

Serine Phosphorylation of Insulin Receptor Substrate 1 by Inhibitor κ B Kinase Complex*[§]

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Insulin resistance contributes importantly to the pathophysiology of type 2 diabetes mellitus. One mechanism mediating insulin resistance may involve the phosphorylation of serine residues in insulin receptor substrate-1 (IRS-1), leading to impairment in the ability of IRS-1 to activate downstream phosphatidylinositol 3-kinase-dependent pathways. Insulin-resistant states and serine phosphorylation of IRS-1 are associated with the activation of the inhibitor κ B kinase (IKK) complex. However, the precise molecular mechanisms by which IKK may contribute to the development of insulin resistance are not well understood. In this study, using phosphospecific antibodies against rat IRS-1 phosphorylated at Ser³⁰⁷ (equivalent to Ser³¹² in human IRS-1), we observed serine phosphorylation of IRS-1 in response to TNF- α or calyculin A treatment that paralleled surrogate markers for IKK activation. The phosphorylation of human IRS-1 at Ser³¹² in response to tumor necrosis factor- α was significantly reduced in cells pretreated with the IKK inhibitor 15 deoxy-prostaglandin J₂ as well as in cells derived from IKK knock-out mice. We observed interactions between endogenous IRS-1 and IKK in intact cells using a co-immunoprecipitation approach. Moreover, this interaction between IRS-1 and IKK in the basal state was reduced upon IKK activation and increased serine phosphorylation of IRS-1. Data from *in vitro* kinase assays using recombinant IRS-1 as a substrate were consistent with the ability of IRS-1 to function as a direct substrate for IKK with multiple serine phosphorylation sites in addition to Ser³¹². Taken together, our data suggest that IRS-1 is a novel direct substrate for IKK and that phosphorylation of IRS-1 at Ser³¹² (and other sites) by IKK may contribute to the insulin resistance mediated by activation of inflammatory pathways.

Many factors implicated in the development of insulin resistance such as TNF- α ¹ (1, 2), free fatty acids (3, 4), and serine

phosphatase inhibitors (5, 6) are able to activate the inhibitor κ B kinase (IKK) complex and its downstream effector, NF κ B (7–10). Interestingly, insulin-sensitizing drugs such as thiazolidinediones inhibit NF κ B activity (11). Adiponectin, a cytokine secreted by adipose cells whose plasma levels are negatively correlated with insulin resistance (12), inhibits IKK activity in cells (13). Moreover, diet-induced insulin resistance is ameliorated in IKK2-deficient mice (14). Because IKK and NF κ B are major components of the intracellular inflammatory pathway, a cross-talk between metabolic and inflammatory signaling pathways may play an important role in the development of insulin resistance and the pathophysiology of major public health problems such as diabetes and obesity. However, molecular mechanisms by which IKK may specifically interact with metabolic insulin signaling pathways are not well understood.

IKK is a serine kinase that controls the activation of NF κ B, a ubiquitous transcription factor closely associated with inflammation (15–18). In addition to inflammation, NF κ B also involves in other biological actions including apoptosis, oncogenesis, and cell differentiation (16, 17). Before activation, NF κ B is bound to the inhibitor κ B (I κ B) protein whose isoforms include I κ B α , I κ B β , and I κ B γ (16, 17). This association between I κ B and NF κ B results in the cytosolic localization of NF κ B. In response to inflammatory stimuli such as TNF- α and interleukin-1, I κ B proteins are degraded in the proteasome, leading to nuclear translocation of NF κ B. I κ B α degradation is controlled by inducible phosphorylation of Ser³² and Ser³⁶ in the I κ B α protein. The phosphorylation of these two serines is directly catalyzed by the IKK complex, which is composed of at least three subunits (IKK1/ α , IKK2/ β , and NEMO/IKK γ) (18). Although both IKK1 and IKK2 can phosphorylate I κ B proteins *in vitro*, IKK2 is indispensable for NF κ B activation *in vivo* (19). Dominant negative mutants of IKK2 are frequently used to block NF κ B pathways in intact cells. Activation of IKK can be initiated by a variety of kinases including protein kinase C (20–22), NF κ B-inducing kinase (23), and MEK kinase 1 (MEKK1) (24). In some cases, NF κ B can be activated in the absence of I κ B protein degradation (*e.g.* vanadium-induced NF κ B activation) (25).

Insulin receptor substrate (IRS) proteins are crucial signaling molecules mediating metabolic actions of insulin (26, 27). Four IRS isoforms (IRS-1, IRS-2, IRS-3, and IRS-4) have been identified that are expressed in a tissue-specific manner (26). The activated insulin receptor phosphorylates IRS proteins on

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplementary Fig. 1.

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¹ The abbreviations used are: TNF- α , tumor necrosis factor- α ; NF κ B, nuclear factor κ B; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEKK, MEK kinase; JNK, c-Jun N-

terminal kinase; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; HA, hemagglutinin; IRS, insulin receptor substrate; IP, immunoprecipitation; GST, glutathione *S*-transferase; pIRS-1, serine-phosphorylated IRS-1; 15dPGJ₂, 15-deoxy-prostaglandin J₂; NEMO, NF κ B essential modulator.

