Insulin Activates Nuclear Factor κB in Mammalian Cells through a Raf-1-mediated Pathway*

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We examined the effect of insulin on nuclear factor κB (NF-κB) activity in Chinese hamster ovary (CHO) cells overexpressing wild-type (CHO-R cells) or kinase-defective insulin receptors mutated at Tyr1162 and Tyr1163 autophosphorylation sites (CHO-Y2 cells). In CHO-R cells, insulin caused a specific, time-, and concentration-dependent activation of NF-κB. The insulin-induced DNA-binding complex was identified as the p50/p65 heterodimer. Insulin activation of NF-κB: 1) was related to insulin receptor number and tyrosine kinase activity since it was markedly reduced in parental CHO cells which proved to respond to insulin growth factor-1 and phorbol 12-myristate 13-acetate; and 2) persisted in the presence of cycloheximide and was blocked by pyrrolidine dithiocarbamate, aspirin and sodium salicylate, three compounds interfering with IκB degradation and/or NF-κB/IκB complex dissociation; 3) was independent of both PMA-sensitive and atypical (z) protein kinases C; and 4) was dependent on Raf-1 kinase activity since insulin-stimulated NF-κB DNA binding activity was inhibited by 8-bromo-cAMP, a Raf-1 kinase inhibitor. Moreover, insulin activation of NF-κB-driven luciferase reporter gene expression was blocked in CHO-R cells expressing a Raf-1 dominant negative mutant. This is the first evidence that insulin activates NF-κB in mammalian cells through a post-translational mechanism requiring both insulin receptor tyrosine kinase and Raf-1 kinase activities.

Insulin exerts a wide array of biological effects including regulation of growth and gene expression. The cytoplasmic events implicated in the transduction of insulin signals from the membrane insulin receptor to the transcriptional machinery in the nucleus are beginning to be understood. Studies using transfected cells overexpressing human insulin receptors and/or various proteins of insulin signaling pathways have shown that, after binding to its receptor, insulin activates insulin receptor tyrosine kinase activity and triggers tyrosine phosphorylation of at least two major substrates, IRS-1 and Shc (1). Once phosphorylated, insulin receptor substrates interact with several proteins including the Grb2/Sos complex which activates the Ras-Raf-1-MAP kinase pathway (1). In contrast, little is known at present as concerns the nuclear transcription factors which are specifically activated by insulin to regulate gene expression.

The nuclear factor κB (NF-κB) was originally described as being present in the cytosol of most cell types as an inactive heterodimer composed of 50-kDa (p50, NF-κB1) and 65-kDa (p65, Rel A) subunits and bound to one of the IκB inhibitor proteins (2–4). Activation of NF-κB involves phosphorylation and degradation of IκB. This results in the dissociation of the NF-κB/IκB complex, a process which can be blocked by inhibitors such as pyrrolidine dithiocarbamate (PDTC), aspirin, and sodium salicylate (5, 6). Thereafter, the active NF-κB p50/p65 heterodimer translocates to the nucleus, where it directly binds to its cognate DNA sequences. NF-κB is a pleiotropic activator which participates in the induction of a wide variety of cellular genes including genes encoding for signaling proteins. Activation of this factor can be achieved in many cell types by agonists of immune and inflammatory responses and also by mitogens (7–9). In this regard, a recent paper (10) reported that the sequence of events triggered by insulin to induce maturation of Xenopus oocytes involves NF-κB activation. This finding prompted us to examine whether insulin was able to activate NF-κB in mammalian cells and, if so, to examine the signal transduction pathway involved. To this end, we used parental Chinese hamster ovary (CHO) cells and CHO cells overexpressing either wild-type human insulin receptors or kinase-defective insulin receptors mutated at Tyr1162 and Tyr1163, two autophosphorylation sites playing a crucial role in receptor activation (11–15). Our study demonstrates that insulin activates NF-κB in mammalian cells through a pathway which requires insulin receptor tyrosine kinase and Raf-1 kinase activities.

