle progression from the G0/G1 to the S and G2/M phases. Interestingly, AP-1-mediated gene expression, that was reported to be negatively regulated by Gsk3β was only weakly increased after IGF-II stimulation. Our present data suggest that the coordinated activation or deactivation of Akt, Gsk3β, and Erk may account for IGF-II mitogenic effects and support an active role for IR^A in IGF-II action. J. Cell. Biochem. 82: 610–618, 2001.

Key words: Gsk3; IGF-II; IR^A; R- cells; Erk1/2; TPA; AP-1

Insulin-like growth factor II (IGF-II) is a 7.5 kDa polypeptide that has been implicated in cell proliferation during fetal growth and tumorigenesis. IGF-II was initially reported to exert its biological effects through the IGF-I tyrosine kinase receptor since IGF-II binds and activates the IGF-I receptor with an affinity similar to that of IGF-I [Krywicki and Yee, 1992]. However, recent studies with null mice bearing single and multiple genetic deletions for the IGF family of ligands and receptors have challenged this concept [Baker et al., 1993]. The analysis of the growth phenotypes in these animal models suggest an important but not an absolute role of IGF-I receptor during fetal development. Genetic and biochemical studies further identified the insulin receptor as the

Abbreviations used: Gsk3, glycogen synthase kinase-3; IGF-II, insulin-like growth factor-II; IR^A, human insulin receptor isoform-A; R-cells, igf1r null mouse embryo fibroblasts; Erk1/2, extracellular signal-regulated kinase; TPA, 12-o-tetradecanoyl-phorbol-13-acetate; AP-1, activator protein-1.

Grant sponsor: The American-Italian Cancer Foundation to Pl.S.; Grant sponsor: The NIH Endocrinology Neurobiology of Aging training grant to E.H.; Grant sponsor: The Associazione italiana Ricerca sul Cancro (AIRC) to R.V.; Grant sponsor: NIH; Grant number: DK51015; Grant sponsor: The American Diabetes Association to C.K.S.

*Correspondence to: Pierluigi Scalia, Department of Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, 2011 Zonal ave., HMR-509, Los Angeles, CA. 90033

E-mail: plscalia@hsc.usc.edu

Received 7 November 2000; Accepted 15 March 2001

functional receptor mediating IGF-II growth effects [Louvi et al., 1997; Morrione et al., 1997]. Employing IGF-I receptor null mouse embryo fibroblasts [Sell et al., 1993], we have recently generated two cell lines, called R^-/IR^A and R^-/IR^B, which stably express the human insulin receptor (IR) isoforms named IR^A (exon 11−) and IR^B (exon11+), respectively [Frasca et al., 1999]. Using these two cell lines, we previously reported that only the short IR isoform IR^A, lacking the 12 amino acids encoded by exon 11 in the IR extracellular α-subunit, binds IGF-II with high affinity and stimulates DNA synthesis [Frasca et al., 1999]. In R^-/IR^A cells, IGF-II was capable of stimulating proximal signaling molecules including insulin receptor autophosphorylation, tyrosine phosphorylation of insulin receptor substrates (IRS) and Shc, and activation of phosphatidylinositol-3-kinase (PI3K). PI3K is critical for mitogenesis triggered by a variety of cell membrane receptors, and the proto-oncogenic protein kinase Akt and glycogen synthase kinase 3 (Gsk3) have been reported to be activated downstream of PI3K [Downward, 1998]. In the present study, we investigated the mitogenic signaling of IGF-II via the Akt/Gsk3β axis in R^-/IR^A cells. Employing flow cytometry we also studied IGF-II-stimulated cell cycle progression via IR^A. These data provide additional evidence of the IGF-II ability to stimulate mitogenesis via the human IR^A and suggest that regulation of the Akt/Gsk3β axis and Erk accounts for this effect.

MATERIALS AND METHODS

Anti-Erk1/2 and anti-Gsk3α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Gsk3β and anti-Akt monoclonal antibodies from Transduction Laboratories (Lexington, KY), and anti-phosphoSer473-Akt and anti-phosphoThr202/Tyr204-Erk were purchased from New England Biolabs (Beverly, MA).

Cell Culture and Growth Factor Treatment

R^-/IR^Acells were previously generated by stable transfection of the parental IGF-I receptor-null (R^-) mouse embryo cell line (provided by Dr. Renato Baserga) with an eukariotic expression vector (pNTK2) containing the human insulin receptor isoform-A (exon 11^-) gene (provided by Dr. Axell Ullrich) and cultured in DMEM, 10% fetal bovine serum and 0.3 mg/ml Puromicin. Human recombinant IGF-II (Calbiochem, San Diego, CA) and insulin (Sigma, St. Louis, MO) were used at 10 nM concentration unless specified otherwise. For all experiments, cells were grown up to 60–70% confluence, serum-deprived for 10–12 h and then stimulated with insulin or IGF-II for the times and at doses specified in figure legends.