multiple tyrosine residues (28) that serve as docking sites for downstream mediators of metabolic actions including phosphatidylinositol-3 kinase (26). IRS proteins also undergo serine phosphorylation, which regulates its function (26, 29). For example, the phosphorylation of rodent IRS-1 at Ser³⁰⁷ or Ser⁶¹² (Ser³¹² and Ser⁶¹⁶ in human IRS-1, respectively) results in the impairment of metabolic insulin signaling pathways (30, 31). IRS-1 is a substrate for multiple serine kinases including JNK (32, 33), ERK (31, 34, 35), mammalian target of rapamycin (36–38), Akt/protein kinase B (39, 40), protein kinase C ζ (41, 42), glycogen synthase kinase-3 (43), and casein kinase II (44). However, IRS-1 has not previously been identified as a direct substrate for kinases that participate in inflammatory pathways related to activation of NF κ B. In the this study, we evaluate the possibility that IRS-1 is a direct substrate for IKK that can provide a mechanism for cross-talk between metabolic and inflammatory signaling pathways.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Cell lines including human hepatoma HepG2 (HB-8065), human embryo kidney (HEK) 293 (CRL-1573) and mouse fibroblast 3T3-L1 (CL-173) were purchased from the American Type Culture Collection. All cells were maintained in Dulbecco's modified Eagle's culture medium supplemented with 10% fetal calf serum. For 3T3 cells, 4 mM glutamine was used in the culture medium. Wild-type and IKK1/2 double knock-out (IKK1/2^{-/-}) cell lines were a gift from Dr. Inder Verma at the Salk Institute (La Jolla, CA). Antibodies against phospho-IRS-1 (Ser³⁰⁷) (number 07-247) and IKK2 (number 05-535), and λ -protein phosphatase number 14-405 were obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies against IRS-1 (sc-7200), IRS-2 (sc-8299), IKK1 (sc-7182), HA (sc-7392), I κ B α (sc-371), I κ B β (sc-945), and pJUN (sc-822) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). β -actin antibody (ab6276) was obtained from Abcam (Cambridge, United Kingdom). Calyculin A (EI-192) and SP600125 (EI-305) were from Biomol (Plymouth Meeting, PA). TNF- α (210-TA-010) was obtained from R&D systems (Minneapolis, MN). 15-Deoxy-prostaglandin J₂ (15dPGJ₂, 538927) was from Calbiochem.

Immunoblotting (45)—Whole cell lysates were made by sonication in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM Hepes, pH 7.8, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, 125 μ M dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Samples (100 μ g of total protein) in 50 μ l of reducing sample buffer were boiled for 3 min and resolved on 6% mini-SDS-PAGE for 90 min at 100 v. The contents of the gel were transferred onto polyvinylidene difluoride membrane (162-0184, Bio-Rad) at 21 v for 120 min. The membrane was pre-blotted in milk buffer for 20 min and then immunoblotted with primary antibody for 1–24 h followed by secondary antibody for 30 min. Horseradish peroxidase-conjugated secondary antibodies (NA934V or NA931, Amersham Biosciences) were used in conjunction with chemiluminescence reagent (NEL-105, PerkinElmer Life Sciences). To detect multiple signals from a single membrane, membranes were treated with a stripping buffer (59 mM Tris-HCl, 2% SDS, 0.75% 2-mercaptoethanol) for 20 min at 37 °C prior to re-blotting with a different antibody.

Dephosphorylation of IRS-1— λ -Protein phosphatase (number 14-405) was used to dephosphorylate IRS-1 in whole cell lysates of calyculin-treated HepG2 cells. The reaction was performed according to the manufacturer's instruction. 100 μ g of protein was incubated with 500 units of enzyme in λ -phosphatase reaction buffer at 30 °C for 20 min. The reaction was stopped by adding Western blot sample buffer and boiling for 3 min. The dephosphorylated proteins were analyzed by immunoblotting.

Immunoprecipitation (IP) and Kinase Assay (46)—Immunoprecipitation was carried out using whole cell lysate (400 μ g of total protein), 2–4 μ g of antibody, and 20 μ l of protein A- or protein G-Sepharose beads (Amersham Biosciences). After treatment, cells were lysed by sonication in a cell lysis buffer (1% Nonidet P-40, 50 mM Hepes, pH 7.6, 250 mM NaCl, 10% glycerol, 1 mM EDTA, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium metabisulfite, 1 mM benzamide hydrochloride, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). IP was conducted by incubating whole cell lysates with antibody for 3–4 h at 4 °C. The immune complex was washed five times in cell lysis buffer before being used for immunoblotting or for the kinase assay. To conduct the kinase assay, the immune complex was washed two more times in a kinase assay buffer (20 mM

Hepes, pH 7.6, 20 mM MgCl₂, 20 mM β -glycerophosphate, 1 mM dithiothreitol, 10 μ M ATP, 1 mM EDTA, 1 mM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, 20 mM creatine phosphate). The kinase assay was conducted at room temperature for 30 min in 20 μ l of kinase assay buffer containing 5 μ Ci of [γ -³²P]ATP. The product was resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane for autoradiography or immunoblotting.

Gel Shift Assay (47, 48)—An oligonucleotide containing the NF κ B binding sequence in the human interleukin-6 gene promoter (–⁷⁴TGG-GATTTTCCCATGAGTCT–⁵⁴) was synthesized as a NF κ B binding probe. Nuclear extracts were prepared with a three-step procedure (48). The protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL). The DNA-protein binding reaction was conducted in a 24- μ l reaction mixture including 1 μ g of poly(dI-dC) (Sigma), 3 μ g of nuclear protein extract, 3 μ g of bovine serum albumin, 4 \times 10⁴ cpm of ³²P-labeled oligonucleotide probe, and 12 μ l of reaction buffer (24% glycerol, 24 mM Hepes, pH 7.9, 8 mM Tris-HCl, 2 mM EDTA, 2 mM dithiothreitol). After the addition of radiolabeled probe, the mixture was incubated for 20 min at room temperature and then resolved on a 5% acrylamide gel that had been pre-run at 170 V for 30 min with 0.5 \times Tris borate buffer. The loaded gel was run at 200 V for 90 min, dried, placed on Kodak X-Omat AR film (Eastman Kodak Co.), and the film was developed after overnight exposure at –70 °C.