EXPERIMENTAL PROCEDURES

Reagents—[γ-32P]ATP (10 Ci/mmol), [γ-32P]dCTP (3000 Ci/mmol), ECL detection kit, Hybond N membranes, and hyperfilms-MP were from Amersham Corp. [20-3H]Phorbol 12,13-dibutyrate (PDBu, 18 Ci/mmol) and acetyl coenzyme A [acetyl-3H], CAT assay grade were obtained from DuPont NEN. Insulin was purchased from Novo Labortatoires and IGF-1 from Calbiochem. Rabbit polyclonal antibodies against p50 and p65 subunits of NF-κB were from Santa Cruz Biotechnology, Inc. MAP kinase R2 antibody. [Ser183/185]PKC-ε pseudosubstrate peptide (PKRQGSSRRRLK) was purchased from Upstate Biotechnology, Inc. [Ser202]PKC-

1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor κB; CHO, Chinese hamster ovary; DTT, dithiothreitol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; RT-PCR, reverse transcriptase-polymerase chain reaction; IGF-1, insulin growth factor-1.
α pseudosubstrate peptide (RFARKGSLRQKVN) was from Life Tech-
nologies, Inc. NF-κB consensus oligonucleotide was from Genosys Biotechnologies and Oct-1 consensus oligonucleotide from Promega. Reverse transcriptase and Taq DNA polymerase were obtained from Perkin-Elmer and DNA polymerase 1 Klenow fragment from New En-
 gland Biolabs. Other reagents were purchased from Sigma.

Cell Transfection—CHO cells were transfected by the method of Dignam et al. (16) with minor modifications, after treatment with or without insulin, IGF-1, or PMA, in the absence or presence of various agents. After two washings in 5 ml of ice-cold phosphate-buffered saline (PBS), cells were harvested in a 1.5 ml tube and centrifuged at 2,000 × g for 5 min in a microcentrifuge. The cell pellet was resuspended in 400 μl of buffer A (10 mM HEPES/KOH, pH 7.9, 70 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 25% glycerol, and the Manufacturer’s instructions. Where indicated, nuclear extracts were
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Insulin Activates NF-κB in CHO-R Cells through Its Own Receptors—Insulin activated NF-κB in CHO-R cells in a time-dependent manner. The effect of the hormone was detected at 1 h, reached a maximum at 6 h, and was no longer detected at 24 h (Fig. 1A). Similarly, Baldwin et al. (7) reported that serum induction of NF-κB in BALB/c3T3 cells culminated at 6 h and was no longer detected at 24 h. The hormone increased the formation of two NF-κB complexes with a major effect on the upper complex designated as band 1 and a minor effect on the lower complex designated as band 2 (Fig. 1A). Insulin activated NF-κB in a concentration-dependent manner (Fig. 1B). The effect of the hormone regularly increased from 10^{-10} M to 10^{-7} M (Fig. 1B). At the latter concentration, the effect of insulin was equivalent to that elicited by 10^{-7} M IGF-1 (Fig. 1B). Two observations reported in our previous (14) and present papers argue for insulin activating NF-κB through its own receptors. First, insulin was potent to activate NF-κB at 10^{-9} and 10^{-8} M, two concentrations at which insulin was unable to displace the binding of 125I-labeled IGF-1 to IGF-1 receptors in CHO-R cells (14). Second, insulin activation was related to insulin receptor number. This is supported by the finding that the sensitivity to insulin exhibited by CHO-R cells for NF-κB activation (Fig. 2B) was markedly decreased in parental CHO cells (Fig. 2). In contrast, these cells proved to be fully responsive to IGF-1 as well as to PMA, a well-known inducer of NF-κB activity in several cell types (2, 3, 5) (Fig. 2). The above results therefore indicate that both insulin and IGF-1 are good inducers of NF-κB in CHO-R cells and that insulin activates this transcription factor in the insulin receptor overexpressing cell line through its own receptors.