Preparation of Whole Cell Lysates

After appropriate stimulation, cells were rinsed with ice-cold PBS, detached by scraping and pelleted in 0.35 ml sonication buffer (50 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 5 mM sodium pyrophosphate, 1 mM DTT, 10 mM beta-glycerophosphate, 10 μg/ml pepstatin A, 10 μ/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM benzamidine, 0.1 mM PMSF, 100 nM okadaic acid). Whole cell lysates were prepared by sonicating cells for 30 sec in ice followed by addition of NP40 at 1% final concentration and further solubilization for 30 min at 4°C with end-over-end rotation. Insoluble cell debris was then discarded by centrifugation for 15 min at 13,000 rpm. The supernatants (whole cell lysates) were either processed immediately or quickly frozen in liquid nitrogen and stored at −70°C until use.

Immunoprecipitation and In Vitro Gsk3 Enzymatic Assay

Whole cell lysates were immunoprecipitated for 2 h at 4°C with either a monoclonal antibody recognizing the amino terminus of rat Gsk3β (1 μg α-Gsk3β for 30 μg cell lysates) or with a polyclonal antibody recognizing the carboxy terminus of rat Gsk3α (1 μg α-Gsk3α for 70 μg cell lysates) followed by 1 h incubation with protein G-Sepharose (Amersham-Pharmacia Biotech, Piscataway, NY). Immunoprecipitates were washed twice with ice-cold sonication buffer (plus 1% NP40) and once with Gsk3 assay buffer (30 mM Tris, pH 7.4, 10 mM Magnesium acetate, 0.1 mg/ml BSA). Beads were resuspended in 30 μl reaction buffer containing 0.1 mM ATP, 20 μM phospho-Glycogen Synthase (pGS)-2 substrate and 3 μCiγ-^{32}P ATP and incubated for 15 min at 30°C. The reaction was stopped by addition of 5 μl of 4 M HCI and aliquots of the reaction mixture were spotted onto P81 paper followed by extensive washings with 150 mM phosphoric acid. P81 papers were air-dried and the radioactivity was measured in a liquid scintillation β-counter.

Western Blot Analysis

Whole cell lysates prepared as described above were denatured in SDS-sample buffer (0.25 M Tris,pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 2.5% β-mercaptoethanol) by heating for 3 min at 95°C and resolved by SDS–PAGE. Proteins were next immobilized on PVDF membrane (Hi-Bond, Amersham-Pharmacia, Piscataway, NY) by semidry transfer and the specific proteins were detected by incubation with primary antibodies (1:2000–1:3000 dilution) in PBST, further incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies and subsequent enhanced chemiluminescence (SuperSignal, Pierce, Rockville, IL).

Luciferase Assay

AP-1 transcriptional activity was evaluated by transient transfection of R^-/IR^A cells (6×10^4 cells) with a luciferase reporter plasmid (1 μg DNA) driven by the AP-1 promoter region (−73/TRE) as described elsewhere [Aroenhei et al., 1997]. Transient DNA transfections were performed with lipofectamine and carried out for 5 h in Optimem media (Life Technologies, Rockville, MD). Cells were then incubated in serum free media (DMEM, 0.1% BSA) with either TPA or IGF-II or insulin at the indicated concentrations for 16 h. Cells were next solubilized in reporter lysis buffer (Promega, Madison, WI), quickly frozen in liquid nitrogen and stored at −70°C. Luciferase activity was assayed in 20 μg of cell lysates following manufacturer's recommendations (Promega).

Flow Cytometric Analysis

Subconfluent R^-/IR^A cells (60–70%) were serum-deprived for 10 h and stimulated with IGF-II or insulin at 10 nM for 2, 4, 6, 12, and 24 h. Serum deprivation was chosen to avoid abnormal changes in metabolism and responsivity associated with other cell synchronization procedures, and serum-deprivation time (10 h) was the same as for the signaling studies. The stimulation medium was replaced every 6 h to minimize the effect of possible IGF-II and insulin degradation. At the end of the stimulation, cells were washed twice with ice-cold PBS, detached by scraping and fixed in 70% ethanol. Ethanol-fixed cells were stored at −20°C for 2–16 h. Ethanol was then removed by pelleting cells at low speed centrifugation followed by cell washes in PBS. DNA staining was performed by incubating cells at 37°C for 2 h in PBS containing 20 mg/ml propidium iodide and 40 mg/ml RNAseA. Stained cells were then analyzed by flow cytometry using a FACStar system (Becton Dickinson, Franklin Lakes, NJ) and the percentage of cells in G0/G1 vs. S and G2/M phases was determined using CellQuest software.

RESULTS

Time Course for Akt Phosphorylation/Activation by IGF-II Via Activation of the Insulin Receptor Isoform-A (IR^A)

The proto-oncogenic serine kinase Akt is a well recognized target in insulin and IGF-I signaling downstream of PI3K. Akt mediates a variety of biological effects including cell growth, cell survival, and glucose metabolism [Downward, 1998]. Full activation of Akt by growth factors requires phosphorylation on Ser473, which tightly correlates with Akt kinase activity [Kohn et al., 1995]. In order to investigate whether Akt was activated in response to IGF-II via IR^A, R^-/IR^A cells were stimulated with either IGF-II or insulin at 10 nM for 1 to 30 min and solubilized. Whole cell lysates were then subjected to SDS–PAGE and subsequent Western blot analysis with an antibody to phosphorylated Akt at Ser473. Akt phosphorylation was rapidly increased by both IGF-II and insulin with a maximal four-fold increase after 10 min (Fig. 1B). Akt phosphorylation by both ligands was sustained up to 30–60 min (Fig. 1A, upper panel). After 8–16 h of treatment, Akt phosphorylation by these two ligands was decreased to the level similar to the basal (data not shown). Increased Akt phosphorylation occurred without changes in its protein levels (Fig. 1A, lower panel). Altough Akt phosphorylation by IGF-II appeared to be higher than by insulin during the first 10 min the difference was not statistically significant as assessed by the ANOVA test.