Plasmid and Transfection—HA-tagged fusion proteins of IKK1, IKK2, IRS-1, IRS-2, and Akt were expressed in HEK 293 cells by transient transfection. The plasmids for HA-IKK1, HA-IKK2, and kinase-dead HA-IKK2 were a gift from Dr. Michael Karin (Department of Pharmacology, University of California, San Diego, CA). The expression plasmids for HA-IRS-1 and HA-IRS-2 were constructed by inserting human IRS-1 or IRS-2 cDNA into pCIS2 expression vector (41). Kinase-dead HA-Akt expression plasmid was from Dr. Bin-Hua Jiang (BMC Cancer Center, West Virginia University). Transient transfection was conducted using LipofectAMINE as reported previously (45). GST-IRS-1 expression plasmid for rat IRS-1 (amino acids 117–513) was from Dr. Xiao-Jian Sun (Endocrinology Division, University of Vermont College of Medicine). The purified GST-IRS-1 was prepared using a protocol described previously (48).

RESULTS

Association between IRS-1 Serine Phosphorylation and IKK Activation—TNF- α treatment of cells causes increased serine phosphorylation of IRS-1 as well as activation of IKK and NF κ B (1, 15). To explore the relationship between IRS-1 serine phosphorylation and IKK activation, HepG2 cells were treated with TNF- α , and IRS-1 phosphorylation at Ser³¹² was examined using the phosphospecific antibody developed against phosphoserine 307 of rat IRS-1 (equivalent to Ser³¹² in human IRS-1). The IRS-1 phosphorylation was induced by TNF- α at 5 min, and this phosphorylation was sustained over 120 min (Fig. 1A, top panel). This phosphorylation of IRS-1 correlated with the disappearance of I κ B α at 5 min (Fig. 1A, third panel), which is an indication of IKK activation. After 30 min, the level of I κ B α started to increase and returned to the basal levels by 60–120 min.

Calyculin is an inhibitor of protein serine phosphatases PP1 and PP2A (49) that induces NF κ B activity by activating IKK (50). In calyculin-treated HepG2 cells, both I κ B α degradation and IRS-1 phosphorylation were observed after 15 min (Fig. 1B, panels 1 and 5). I κ B β expression was also reduced slightly after 30 min of calyculin treatment. In addition, a mobility shift in IKK1 and IKK2 was observed at 30 and 60 min that is consistent with the serine phosphorylation and activation of these two kinases by calyculin (Fig. 1B, panels 3 and 4). The IKK1 signal also increased in a time-dependent manner after calyculin treatment. The serine-phosphorylated IRS-1 exhibited reduced mobility on the gel, and this change resulted in two bands detected by the phosphospecific antibody. One band migrated at a level of ~180 kDa, whereas the other band migrated at the level of 165 kDa. The intensity of both bands increased in a time-dependent manner. In addition, of the two forms of serine-phosphorylated IRS-1 (pIRS-1) seen in Fig. 1B, only the bottom band, not the top band, was recognized by the

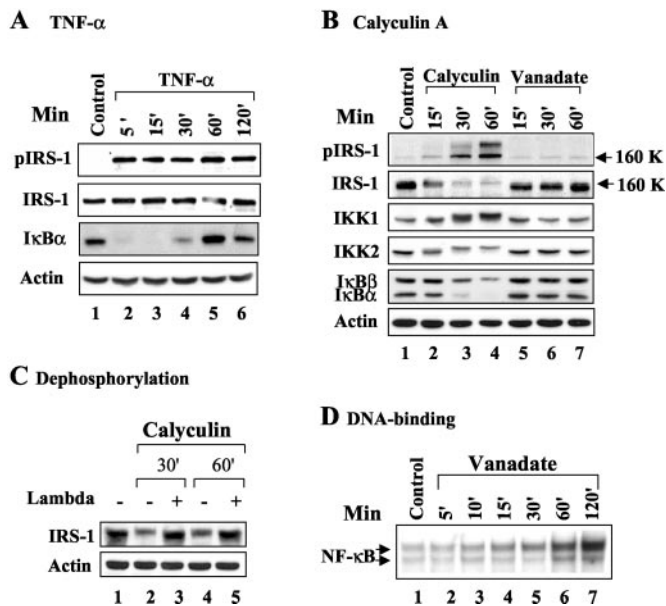


FIG. 1. Association between Ser³¹² phosphorylation of human IRS-1 and activation of IKK2. Ser³¹² phosphorylation of human IRS-1 was induced by TNF- α (25 ng/ml) or calyculin (50 nM). HepG2 cells were plated in 24-well plates and serum-starved overnight before TNF- α treatment. Whole cell lysates were resolved by 6% SDS-PAGE and immunoblotted using antibodies against phospho-IRS-1 (Ser³⁰⁷), IRS-1, IKK2, I κ B α , I κ B β , and actin. **A**, TNF- α -treated HepG2 cells. **B**, calyculin and vanadate-treated HepG2 cells. Vanadate (NaVO₃, 400 μ M) was used in the treatment. **C**, restoration of gel mobility and antibody affinity of IRS-1 by serine dephosphorylation. λ -protein phosphatase was used to dephosphorylate IRS-1 in the whole cell lysate of calyculin-treated HepG2 cells. **D**, time-dependent activation of NF κ B by vanadate. The nuclear protein from vanadate-treated HepG2 cells was extracted and used in the gel shift assay.

regular IRS-1 antibody. The bottom band was reduced significantly at the time points of 30 and 60 min when the serine phosphorylation was increased dramatically.