Characterization of Insulin-induced NF-κB-DNA Complexes—The specificity of bands 1 and 2 was assessed in competition experiments. Both bands disappeared in the presence of the unlabeled oligonucleotide containing the NF-κB-binding site whereas they remained unchanged in the presence of the unlabeled oligonucleotide containing the unrelated OCT-1 consensus sequence (Fig. 3A). We further characterized the insulin-induced DNA-protein complexes by using antibodies directed against the p50 and p65 NF-κB subunits. Incubation of nuclear extracts (30 min at 4°C) from insulin-treated cells (6 h, 10^{-7} M) with anti-p50 antibody abolished band 2 with a concomitant supershift, and reduced the amount of band 1, suggesting the presence of p50 in the two protein complexes (Fig. 3B). In contrast, the anti-p65 antibody had no effect on band 2, suggesting that p65 was absent from the band 2 DNA-protein complex. However this antibody, like the p50 antibody, markedly reduced the intensity of band 1, indicating that band 1 corresponds to a DNA-protein complex composed of both p50 and p65 NF-κB subunits (Fig. 3B). These experiments enabled us to identify the major and minor DNA-protein NF-κB complexes induced by insulin as the p50/p65 heterodimer (band 1) and p50 homodimer (band 2), respectively. This finding is of particular interest since the p50/p65 heterodimer is known as the main effective and also the major inducible form of NF-κB (22), whereas the p50 homodimer has been reported to occur as constitutive factor in nuclei of certain cell types (2, 3, 23).

Insulin Activates NF-κB-dependent Luciferase Reporter Gene Expression—To determine whether insulin enhances NF-κB-driven reporter gene expression, CHO-R cells were transfected with a plasmid expressing the luciferase reporter gene driven by the NF-κB consensus sequence. Transfection of CHO-R cells with this plasmid results in the expression of luciferase activity driven by the NF-κB consensus sequence (Fig. 4). The effect of insulin was observed at 6 h, reached a maximum at 24 h, and was no longer detected at 24 h (Fig. 4A). The hormone increased the luciferase activity, as described under “Experimental Procedures.” This is a representative experiment independently performed three times.

RESULTS AND DISCUSSION

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Insulin Activation of NF-κB

To investigate the role of the insulin receptor tyrosine kinase in the activation of NF-κB by insulin, we studied the effect of insulin in CHO-Y2 cells expressing insulin receptors made kinase defective by mutation at Tyr<sup>1162</sup> and Tyr<sup>1163</sup>, two major autophosphorylation sites of the insulin receptor tyrosine kinase domain playing a crucial role in receptor activation (25). Nuclear extracts from CHO-Y2 cells which had been treated with 10<sup>−7</sup> M insulin for various time periods (0–6 h) or with graded concentrations (0–10<sup>−7</sup> M) of insulin for 6 h were tested for NF-κB activity. As shown in Fig. 5, the effect of insulin in CHO-Y2 cells was dramatically reduced as compared to that observed in CHO-R cells (Fig. 1). Similar reduction was previously reported for insulin receptor tyrosine kinase activity (14). This finding further argues for an effect of insulin being mediated by insulin and not IGF-1 receptors, since we previously reported that CHO-Y2 cells expressed the same number of IGF-1 receptors as CHO-R cells (13). Most importantly, this finding shows that insulin receptor autophosphorylation sites Tyr<sup>1162</sup> and Tyr<sup>1163</sup> play a pivotal role in insulin activation of NF-κB and therefore indicate that this process takes place among the multiple insulin-stimulated pathways requiring the integrity of the insulin receptor tyrosine kinase activity (25). Otherwise, the finding that the activation of NF-κB by insulin was lower in CHO-Y2 cells than in CHO cells most probably reflects a dominant negative effect exerted by the overexpressed mutated insulin receptors on endogenous insulin receptors or their downstream targets, as has been previously found for other insulin-responsive pathways (12, 14).

Insulin Activation of NF-κB Involves a Post-translational Mechanism—To approach the mechanism whereby insulin induced NF-κB in CHO-R cells, nuclear extracts were prepared from CHO-R cells which had been treated for 6 h with 10<sup>−7</sup> M insulin, transfection included 1 μg of pRSV-CAT plasmid as a monitor for transfection efficiency and were carried out at a constant amount of DNA (11 μg/dish). After transfection, cells were maintained for 36 h in culture medium containing 0.3% FCS and then for 12 h in the absence or presence of 10<sup>−7</sup> M insulin. Forty-eight h post-transfection, cell extracts were prepared and assayed for luciferase and CAT activities as described under “Experimental Procedures.” The results, presented as normalized luciferase activity (ratio of NF-κB–luciferase activity to the CAT activity measured in each extract) are the means ± S.E. from three independent experiments, each performed in triplicate.