Time Course for Gsk3β Deactivation and Differential Regulation of Gsk3α and Gsk3β Isozymes by IGF-II Via IR^A

Gsk3 consists of two isozymes, Gsk3α and Gsk3β, which share 85% homology at the amino acid level [Woodgett, 1990]. Both Gsk3 isozymes share similar substrate specificity and have been reported to be deactivated by insulin and

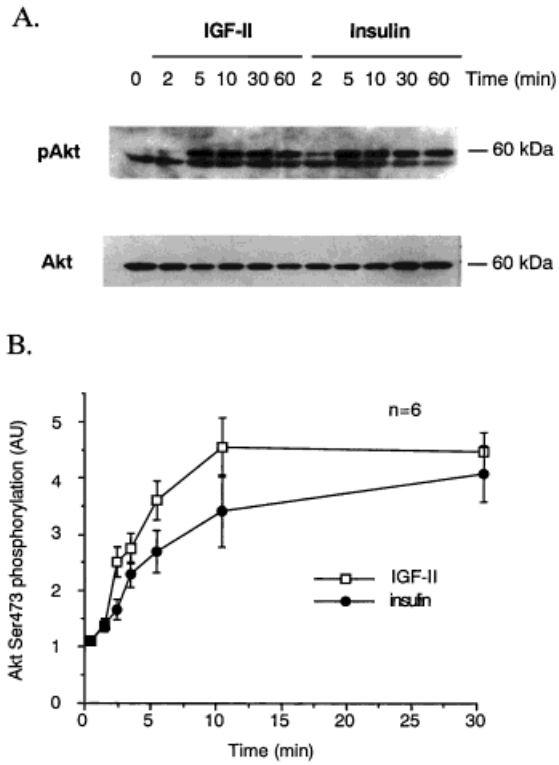

Fig. 1. Akt phosphorylation/activation by IGF-II and insulin in R^-/IR^A cells. Subconfluent R^-/IR^A cells were serum-deprived for 10 h, stimulated with either IGF-II or insulin at 10 nM for 0, 1, 2, 3, 5, 10, and 30 min and solubilized. Whole cell lysates (20 μg protein/lane) were subjected to SDS–PAGE followed by Western blot analysis using specific antibodies recognizing the Akt protein (**A**, **lower panel**) or its phosphorylated active form (A, **upper panel**). A representative blot with cells that were stimulated up to 60 min is shown. (**B**), intensities of the phospho-Ser473 Akt bands were quantified by a laser densitometer. Data are presented as average±SE of six independent experiments. AU, arbitary units.

IGF-I [Plyte et al., 1992; Sutherland et al., 1993; Welsh and Proud, 1993; Cross et al., 1994]. In order to study Gsk3 (α and β) protein expression and their enzyme activities in R^-/IR^A cells, we first prepared whole cell lysates from unstimulated cells after 10 h of serum deprivation. Whole cell lysates were then subjected to immunoprecipitation with either anti-Gsk3α and anti-Gsk3β antibodies and their enzymatic activities against the Gsk3 substrate pGS2 were assayed in vitro as previously described [Sung et al., 1998]. Both Gsk3α and Gsk3β were expressed in R^-/IR^A cells as evidenced by their activities in unstimulated cells. In these cells Gsk3β activity at basal states was approximately five times higher than Gsk3α activity (data not shown). We next studied the ability of Gsk3 isozymes to respond to insulin and IGF-II in R^-/IR^A cells. Following 10 h serum deprivation, cells were stimulated with IGF-II or insulin at 10 nM for 2 min. Cells were subsequently solubilized, whole cell lysates were subjected to immunoprecipitation for both Gsk3α and Gsk3β and the kinase activities recovered in the immunoprecipitates were assayed as described in Materials and Methods. Both IGF-II and insulin induced up to 70–80% deactivation of Gsk3β after 2 min stimulation whereas they deactivated Gsk3α much less effectively (i.e., 25–35%, Table I). The rate of IGF-II- and insulin-induced deactivation of Gsk3α observed after 2 min was not modified after 5, 10, and 30 min exposure to ligands (data not shown). Since Gsk3β was the major IGF-II- and insulin-responsive Gsk3 isozyme in R^-/IR^A cells, we studied in more detail its time course of deactivation after growth factor stimulation. Gsk3β activity was markedly reduced as early as 1 min after growth factor stimulation and maximal inhibition occurred at 2–3 min reaching 70–80% inhibition (Fig. 2A). No significative difference was present between the two ligands. Gsk3β deactivation by IGF-II and insulin was maintained up to 30 min. No significant difference in Gsk3β protein amount was observed during 30 min stimulation with both growth factors (Fig. 2B). The comparison of time courses of Akt activation and Gsk3β deactivation in R^-/IR^A cells consistently showed that Akt reached its maximal activity shortly after the maximal deactivation of Gsk3β (Figs. 1 and 2).