In calyculin-treated cells, the signal intensity of the total IRS-1 was reduced at 15, 30, and 60 min (Fig. 1B, panel 2). This reduction may result from decreased antibody affinity to the pIRS-1 or a reduction in IRS-1 protein abundance. To distinguish between these two possibilities, whole cell lysates derived from calyculin-treated HepG2 cells were incubated with λ -protein phosphatase to dephosphorylate pIRS-1. The result shows that the IRS-1 protein signal was restored along with the gel mobility after dephosphorylation at serine residues (Fig. 1C), suggesting that the loss of IRS-1 signal is a result of reduced antibody affinity to the phosphorylated IRS-1. This is also supported by studies using a different IRS-1 antibody or using proteasome inhibitor (data not shown).

Vanadate is a tyrosine phosphatase inhibitor that induces the DNA binding of NF κ B through an alternative pathway in which IKK is not activated (25). As expected, in vanadate-treated cells, I κ B α protein levels and IKK mobility were not altered (Fig. 1B), whereas the DNA binding of NF κ B was induced (Fig. 1D). Moreover, vanadate treatment did not result in phosphorylation of IRS-1 at Ser³¹² (Fig. 1B).

To investigate the relationship between serine phosphorylation of IRS-1 and IKK activity more directly, we used the IKK inhibitor 15dPGJ₂ (51). When HepG2 cells were pre-treated with 15dPGJ₂, the phosphorylation of IRS-1 at Ser³¹² in response to TNF- α treatment was inhibited (Fig. 2A). In line with previously published results (30, 32), the JNK inhibitor SP600125 (52) also inhibited the phosphorylation of IRS-1 at Ser³¹² (Fig. 2A). Pre-treatment with both 15dPGJ₂ and SP600125 resulted in more complete inhibition of serine phosphorylation on IRS-1 than treatment with either inhibitor

alone (Fig. 2A). Consistent with the ability of 15dPGJ₂ to inhibit IKK activity in a specific manner, I κ B α degradation in response to TNF- α treatment was inhibited by 15dPGJ₂ but not SP600125 (Fig. 2B). Similarly, SP600125 but not 15dPGJ₂ inhibited phosphorylation of c-JUN in response to TNF- α (Fig. 2C). Thus, specific suppression of IKK activity is associated with the inhibition of IRS-1 phosphorylation at Ser³¹² in response to TNF- α .

Association between IRS-1 and IKK Complex in Intact Cells—Based on the close relationship between IKK activity and serine phosphorylation of IRS-1 described above, we next inquired whether IRS-1 could serve as a direct substrate for IKK. As a first step to test this hypothesis, the physical association between IRS-1 and the IKK complex was examined using co-immunoprecipitation (Fig. 3). In HepG2 cells, IRS-1 was detected in the immune complex precipitated with anti-IKK-1 antibody (Fig. 3A). Moreover, the association between IKK-1 and IRS-1 observed in the basal state was reduced by calyculin treatment but not by vanadate treatment (Fig. 3A). Consistent with immunoblotting results shown in Fig. 1B, the intensity of the IKK1 signal was increased and the intensity of the IRS-1 signal was decreased by calyculin in a time-dependent manner. As expected, IKK2 was also detected in the IKK1 immunoprecipitate. Similar results were obtained when IRS-1 immunoprecipitates were probed with the IKK antibody (Fig. 3B). These results suggest an association between IRS-1 and IKK1 that was observed in the basal state and reduced dramatically after calyculin treatment. It is interesting to note that the dynamics of the association between IRS-1 and IKK1 were different depending on whether anti-IKK1 or anti-IRS-1 antibody was used for immunoprecipitation. With the anti-IKK1 antibody, the association of IRS-1 disappeared at 60 min (Fig. 3A). By contrast, with the anti-IRS-1 antibody, the association of IKK1 became undetectable at 5 min (Fig. 3B). These differences may be attributed to a change in antibody affinity for the phosphorylated IRS-1 as described above (Fig. 1). Thus, less IRS-1 may be immunoprecipitated with anti-IRS-1 antibody after calyculin treatment. Consistent with this explanation is the fact that the intensity of the IRS-1 signal detected by the regular IRS-1 antibody was decreased in the IRS-1 immunoprecipitate after calyculin treatment (Fig. 3B). In 293 cells transiently transfected with HA-tagged IKK2, both IRS-1 and IRS-2 were detected in the anti-HA immunoprecipitate (Fig. 3C). Similar to results from HepG2 cells, this association between IKK2 and IRS proteins was abolished after calyculin treatment of the cells. TNF- α treatment also abolished the interaction between IKK and IRS proteins (data not shown). To demonstrate that the immunoprecipitation results are specific, non-immune IgG as well as an unrelated antibody against p38 was used to immunoprecipitate samples in control experiments (Fig. 3D). These two controls did not give the results observed with the IRS-1 or IKK1 antibody. Taken together, these data suggest that IRS-1 and IKK directly interact in intact cells and that this interaction is reduced by activation of IKK and serine phosphorylation of IRS-1.