Fig. 3. Characterization of insulin-induced NF-κB-DNA complexes. Nuclear extracts prepared from control or insulin-stimulated (6 h, 10<sup>−7</sup> M) CHO-R cells were assayed for NF-κB activation (25). Nuclear extracts from CHO-Y2 cells which had been treated with 10<sup>−7</sup> M insulin for various time periods (0–6 h) or presence of unlabeled oligonucleotides containing the NF-κB site (lane 3) or the OCT-1 (lane 4) consensus sequences (A) or after a 30-min incubation without (lane 1) or with anti-p50 (lane 2) or anti-p65 (lane 3) antibodies (B). EMSA was performed as described under “Experimental Procedures.” This is a representative experiment independently performed three times.

Fig. 4. Insulin induction of NF-κB-mediated luciferase reporter gene activity in CHO-R cells. CHO-R cells were co-transfected by the CaPO<sub>4</sub> method with 5 μg of either control (conaluc A) or test (Ig<sub>κ</sub>κ3-conaluc, B) plasmids, in the absence or presence of the expression plasmid for the Raf-1 dominant negative mutant Raf-C4. All transfections included 1 μg of pRSV-CAT plasmid as a monitor for transfection efficiency and were carried out at a constant amount of DNA (11 μg/dish). After transfection, cells were maintained for 36 h in culture medium containing 0.3% FCS and then for 12 h in the absence or presence of 10<sup>−7</sup> M insulin. Forty-eight h post-transfection, cell extracts were prepared and assayed for luciferase and CAT activities as described under “Experimental Procedures.” The results, presented as normalized luciferase activity (ratio of NF-κB–luciferase activity to the CAT activity measured in each extract) are the means ± S.E. from three independent experiments, each performed in triplicate.

Fig. 5. Loss of NF-κB activation by insulin in CHO-Y2 cells. Experiments described in Fig. 1, A and B, with insulin were performed in CHO-Y2 cells. Maximal insulin activation of NF-κB in CHO-R cells is given as a control. This is a representative experiment independently performed three times.
Insulin in the presence or absence of the protein synthesis inhibitor cycloheximide (10 μg/ml). This cycloheximide treatment was found to inhibit 90–95% of basal and insulin-stimulated protein synthesis in CHO-R cells, in accordance with previous results (14). Cycloheximide alone increased NF-κB activity in CHO-R cells (Fig. 6A), as previously reported in other cell types (5, 7, 26). The mechanism underlying this increase is unknown but may involve inhibition of IκB synthesis, as has been suggested by others (5, 8, 26). Most importantly, we observed that activation of NF-κB by insulin was preserved in the presence of cycloheximide, indicating that this process did not require de novo protein synthesis (Fig. 6A). This finding, together with the finding that NF-κB activation by insulin occurred within the first hour of treatment, argue for a post-translational effect of insulin on NF-κB activity. This may proceed from the ability of the hormone to promote dissociation of the NF-κB-IκB complex.

To test this hypothesis, we examined the effect of: 1) PDTC, a thiol compound scavenging reactive oxygen intermediates and, thereby, impeding the release of IκB from NF-κB (5); and 2) aspirin and sodium salicylate, two drugs interfering with a pathway that leads to IκB phosphorylation and/or degradation or both (6). As shown in Fig. 6B, PDTC (0.1 mM) completely abolished maximal insulin activation of NF-κB, as indicated by the complete disappearance of band 1 and band 2 DNA-binding complexes in nuclear extracts prepared from CHO-R cells treated with insulin (6 h, 10−7 M). PDTC was reported to cause similar inhibition in Jurkat cells exposed to various NF-κB activators including lipopolysaccharide, tumor necrosis factor-α, interleukin-1, and PMA (5). As shown in Fig. 6C, sodium salicylate (10 mM) and aspirin (5 mM) were also potent to inhibit insulin induction of NF-κB in CHO-R cells. In accordance with these results, sodium salicylate and aspirin were recently shown to inhibit activation of NF-κB by lipopolysaccharide, tumor necrosis factor-α, and phytohemagglutinin plus PMA in Jurkat cells (6). The mechanisms involved in sodium salicylate or PDTC inhibitory effects in CHO-R cells could be identical to those previously described in the above studies, i.e. impairment of a pathway leading to IκB degradation and/or NF-κB-IκB complex dissociation.