TABLE I. Effects of IGF-II and Insulin on Gsk 3 Activity in R-IR^A Cells

	Gsk3α (%)	Gsk3β (%)
Basal	100	100
IGF-II	76.0±13.2	31.4±10.4
Insulin	66.0±19.6	20.5±4.2

Cells were serum-deprived for 10 h, stimulated with either IGF-II or insulin at 10 nM for 2 min and solubilized. Whole cell lysates (30 μg for Gsk3β, 70 μg for Gsk3α) were subjected to immunoprecipitation with specific antibodies to Gsk3 isozymes and resultant immunoprecipitates were assayed for enzymatic activity against pGS2 substrate as described in Materials and Methods. Data are expressed as percentages of Gsk3α and Gsk3β basal activity and are average±SE of three and four independent experiments, respectively.

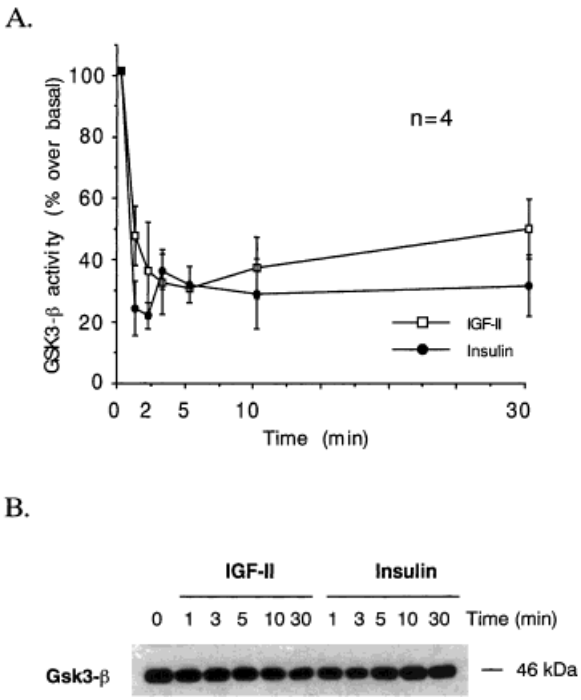

Fig. 2. Time courses for Gsk3β deactivation by IGF-II and insulin in R^-/IR^A cells. **A:** The same cell lysates prepared in Figure 1 were immunoprecipitated with anti-Gsk3β antibody as described in Materials and Methods. These immunopecipitates were next assayed for enzymatic activity against pGS2 substrate. Data are presented as percent of basal activity and are average±SE of four independent experiments; **B:** Aliquots of the cell lysates were subjected to SDS–PAGE followed by western blot analysis for Gsk3β protein content (B). A representative blot is shown.

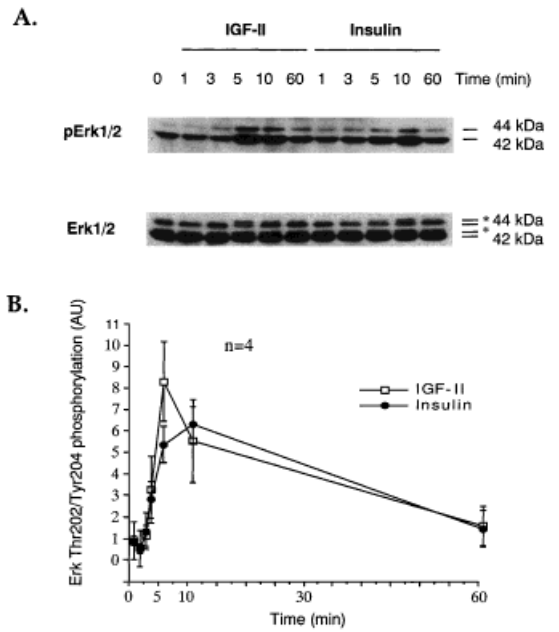

Fig. 3. Erk$\frac{1}{2}$ phosphorylation/activation by IGF-II and insulin in R^-/IR^A cells. Subconfluent cells were serum-deprived for 10 h and stimulated with either IGF-II or insulin at 10 nM for 0, 1, 3, 5, 10, and 60 min. Whole cell lysates (20 μg/lane) were resolved by SDS–PAGE. Erk$\frac{1}{2}$ content and activation were detected by Western blot analysis using specific antibodies recognizing Erk1 (44 kDa) and Erk2 (42 kDa) (Fig. 3A, lower panel; asterisks, Erk$\frac{1}{2}$ phosphorylative shift) or their activated forms that were phosphorylated on Thr202/Tyr204 (Fig. 3A, upper panel). Representative blots are shown. Intensities of the phosphorylated Erk1 and Erk2 bands were quantified using a laser densitometer and combined (Fig. 3B). Data are presented as average±SE of four independent experiments. AU, arbitrary units.