Phosphorylation of IRS-1 by IKK Complex in Vitro—To further examine the possibility that IRS-1 is a novel substrate for IKK, we performed immune complex kinase assays using recombinant IKK2 derived from transiently transfected 293 cells. Expression vectors for HA-tagged IRS-1 and HA-tagged IKK2 were co-transfected into HEK 293 cells, and then recombinant proteins were recovered by immunoprecipitating with the anti-HA antibody. After being extensively washed, the immune complex was used in an *in vitro* kinase assay (46). Three major phosphoproteins were observed in the HA immunoprecipitates when wild-type IKK2 was used (Fig. 4A). The identity of these

FIG. 2. Regulation of IRS-1 phosphorylation by IKK inhibitor. Phosphorylation of IRS-1 at Ser³¹² in response to TNF- α (30 min) in HepG2 cells was impaired by pre-treatment with 15dPGJ₂ (15 μ M) or SP600125 (50 μ M) for 30 min. Ser³¹² phosphorylation of IRS-1 was monitored with pIRS-1 antibody. I κ B α degradation and c-JUN phosphorylation were examined to evaluate the specificity of the two inhibitors. *A*, inhibition of IRS-1 phosphorylation. *B*, inhibition of I κ B α degradation by 15dPGJ₂. *C*, inhibition of c-JUN phosphorylation by SP600125.

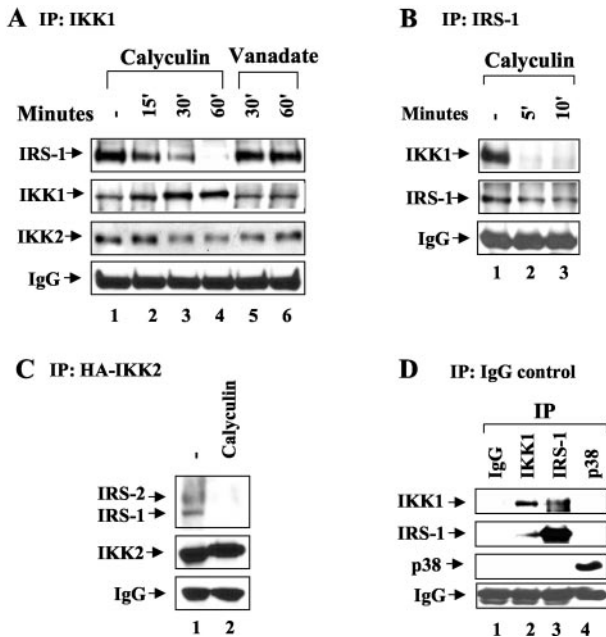


FIG. 3. Analysis of IRS/IKK interaction by immunoprecipitation. HepG2 cells were plated in 100-mm plates overnight and treated without or with calyculin or vanadium as indicated. 400 μ g of total protein was immunoprecipitated with the indicated antibody, and the immune complex was resolved by 6% SDS-PAGE and immunoblotted with various antibodies as indicated. *A*, IP with anti-IKK1 antibody (4 μ g/reaction). *B*, IP with anti-IRS-1 antibody (4 μ g/reaction). *C*, IP of recombinant HA-IKK2. HA-tagged IKK2 was overexpressed in HEK 293 cells by transient transfection. Cells were treated without or with calyculin for 30 min before harvesting at 36 h. HA-IKK2 was immunoprecipitated with anti-HA antibody (2 μ g/reaction). The presence of IRS-1, IRS-2, and IKK2 was determined by immunoblotting. *D*, IP with non-immune rabbit IgG or anti-p38 antibody. Whole cell lysate from untreated HepG2 cells was immunoprecipitated with 4 μ g of non-immune rabbit IgG or anti-p38 antibody and immunoblotted as indicated.

phosphoproteins was determined by immunoblotting. They are HA-IRS-1 with a mobility of 160 kDa, HA-IKK2 with a mobility of 89 kDa, and NEMO/IKK γ with a mobility of 48 kDa. Importantly, these phosphoproteins were absent or significantly reduced when an empty vector or the kinase-dead IKK2 was used instead of wild-type IKK2 (Fig. 4A). Similar results were obtained with the wild type IKK1 with the exception that phosphorylation of IKK2 and NEMO was much weaker (Fig. 4A, lane 4). The phosphorylation of IKK2 is consistent with autophosphorylation (53), and NEMO is known to be a substrate for IKK2 (54). More importantly, these results suggest that IRS-1 is capable of functioning as a direct substrate for IKK *in vitro* because IRS-1 phosphorylation was only observed in the immune complexes with wild-type IKK2 but not with kinase-dead IKK2 or the empty vector.

The effect of calyculin treatment on the ability of IRS-1 to undergo phosphorylation by IKK was also investigated (Fig.