Insulin Activation of NF-κB Appears to be Independent of PMA-sensitive PKC Isoforms—Because PKC is a known inducer of NF-κB (2–4) and PMA activates PKC is a known inducer of NF-κB (2–4) and PMA activates...

**Fig. 6. Effect of cycloheximide and inhibitors of NF-κB-IκB complex dissociation on NF-κB activation by insulin in CHO-R cells.** Nuclear extracts were prepared from CHO-R cells which had been incubated for 6 h with or without 10−7 M insulin in the absence or presence of 10 μg/ml cycloheximide (CHX) (A) or 0.1 mM PDTC (B) or 5 mM aspirin or 10 mM sodium salicylate (NaSal) (C). EMSA was performed as described under “Experimental Procedures.” This is a representative experiment independently performed three times.

NF-κB in CHO cells (Fig. 2), we next examined PKC involvement in insulin activation of NF-κB. We thus tested the effect of insulin (6 h, 10−7 M) on NF-κB activity in CHO-R cells which had been treated for 24 h in the presence or absence of 2.5 μM PMA. This treatment was previously found to produce efficient down-regulation of PMA-sensitive PKC isoforms, as evaluated by measuring the specific binding of [3H]PDBu to whole cells (12). As shown in Fig. 7A, the activation of NF-κB by insulin was similar in untreated and PMA-treated CHO-R cells, indicating that insulin signaling of NF-κB activity was mediated by a pathway which was independent of PMA-sensitive PKCs, i.e. classical and novel PKC isoforms. However, PKC-ζ, an atypical PMA-insensitive PKC isoform (27), was recently reported to...
induce phosphorylation of IκB in vitro (28) and also to be involved in insulin-induced maturation of Xenopus oocytes, a Ras-mediated process associated with NF-κB activation (10). We therefore evaluated the level of expression of this PKC isoform in CHO-R cells by the RT-PCR technique. The data indicated that PKC-ζ was detectable in CHO-R cells but at a very low level as compared to that observed in control Caco-2 and BCl1 cells (Fig. 7B). Since this finding raised the possibility that PKC-ζ may be involved in insulin stimulation of NF-κB activity in CHO-R cells, we next examined whether insulin was able to increase PKC-ζ activity under conditions where it activated NF-κB in CHO-R cells. To this end, we took advantage of a recently described in situ PKC-ζ activity assay (20). In this assay, the phosphorylation of the PKC-ζ peptide, the most efficient substrate for PKC-ζ (29), was measured in digitonin-permeabilized cells either in the presence or in the absence of the PKC-ζ inhibitor pseudosubstrate peptide. As could be expected from the results presented in Fig. 7B, the level of PKC-ζ activity in CHO-R cells (0.9 nmol of 32P transferred to PKC-ζ peptide/10⁵ cells) was far lower than that measured in Caco-2 cells (3.5 nmol of 32P transferred to PKC-ζ peptide/10⁵ cells). Insulin (10⁻⁷ M) had no effect on PKC-ζ activity (90–100% of control) in CHO-R cells, whatever the time of incubation examined (30 min, 1, 2, and 6 h). Similarly, PMA (1.62 × 10⁻⁷ M, 15 min) was ineffective on PKC-ζ activity in CHO-R cells (110% of the control value), as previously shown in other cell types (27). In contrast, PMA (1.62 × 10⁻⁷ M) caused a marked increase in PKC activity (130 and 200% of the control value at 5 and 15 min, respectively) when the same permeabilization procedure was run out with the PKC-ζ peptide, a good substrate for classical and novel PKCs (29). Taken as a whole, the above results argue against the involvement of PKC-A-sensitive PKC isoforms in insulin activation of NF-κB in CHO-R cells and do not favor the hypothesis that the PKC-A-insensitive PKC-ζ isoform may play a role in this process.