Time Course for Erk Phosphorylation/ Activation by IGF-II Via IR^A

Erk has been implicated in cell proliferation by various growth factors. Here we studied the activation of Erk in R^-/IR^A cells under the same conditions used to study the Akt/Gsk3β axis. First, employing antibodies to phosphorylated Erk on thr202/tyr204 (Fig. 3A, upper panel), we studied Erk1/2 phosphorylation at these sites that is required for full Erk activation by growth factors [Marshall, 1995]. Second, we examined the mobility shift of Erk 1/2 using antibodies to Erk protein (Fig. 3A. lower panel). As shown by increased phosphorylation and mobility shift (Fig. 3A), IGF-II and insulin at 10 nM induced a rapid, but transient activation of both Erk 1 (44 kDa) and Erk 2 (42 kDa) in R^-/IR^A cells with maximal activation at 5–10 min (Fig. 3B).

AP-1 is a Weak Transcriptional Target for IGF-II and Insulin in R-IR^A Cells

The transcription factor c-jun has been shown to be negatively regulated upon its phosphorylation by Gsk3β [Boyle et al., 1991; De Groot et al., 1993; Nikolakaki et al., 1993]. Gsk3β deactivation by growth factors may therefore represent a potential mechanism to upregulate c-Jun/AP-1 activity in vivo. To study whether IGF-II stimulates AP-1 activity via the IR^A, we transiently transfected R^-/IR^A cells with a luciferase reporter construct driven by the AP-1 promoter (−73/TRE-Luc). After 10 h of serum deprivation, cells were treated for 16 h with either IGF-II or insulin or TPA as positive control. IGF-II at 10 and 100 nM increased AP-1 transcriptional activity by 1.5–1.6-fold, whereas insulin at 10 and 100 nM increased AP-1

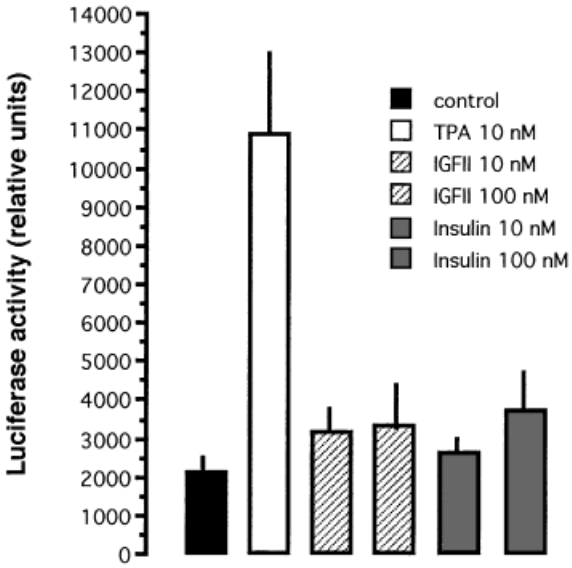

Fig. 4. AP-1 transcriptional activation by TPA, IGF-II, and insulin in R^-/IR^A cells. Subconfluent R-IR^A cells in six well plates were transiently transfected with −73/AP-1-luciferase reporter plasmid (1 μg DNA) in low serum-containing media. After 5 h incubation at 37°C, the transfection media was replaced with serum free media complemented with growth factors as described in Materials and Methods. After additional 16 h incubation, cells were harvested in reporter lysis buffer (Promega). Cell lysates (20 μg) were used to measure luciferase activity. Data are presented as relative units of luciferase activity and are the average±SE of two independent experiments with each experiment performed in triplicate.

activity by 1.3–1.7-fold (Fig. 4). Under the same conditions, TPA at 10 nM induced a 5.5-fold stimulation of AP-1 transcriptional activity in R^-/IR^A cells.

IGF-II Triggers Cell Cycle Progression Via IR^A

We previously showed that IGF-II stimulates DNA synthesis in R^-/IR^A cells as assessed by thymidine incorporation and that antibodies against the insulin receptor can suppress the proliferative effect of IGF-II in breast cancer cells [Frasca et al., 1999; Sciacca et al., 1999]. In order to better define the mitogenic potential of IGF-II via IR^A activation, we investigated the abilities of both IGF-II and insulin to stimulate the cell cycle progression from G1/G0 to S and G2/M phases in R^-/IR^A cells employing flow cytometry. Subconfluent R^-/IR^A cells were serum-deprived for 10 h and stimulated with IGF-II or insulin at 10 nM for 2, 4, 6, 12, and 24 h. At the end of stimulation, the cellular DNA was stained with propidium iodide and cells were subjected to flow cytometric analysis (Fig. 5). Although a substantial population of serum-deprived cells (<10%) displayed an S-phase DNA content, this percentage was similar in all paired experiments. After 10 h of serum deprivation, 70% of the cells were arrested at the G0/G1 phase, while 30% of the cells were distributed at the S and G2/M phases (10% at S phase + 20% at G2/M phase). IGF-II induced a time-dependent G1/S transition by decreasing cell population at G0/G1 and increasing cells at S and G2/M phases. After 12 h of growth factor treatment, IGF-II decreased the G0/G1 cell population from 70 to 54%, and increased both the S population from 10 to 20% and the G2/M cell population from 20 to 26%. Insulin similarly decreased G0/G1 cell population from 70 to 56%, and increased both the S population from 10 to 19% and the G2/M population from 20 to 25%. After 24 h, IGF-II further decreased the G0/G1 population to 46% and increased the overall population at S and G2/M phases to 54% (16% at S phase and 38% at G2/M phase). Similarly, insulin induced a further decrease in the G0/G1 population to 49% after 24 h and caused a parallel increase of the population at S and G2/M to 51% (15% at S phase and 36% at G2/M phase). These results suggest that IGF-II, like insulin, effectively induced cell cycle progression from G0/G1 to S via IR^A and doubled the percentage of cell population at S phase after 12 h of treatment. In control cells incubated in the absence of growth factors, there was no significant increase in both S and G2/M populations (data not shown).