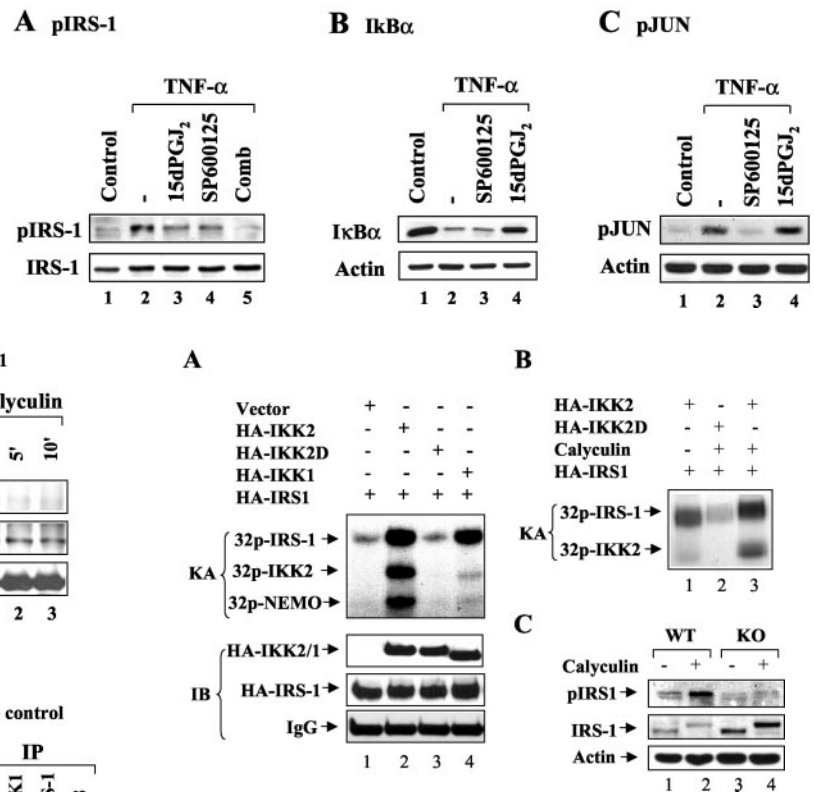


FIG. 4. Phosphorylation of IRS-1 by IKK complex *in vitro*. *A*, *In vitro* kinase assay (KA). Expression vectors for HA-tagged IKK2 (5 μ g) and HA-IRS-1 (5 μ g) were co-transfected into HEK 293 cells. Recombinant IKK2 and IRS-1 were immunoprecipitated with anti-HA antibody (2 μ g/reaction). The IP product was subjected to an *in vitro* kinase assay. The kinase assay product was resolved by SDS-PAGE, and phosphorylation was examined by autoradiography. The presence of recombinant proteins including IRS-1, IKK2, dominant negative form of IKK2 (IKK2D), and IKK1 was confirmed by immunoblotting (IB). This kinase assay was independently repeated five times with comparable results. *B*, effect of calyculin on kinase activity of IKK complex. The kinase assay was conducted as stated in *A* using samples derived from cells treated without or with calyculin. *C*, reduction of IRS-1 serine phosphorylation in IKK1 and IKK2 double knock-out cells (KO). KO cells were treated with calyculin for 30 min, and levels of pIRS-1 (Ser³¹²) and total IRS-1 were determined by immunoblotting. WT, wild type.

4B). Calyculin treatment resulted in increased IRS-1 phosphorylation by wild-type but not kinase-dead IKK2. This finding suggests that calyculin increased the activity of IKK2 to phosphorylate IRS-1. Consistent with this possibility, the autophosphorylation of IKK2 was increased by treatment with calyculin (Fig. 4B). Like endogenous IRS-1, HA-IRS-1 also exhibited a reduced mobility in response to calyculin. This reduction in mobility occurred before IRS-1 was phosphorylated by IKK *in vitro* because this phenomenon was also observed with kinase-dead IKK2 (Fig. 4B). Thus, there may be other kinases that are activated by calyculin that also phosphorylate IRS-1. Nevertheless, the mobility of IRS-1 was further reduced after phosphorylation by IKK *in vitro*. These results suggest that the magnitude of the mobility shift of IRS-1 is related to the level of serine phosphorylation in IRS-1.

The role of IKK in phosphorylation of IRS-1 was further investigated in intact cells using an IKK1 and IKK2 double knock-out cell line (IKK1/2^{-/-}) (Fig. 4C). The absence of IKK1 and IKK2 in these cells was confirmed by immunoblotting (data not shown). After calyculin treatment, the pIRS-1 level was much lower in the IKK1/2^{-/-} cells as compared with wild-type cells, although the IRS-1 protein abundance was significantly higher in the knock-out cells. It is noteworthy that in the