Insulin Activation of NF-κB Is Inhibited by 8-Bromo-cAMP, An Agent Interfering with Growth Factor Activation of Raf-1 Kinase—The recent study of Li and Sedivy (30) reported the ability of Raf-1 kinase to activate NF-κB by dissociating the NF-κB-IκB complex. Moreover, several papers provided evidence that, in mammalian cells, the Ras-Raf-1 pathway mediated the activation of NF-κB by various stimuli or inducers (31–33). We judged these findings of particular interest since we (34) and others (35, 36) previously reported the capacity of insulin to activate this pathway in CHO-R cells. We therefore investigated whether insulin activation of NF-κB in these cells was affected when inhibiting Raf-1 kinase by 8-bromo-cAMP. This cell-permeable cAMP analogue is an activator of protein kinase A, a kinase which, in some cell types (37, 38), inhibits Ras-dependent activation of Raf-1 kinase by phosphorylating the enzyme on its kinase domain (39). As a preliminary to this experiment, we examined insulin activation of MAP kinase, a downstream substrate of Raf-1, by evaluating MAP kinase nuclear translocation in CHO-R cells (40).

Insulin (6 h, 10⁻⁷ M) increased the amount of immunoreactive MAP kinase associated with the nuclear fraction (225% of the value measured in the nuclear fraction from control cells), indicating the ability of the hormone to induce a long term MAP kinase translocation in the nucleus of CHO-R cells. Treatment of CHO-R cells with 8-bromo-cAMP (0.25 mM) prevented the insulin-induced increase in nuclear immunoreactive MAP kinase (92% of the control value). In view of these data, we investigated the effect of 8-bromo-cAMP on NF-κB activity by insulin. Fig. 8 shows that 8-bromo-cAMP (0.25 mM) did not increase the DNA binding activity of NF-κB, making unlikely in vivo activation of this transcription factor by protein kinase A in CHO-R cells, in contrast to what has been found in in vitro experiments using cytosolic fractions from 70 Z/3 cells (41). This compound inhibited insulin activation of NF-κB in a concentration-dependent manner, with the maximal inhibitory effect being observed at 0.25 mM (Fig. 8). In contrast, even at a 2-fold higher concentration (0.5 mM), 8-bromo-cGMP poorly modified insulin-stimulated NF-κB DNA-binding activity in CHO-R cells (Fig. 8), demonstrating the specificity of 8-bromo-cAMP inhibitory effect. These experiments showed that: 1) under conditions where insulin induced the maximal stimulation of NF-κB activity, it activated Raf-1 kinase, as assessed by MAP kinase nuclear translocation, a process shown to be associated with growth factor signaling (40); and 2) 8-bromo-cAMP activation of protein kinase A inhibited insulin stimulation of both Raf-1 kinase and NF-κB DNA binding activity. Such findings argue for the notion that Raf-1 kinase mediates the activation of NF-κB by insulin in CHO-R cells.

Insulin Activation of NF-κB Is Blocked in CHO-R Cells Expressing a Raf-1 Dominant Negative Mutant—To strengthen the above hypothesis, we examined the activation by insulin of NF-κB-mediated luciferase gene expression in CHO-R cells transfected with the Raf-1 dominant negative mutant Raf-C4. This mutant encodes the cysteine-rich amino-terminal regulatory domain of Raf-1 but lacks the kinase domain and a serine/threonine region which is believed to function as a regulatory phosphorylation site (42). In these experiments, CHO-R cells were transfected with the (Igκ)3-conaluc (Fig. 4B) was no longer observed in cells which had been co-transfected with Raf-C4 (0.90 ± 0.07-fold) (Fig. 4B). These results provide clear evidence that the pathway whereby insulin activates NF-κB-mediated gene expression in CHO-R cells involves Raf-1 kinase.

The question of whether insulin activation of NF-κB in these cells is a MAP kinase-mediated process cannot be answered by...
the results presented here. A recent paper (33) studying activation of NF-κB by hypoxia concluded to the involvement of Ras and Raf-1 but not of MAP kinase in this process. Whether or not this may be the case for insulin activation of NF-κB remains to be determined. As well, it would be interesting to examine whether, in other cell lines, a pathway bifurcating from Ras and involving PKC-ζ may, in parallel to the Raf-1 kinase pathway, participate in NF-κB activation by insulin. In this regard, both Raf-1 and PKC-ζ were reported to initiate parallel pathways downstream of Ras for regulation of mouse fibroblast proliferation (43).

In conclusion, our study provides the first evidence that insulin specifically activates NF-κB in mammalian cells. This process involves a post-translational mechanism requiring both insulin receptor tyrosine kinase and Raf-1 kinase activities.

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