DISCUSSION

Recent evidence indicates that the insulin receptor isoform A is a high affinity receptor for both insulin and IGF-II [Frasca et al., 1999]. IGF-I, in contrast, binds and activates IR^A with much lower affinity. Mild quantitative and time course differences have been observed in intracellular signaling molecules after either insulin or IGF-II binding to IR^A. These differences include phosphorylation of intracellular signaling molecules like IRS-1 and -2, Shc, and PI3K [Frasca et al., 1999]. In addition, the two ligands elicit mildly different biological responses: activation of IR^A by insulin primarily leads to metabolic effects while, when activated by IGF-II, mitogenic effects prevail.

In the present study, we performed time course experiments using R^-/IR^A cells (that

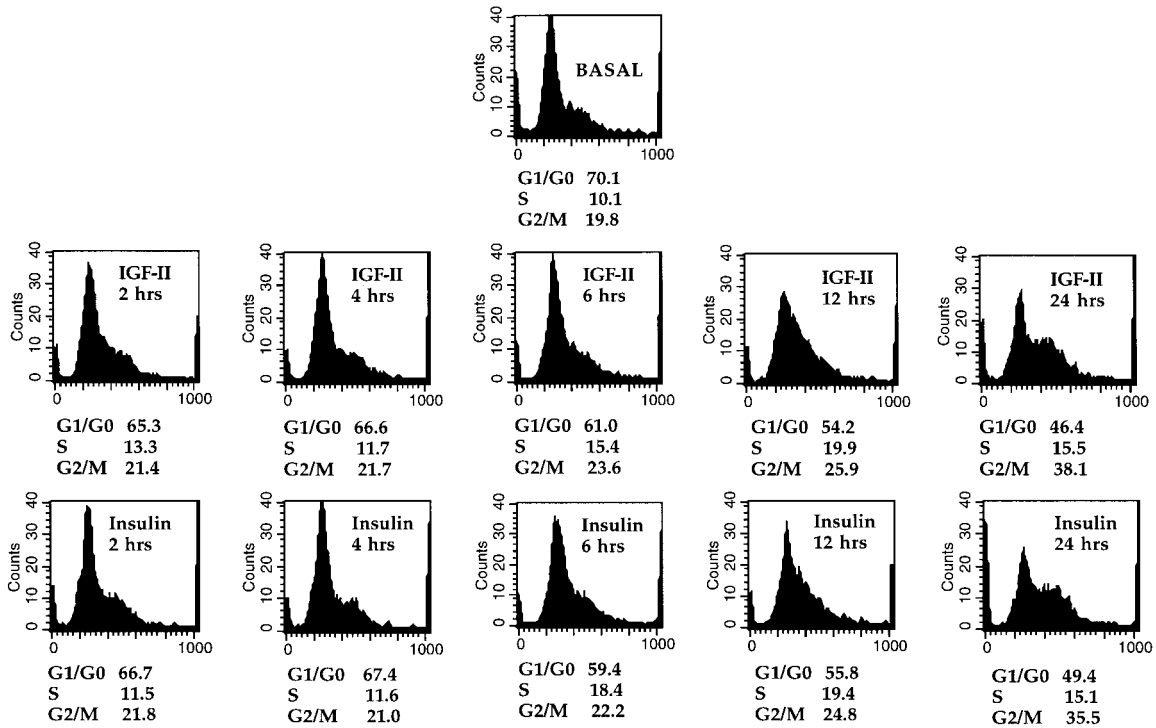

Fig. 5. Effects of IGF-II and insulin on cell cycle progression from G1/G0 to S and G2/M phases in R^-/IR^A cells. Subconfluent R-IR^A cells were serum-deprived for 10 h (basal, top panel) and stimulated with either IGF-II (middle panels) or insulin (bottom panels) at 10 nM for 2 to 24 h. Cells were then fixed in ethanol and resuspended in PBS. Next, DNA was stained with propidium iodide and cells were analyzed by flow cytometry. The analysis was performed with CellQuest software (Becton Dickinson). The percentages of cells found in G0/G1, S and G2/M phases are indicated in each picture. A representative profile of three independent experiments is shown.

only express the IR isoform A) to define mitogenic targets of IGF-II via IR^A and downstream of PI3K and also to establish a hierarchy for the activation of mitogenically relevant signaling molecules. Since the protooncogenic serine kinase Akt lies downstream of PI3K and is known to play an important role for cell growth, we studied the effect of IGF-II on Akt activation. The full activation of Akt requires phosphorylation at both Thr-308 and Ser-473, which is mediated by distinct phosphoinoside dependent kinases [Alessi et al., 1997]. Here, we show that Akt is activated by IGF-II via activation of IR^A. This result is consistent with the concept that both IGF-II and Akt regulate cell growth and survival [Cristofori et al., 1994; Kulic et al., 1997].