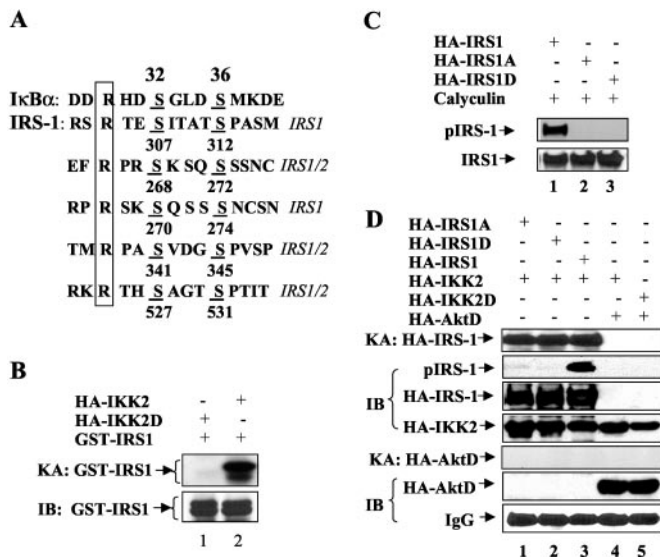


FIG. 5. IKK2 phosphorylation sites in IRS-1. *A*, alignment of phosphoacceptor sequences of human IκBα and potential IKK phosphorylation sites in human IRS-1. Sequences conserved between human IRS-1 and IRS-2 are indicated by IRS-1/2. *B*, phosphorylation of GST-IRS-1 (amino acids 117–513) protein by IKK2 immunoprecipitate. GST-IRS-1 fusion protein (2 μg/reaction) was purified from bacterial lysate using glutathione-Sepharose 4B beads. Immunoprecipitate of HA-IKK2 from transfected HEK 293 cells was used as a source of kinase. *C*, point mutation of Ser³¹² in IRS-1. Alanine (*HA-IRS1A*) or aspartate (*HA-IRS1D*) was substituted for Ser³¹² in IRS-1 by site-directed mutagenesis. The mutated HA-IRS1 proteins were expressed in HEK 293 cells by transient transfection. Phosphorylation of IRS-1 was determined by immunoblotting with pIRS-1 antibody. *D*, phosphorylation of mutated IRS-1 by IKK2. Kinase assays were carried out as stated in the legend for Fig. 4A. HA-tagged Akt mutant (*HA-AktD*) was used as a negative control substrate for the IKK kinase assay. Comparable recovery of recombinant IRS-1, IKK2, IKK2D, and AktD was confirmed by immunoblotting. *KA*, kinase assay; *IB*, immunoblotting.

IKK knock-out cells, IRS-1 still exhibited a mobility shift after calyculin stimulation. This mobility shift is probably because of phosphorylation of IRS-1 by serine kinases other than IKK.

Phosphorylation of IRS-1 by IKK at Multiple Serine Residues—The amino acid sequence of IRS-1 was analyzed to identify potential sites of phosphorylation by IKK. A putative IKK consensus sequence for phosphorylation was derived from the amino acid sequence of IκBα that contains two IKK phosphorylation sites at Ser³² and Ser³⁶ (Fig. 5A). Based on sequence homology, five potential IKK phosphorylation sites were identified in the human IRS-1 protein. All of these sites contain an arginine (boxed) followed by two serines (underlined). The two serine residues are separated by three amino acid residues with the exception that in one instance where four amino acid residues are found between the two serines. Among these five putative IKK phosphorylation sites in IRS-1, three are conserved in human IRS-2 (indicated by IRS-1/2). Thus, in addition to Ser³¹², it is possible that other serine residues on IRS-1 may be phosphorylated by IKK. Our data generated with the phosphospecific IRS-1 antibody (pIRS-1 Ser³⁰⁷) suggest that inhibition of IKK activity or the absence of IKK results in decreased phosphorylation of Ser³¹² (Figs. 2A and 4C). To provide more evidence that serine residues identified by our sequence analysis represent *bona fide* phosphorylation sites for IKK, we used a GST fusion protein containing amino acids 117–513 from rat IRS-1 as a substrate for IKK in an *in vitro* kinase assay. This fragment of IRS-1 contains four potential IKK phosphorylation sites identified by our sequence analysis (including Ser³¹²). Immunoblots of the GST-IRS-1 fragment revealed two bands with an apparent molecular mass of ~70 kDa. Both forms of the peptide were phosphorylated by wild-

type but not kinase-dead IKK2 in the *in vitro* kinase assay (Fig. 5B). However, the larger form of the GST-IRS-1 peptide was phosphorylated to a much greater extent.

To further evaluate the phosphorylation of IRS-1 on Ser³¹² by IKK, we made point mutants of IRS-1 with the substitution of Ala or Asp for Ser³¹². These mutants also contain a HA epitope fused to the C terminus of IRS-1. The IRS-1 mutants were expressed in 293 cells, immunoprecipitated with anti-HA antibody, and immunoblotted with the phosphospecific IRS-1 antibody (Fig. 5, C and D). As expected, the calyculin treatment of cells resulted in the phosphorylation of wild-type IRS-1. However, the anti-pIRS-1 antibody did not recognize the mutant IRS-1 proteins in cells treated with calyculin (Fig. 5C). This serves to confirm the specificity of our phosphospecific antibody as well as the substitutions of Ala and Asp at position 312 in the IRS-1 mutants. When these mutants were used as substrates in IKK kinase assays, both mutants retained the ability to be phosphorylated by IKK (Fig. 5D). Compared with wild-type IRS-1, there was no significant reduction in the phosphorylation of the mutants by IKK. In the same kinase assay, wild-type IRS-1 was phosphorylated at Ser³¹² as demonstrated by immunoblotting with pIRS-1 antibody (Fig. 5D). Comparable recovery of IRS-1 and IKK2 proteins in each sample was confirmed by immunoblotting. In addition, we used HA-tagged Akt (dominant negative mutant) as a negative control in the kinase assay. As expected, Akt was not phosphorylated by IKK2 (Fig. 5D). Thus, it is probable that additional IKK phosphorylation sites on IRS-1 exist in addition to Ser³¹².

DISCUSSION

In this study, we obtained substantial evidence supporting that IRS-1 may be a potential new substrate for IKK2. The data consistently support the possibility that IRS-1 can be phosphorylated by IKK2 in cells and in a cell-free system. In cells, serine phosphorylation of IRS-1 was induced upon IKK activation (Fig. 1, A and B) and was reduced upon IKK inactivation by 15dPGJ₂ (Fig. 2). The serine phosphorylation was significantly reduced in the IKK null cells (Fig. 4C). If IKK activity was not induced when NFκB was activated (Fig. 1D), IRS-1 was not phosphorylated as observed in cells treated with vanadate (Fig. 1B). These results confirm that IRS-1 can be phosphorylated by IKK in cells. The serine phosphorylation may lead to some biochemical changes in IRS-1 protein. In calyculin-treated cells, the phosphorylation reduced IRS-1 gel mobility and decreased IRS-1 affinity to the IRS-1 antibody utilized. Although these results are from HepG2 cells, similar data were also obtained in 3T3-L1 and 293 cells (data not shown). These effects of serine phosphorylation are supported by the dephosphorylation study (Fig. 1C). It is noted that IRS-1 protein signal became weak when the phosphorylation signal was strong (Fig. 1B). The reduced protein signal might be due to a loss of protein abundance or loss of protein affinity to the IRS-1 antibody used. The dephosphorylation study supports the affinity change.