Gsk3β has been previously shown to be directly phosphorylated by Akt [Cross et al., 1995]. Thus, we studied the ability of IGF-II to regulate Gsk3β activity in R-IR^A cells. Gsk3 was initially reported to phosphorylate and inhibit glycogen synthase [Embi et al., 1980]. Subsequent molecular cloning of Gsk3 identified two distinct isozymes, Gsk3α and Gsk3β [Woodgett, 1990]. Growth factors induce phosphorylation and deactivation of Gsk3 [Sutherland et al., 1993; Cross et al., 1994]. Insulin and other mitogenic stimuli have been shown to inhibit Gsk3β by ~50% [Borthwick et al., 1995; Staal et al., 1999]. Here, we report that in R^-/IR^A cells both IGF-II and insulin induce up to 70–80% deactivation of Gsk3β enzymatic activity. These data suggest that, at least in these embryonic cells, there may exist a more abundant pool of Gsk3β that is readily available for modulation by insulin and IGF-II via the IR isoform A. Interestingly, in these cells IGF-II and insulin deactivated Gsk3α much less effectively than Gsk3β. Furthermore, our study demonstrates that maximal Gsk3β deactivation occurs within 1–3 min after insulin or IGF-II treatment. Akt activation, however, reaches its maximum at 5–10 min in these cells. The rapid maximal deactivation of Gsk3β preceding maximal Akt activation raises the possibility that maximal Gsk3β deactivation does not require full Akt activation and/or that Gsk3β may be regulated

by additional signaling components other than Akt. In agreement with the latter hypothesis, Delcommenne et al. [1998] have reported that Gsk3β could be directly regulated by the integrin-linked kinase in an insulin and wortmannin-sensitive pathway. Gsk3 has a broad substrate specificity for a variety of cellular targets, including transcription factors such as c-jun, c-myc, c-myb, and CREB, the microtubule-associated protein Tau, the translation factor eIF-2B and the regulatory subunit of cyclic AMP-dependent protein kinase [Plyte et al., 1992]. Although the exact role of Gsk3 in cell growth is still unclear, its ability to phosphorylate a number of molecules implicated in mitogenesis suggest a critical role for Gsk3 in the regulation of cell growth. Under the experimental conditions used for this study, both IGF-II and insulin similarly induced the rapid and transient phosphorylation/activation of Erk 1 and 2. This is consistent with our previous report [Frasca et al., 1999].

Insulin and IGF-I increase stimulation of AP-1 transcriptional activity [Monier et al., 1994; Griffiths et al., 1998]. The proto-oncogenic protein c-jun is the major component of the AP-1 transcriptional complex. c-jun is directly phosphorylated by Gsk3β in vitro on sites that negatively regulates its DNA binding [Boyle et al., 1991; Nikolakaki et al., 1993]. Cotransfection experiments have also shown that overexpression of Gsk3β or its Drosophila homologue shaggy negatively regulates c-jun transcriptional activity [De Groot et al., 1993]. Moreover, a Gsk3β kinase defective mutant is able to prevent the inhibitory effect of Gsk3β on c-jun [Nikolakaki et al., 1993]. Therefore, we hypothesized that c-jun/AP-1 represents a relevant target of IGF-II signaling via Gsk3β deactivation in R^-/IR^A cells. However, we found that IGF-II and insulin induce only a slight increase of the AP-1 transcriptional activity suggesting that additional factors may be required for full activation of AP-1 in vivo. In this regard, it should be noted that c-jun activity can be regulated by differential phosphorylation on both Gsk3 and Erk-sensitive sites. The coordinated c-jun phosphorylation at these sites may be required for its full activation [Hesketh, 1997].

We have previously reported that IGF-II stimulated cell growth in R^-/IR^A cells as assessed by thymidine incorporation into DNA and that IGF-II induced DNA synthesis more efficiently than insulin in cells serum-deprived for 48 h [Frasca et al., 1999]. In the present study, we further investigated the IGF-II mitogenic potential in R-IR^A cells by assessing the cell cycle progression following IGF-II treatment. Under our experimental conditions, IGF-II at 10 nM doubled the cell population at the synthetic (S) phase after 12 h and that at the mitotic phase (G2/M) after 24 h. Insulin increased the cell populations at S and G2/M phases with a similar time course. These data also showed time-dependent continuous decreases in the G1/G0 cell population concomitant with increases in the G2/M cell population. When S and G2/M cell populations were combined, a similar time-dependent continuous increase in response to the two ligands is found. However, the analysis of the S phase cell population alone (representing the DNA synthetic process) provides a different profile peaking at 12 h and varying at times following treatments.

In summary, our studies strongly support the role of IR^A in the IGF-II-mediated mitogenic signaling and further suggest that the activation of the Akt/Gsk3β axis and Erk are intrinsic elements of this process. Further studies are required to fully characterize the upstream regulation and the specific role of Gsk3β in IGF-II-mediated cell growth.

ACKNOWLEDGMENTS

We thank Dr. Ebrahim Zandi for providing the −73/TRE-Luc reporter gene construct and Hal Saucier of the Flow Cytometry Core Facility of the University of Southern California, Keck School of Medicine for his technical assistance.

REFERENCES

Alessi DR, James S, Downes CP, Holmes A, Gafney P, Reese C, Cohen P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B-α. Curr Biol 7:261–269.

Aroenhei A, Zandi E, Henneman H, Elledge SJ, Karin M. 1997. Isolation of an AP-1 repressor by a novel method for detecting protein interactions. Mol Cell Biol 17:3094–3102.

Baker J, Liu JP, Robertons EL, Efstratiadis A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75:73–82.

Borthwick AC, Wells AM, Rochford JJ, Hurel SJ, Turnbull DM, Yeaman SJ. 1995. Inhibition of glycogen synthase

kinase-3 by insulin in cultured human skeletal muscle myoblasts. Biochem Biophys Res Commun 210:738–745.
Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, Hunter T. 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell 64:573–584.
Cristofori G, Naik P, Hanahan D. 1994. A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. Nature 369:414–418.
Cross DA, Alessi DR, Vandenheede JR, McDowell HE, Hundall HS, Cohen P. 1994. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor I in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. Biochem J 303:21–26.
Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated protein kinase B. Nature 378:785–789.
De Groot RP, Auwerks J, Bouoruis M, Sassone-Corsi P. 1993. Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the Drosophila kinase shaggy. Oncogene 8:841–847.
Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S. 1998. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/Akt by the integrin-linked kinase. Proc Natl Acad Sci USA 95:11211–11216.
Downward J. 1998. Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 10:262–267.
Embi N, Rylat DB, Cohen P. 1980. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur J Biochem 107:519–527.
Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A, Vigneri R. 1999. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol 19:3278–3288.
Griffiths MR, Black AJ, Culbert AA, Dickens M, Shaw PE, Gillespie DA, Tavare' JM. 1998. Insulin-stimulated expression of c-fos, fra-1 and c-jun accompanies the activation of the activator protein 1(AP-1) transcroptional complex. Biochem J 335:19–26.
Hesketh R. 1997. The oncogene and tumor suppressor gene facts book, 2nd Ed. London: Academic Press. p 226–233.
Kohn AD, Kovacina KS, Roth RA. 1995. Insulin stimulates the kinase activity of Rac-PK, a pleckstrin homology domain containing Ser/Thr kinase. EMBO J 14:4288–4295.
Krywicki RF, Yee D. 1992. The insulin-like growth factor family of ligands, receptors, and binding proteins. Breast Cancer Res Tr 22:7–19.
Kulic G, Klippel A, Weber MJ. 1997. Antiapoptotic signaling by the insulin-like growth factor receptor, phosphatidylinositol-3kinase and Akt. Mol Cell Biol 17:1595–1606.
Louvi A, Accili D, Estratiadis A. 1997. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev Biol 189:33–48.
Marshall CJ. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179–185.
Monier D, Boutilier AL, Giraud P, Chiur R, Aunis D, Feltz P, Zwiller J, Loeffler JP. 1994. Insulin-like growth factor-I stimulates c-fos and c-jun transcription in PC12 cells. Mol Cell Endocrinol 104:139–145.
Morrione A, Valentinis B, Xu G, Yumet A, Louvi A, Efstratiadis A, Baserga R. 1997. Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc Natl Acad Sci USA 94:3777–3782.
Nikolakaki E, Coffer PJ, Homelsoet R, Woodgett JR, Defize LH. 1993. Glycogen synthase kinase 3 phosphorylates Jun family members in vitro and negatively regulates their transactivating potential in intact cells. Oncogene 8:841–847.
Plyte SE, Hughes K, Nikolakaki E, Pulverer BJ, Woodgett JR. 1992. Glycogen synthase kinase-3: functions in oncogenesis and development. Biochem Biophys Acta 1114:147–162.
Sciacca L, Costantino A, Pandini G, Mineo R, Frasca F, Scalia P, Sbraccia P, Goldfine ID, Vigneri R, Belfiore A. 1999. Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism. Oncogene 18:2471–2479.
Sell C, Rubini R, Rubin JP, Liu P, Efstratiadis A, Baserga R. 1993. Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. Mol Cell Biol 14:3604–3612.
Staal FJT, Burgering MT, Van de Wetering M, Clevers H. 1999. Tcf-1-mediated transcription in T lymphocytes: differential role for glycogen synthase kinase-3 in fibroblasts and T cell. Int Immunol 11:317–323.
Sung CK, Choi WS, Scalia P. 1998. Insulin-stimulated glycogen synthase in cultured hepatoma cell: differential effect of inhibitors of insulin signalin molecules. J Rec Sig Trans Res 18:243–263.
Sutherland C, Leighton I A, Cohen P. 1993. Inactivation of glycogen synthase kinase-3-β by phosphorylation: new kinase connection in insulin and growth-factor signaling. Biochem J 296:15–19.
Welsh G, Proud C G. 1993. Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor elF-2B. Biochem J 294:625–629.
Woodgett JR. 1990. Molecular cloning and expression of glycogen synthase kinase-3 /factor A. EMBO J 9:2431–2438.