The serine phosphorylation also influences the association of IRS-1 with IKK complex. In co-precipitation study, endogenous IRS-1 was detected in the immunoprecipitate of IKK using IKK1 antibody or HA antibody (Fig. 3, A and C). Similarly, endogenous IKK1 was observed in the immunocomplex of IRS-1 antibody (Fig. 3B). These results suggest a protein-protein association between IRS-1 and IKK complex in cells. Interestingly, this association was reduced after IRS-1 was phosphorylated by IKK as revealed by the time-dependent reduction in the association observed in the co-immunoprecipitation analysis (Fig. 3, A and B). A discrepancy in the kinetic of the disassociation is noted in the immunoprecipitation using IRS-1 versus IKK1 antibody. This discrepancy may be the result of

affinity change of phosphorylated IRS-1 to the IRS-1 antibody as supported by the dephosphorylation study. The IRS-1/IKK association was not observed in the immunoprecipitates of the control IgG and antibody to p38 (Fig. 3D). Physiological significance of the IRS-1/IKK disassociation remains to be investigated. One possibility might be that the disassociation eventually leads to degradation of IRS-1 in proteasome (55, 56).

IRS-1 was phosphorylated by IKK complex *in vitro*. In the kinase assay, immunoprecipitates of IKK-2 resulted in the phosphorylation of recombinant IRS-1 proteins expressed in mammalian cells or in bacteria (Figs. 4A and 5B). The phosphorylation was dependent on catalytic activity of IKK-2 and was promoted when IKK-2 activity was induced (Fig. 4, A and B). IKK-mediated phosphorylation contributes to the mobility change of IRS-1 (Fig. 1B). However, the mobility change seems to be controlled not only by IKK-mediated phosphorylation but also by phosphorylation induced by other serine kinases. This is supported by the fact that after calyculin treatment, IRS-1 mobility was reduced in cells expressing the dominant negative IKK2 (Fig. 4B, lane 2) or in the IKK null cells (Fig. 4D, lane 4). IKK is able to reduce the mobility further through serine phosphorylation (Fig. 4B, lane 3). Because a negative control, *i.e.* recombinant Akt, was not phosphorylated by the IKK complex in the same kinase assay condition (Fig. 5D, lane 4), *in vitro* phosphorylation of IRS-1 by IKK may not be a result of random protein interaction. A cofactor such as NEMO/IKK γ might be required for the physical association of IRS/IKK. NEMO is shown to mediate molecular association of I κ B α with IKK and is required for I κ B α phosphorylation by IKK (57).

In this study, the phosphorylation of serine 312 of IRS-1 was monitored with the phosphospecific IRS-1 (pSer³⁰⁷) antibody in phosphorylation by IKK. However, the data suggest that there are other potential IKK phosphorylation sites in IRS-1 in addition to Ser³¹². Five potential IKK phosphorylation motifs were identified in the human IRS-1 using the consensus sequence derived from I κ B α (Fig. 5A). Three of the motifs are conserved in the human IRS-2. Since mutation of Ser³¹² did not prevent IRS-1 phosphorylation by IKK, the result suggests existence of those potential phosphoacceptors of IKK.

Although TNF- α and calyculin both activate IKK, they exhibited certain differences in activities. For example, IRS-1 did not exhibit a significant change in mobility and antibody affinity in the TNF- α -treated HepG2 cells but did in calyculin-treated HepG2 cells (Fig. 1, A and B). It is not clear what is the exact molecular basis of this difference. One possibility is that calyculin may induce phosphorylation of more serine/threonine residues per IRS-1 molecule. There are >50 serine/threonine residues in each human IRS-1 molecule (58). It is possible that some of the serine/threonine residues can be phosphorylated in response to calyculin but not TNF- α . It is known that some serine kinases such as ERK (31, 35) are able to phosphorylate IRS-1 at serine residues. Because calyculin is able to activate ERK as well (59), ERK may contribute to more serine phosphorylation per IRS-1 molecule by calyculin. Interestingly, both TNF- α and calyculin treatment are known to be associated with insulin resistance, whereas vanadate has insulin-sensitizing activity (1, 60–62). The role of IKK may help us to understand the activities of these agents.

In summary, our data suggest consistently that IRS-1 may be phosphorylated by IKK at serine residues. Multiple serine residues on IRS-1 including Ser³¹² will probably undergo phosphorylation by IKK. Because IRS-2 also associates with IKK and contains putative IKK phosphorylation sites, it may also be phosphorylated by IKK. These results suggest that the IRS protein family may represent a novel class of substrates for IKK kinases. The phosphorylation of Ser³⁰⁷ on rat IRS-1 inhib-

its the tyrosine phosphorylation of IRS-1 by the insulin receptor and impairs metabolic insulin signaling pathways (30). Because the human Ser³¹² is equivalent to the rat Ser³⁰⁷, our data suggest that IKK-mediated phosphorylation may represent a new mechanism through which inflammatory pathways contribute to insulin resistance. This mechanism may also play a role in the insulin resistance associated with lipid disorder.

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