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Insulin Resistance Due to Phosphorylation of Insulin Receptor Substrate-1 at Serine 302*

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Inhibitory serine phosphorylation is a potential molecular mechanism for insulin resistance. We have developed a new variant of the yeast two-hybrid method, referred to as disruptive yeast tri-hybrid (Y3H), to identify inhibitory kinases and sites of phosphorylation in insulin receptors (IR) and IR substrates, IRS-1. Using IR and IRS-1 as bait and prey, respectively, and c-Jun NH_2-terminal kinase (JNK1) as the disruptor, we now show that phosphorylation of IRS-1 Ser-307, a previously identified site, is necessary but not sufficient for JNK1-mediated disruption of IR/IRS-1 binding. We further identify a new phosphorylation site, Ser-302, and show that this too is necessary for JNK1-mediated disruption. Seven additional kinases potentially linked to insulin resistance similarly block IR/IRS-1 binding in the disruptive Y3H, but through distinct Ser-302- and Ser-307-independent mechanisms. Phosphospecific antibodies that recognize sequences surrounding Ser(P)-302 or Ser(P)-307 were used to determine whether the sites were phosphorylated under relevant conditions. Phosphorylation was promoted at both sites in Fao hepatoma cells by reagents known to promote Ser/Thr phosphorylation, including the phorbol ester phorbol 12-myristate 13-acetate, anisomycin, calyculin A, and insulin. The antibodies further showed that Ser(P)-302 and Ser(P)-307 are increased in animal models of obesity and insulin resistance, including genetically obese *ob/ob* mice, diet-induced obesity, and upon induction of hyperinsulinemia. These findings demonstrate that phosphorylation at both Ser-302 and Ser-307 is necessary for JNK1-mediated inhibition of the IR/IRS-1 interaction and that Ser-302 and Ser-307 are phosphorylated in parallel in cultured cells and *in vivo* under conditions that lead to insulin resistance.

Insulin resistance is the condition in which target tissues fail to respond appropriately to circulating insulin. Although genetics may play a role in the pathogenesis of type 2 diabetes, it has become increasingly clear that acquired, non-genetic causes of insulin resistance represent a critical link between the rapidly growing national and worldwide epidemics in obesity and type 2 diabetes (1–5). Obesity, fatty diet, and sedentary lifestyle directly promote insulin resistance, and exercise and weight loss reverse it. Obesity and insulin resistance are also associated with and exacerbate hypertension and hyperlipidemia in addition to predisposing to the development of type 2 diabetes. This constellation of conditions, referred to collectively as either the metabolic or dysmetabolic syndrome, represents a third interrelated epidemic with a prevalence of ~24% of adults in the United States (6). Individuals with the metabolic syndrome have a seriously increased risk of developing atherosclerotic cardiovascular disease. Elucidating the molecular pathways that connect obesity to pathogenesis of insulin resistance clearly has great public health importance.

Of hypothesized mediators of insulin resistance, recent findings have profiled potential roles for inflammation and proinflammatory cytokines, other fat cell-derived cytokines, free fatty acids, and inhibitory serine/threonine (Ser/Thr) phosphorylation of upstream elements of insulin signaling (7–14). In fact, inflammation- and free fatty acid-mediated mechanisms may converge at the level of Ser/Thr phosphorylation of insulin receptors (IRs)[1] and insulin receptor substrates (IRSs) to provide potentially unifying mechanisms for insulin resistance (15–20). Consistent with this, insulin-sensitizing, anti-inflammatory salicylates reverse Ser/Thr phosphorylation of IR and IRSs in insulin-responsive tissues in obesity-, diet- and free fatty acid-induced models of insulin resistance (15, 16).

Although IR is a tyrosine kinase, insulin also stimulates Ser/Thr phosphorylation of numerous signaling enzymes and other proteins (21, 22). Many are Ser/Thr kinases involved in kinase cascades. Those mediating some of the insulin cellular actions include Raf, MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase), MAPK, p90RSK, phosphatidylinositol 3-kinase, phosphoinositide-dependent protein kinase 1, protein kinase B/AKT, mTOR, p70 S6 kinase, GSK3β, PKCβ1, PKCζ, and PKCλ. Overexpression of many of these kinases paradoxically inhibits insulin signaling as opposed to activating it, suggesting that the same kinases that mediate insulin signaling might also play roles in negative feedback of it (23). In fact, IR and IRSs are themselves Ser/Thr-phosphorylated in response to insulin, providing a potential mechanism for negative feedback.

Basal levels of IRS-1 Ser/Thr phosphorylation are increased in cells under various conditions, leading to observable shifts in electrophoretic mobility (15, 24–27). The magnitudes of the shifts demonstrate that multiple sites are phosphorylated. In

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[1] The abbreviations used are: IR, insulin receptor; IRS, IR substrate; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; GSK, glycogen synthase kinase; PKA and PKC, protein kinase A and C, respectively; JNK, c-Jun NH_2-terminal kinase; Y3H, yeast tri-hybrid; WT, wild type; IKK, IκB kinase complex.

fact, IRS-1 contains 232 serines and threonines, nearly 19% of its 1231 residues, providing great potential for multisite phosphorylation. The rapid accumulation of "hyper-phosphorylated" IRS-1 upon treatment with the Ser/Thr phosphatase inhibitors okadaic acid or calyculin A further indicates that it is constantly being Ser/Thr-phosphorylated. Normally this is countered by phosphatases like PP2A and PP1, such that under steady-state conditions there should be a given "responsiveness" to the system. The balance might be shifted by activating Ser/Thr kinases that phosphorylate IRS or by inhibiting a relevant phosphatase. The net result in these cases would be the same; that is, Ser/Thr phosphorylation of IRS-1, diminished insulin signaling, and the development of insulin resistance. Conversely, insulin signaling should be sensitized either by inhibiting the relevant Ser/Thr kinase(s) or activating the appropriate phosphatase(s).

A host of Ser/Thr kinases can be shown to attenuate upstream insulin action in cultured cells, including PKA (28–30), AKT/protein kinase B (31, 32), AMP-dependent protein kinase (33), PKCs (34–38), MAPK (39), GSK3β (40, 41), casein kinase II, JNK (17, 18, 20, 42), mTOR (43, 44), phosphatidylinositol 3-kinase (45, 46, 46–51), Rho kinase (ROK) (52), and salt-inducible kinase (SIK1/2) (53). Specific Ser/Thr phosphorylation sites in IRS-1 identified *in vitro* include Ser-307 (17, 18, 20, 42, 51, 54–56), Ser-612 (37), Ser-636 and Ser-639 (44), Ser-731 (57), and Ser-789 (33, 58). Of these, Ser-307[2] phosphorylation has been studied most intensively as a mechanism for disrupting IR/IRS-1 interactions (17, 18, 42, 51, 54–56, 59–61). Originally identified as a target of JNK in cells (17), Ser-307 was subsequently found to be phosphorylated as well in cells treated with tumor necrosis factor-α, PMA, insulin, or insulin-like growth factor-1 (42, 51, 54–56, 59, 61). Importantly, Ser-307 has been found to be phosphorylated *in vivo* in insulin-resistant rodent models (20) and in human skeletal muscle (54). We have shown previously that Ser-307 phosphorylation blocks IR/IRS-1 binding in disruptive yeast tri-hybrid (Y3H) experiments (18). We have now used the disruptive Y3H method to identify an additional serine (Ser-302) in IRS-1 that is equally necessary for JNK-mediated disruption. We also show that Ser-302 and Ser-307 are phosphorylated in cells and *in vivo* under equivalent conditions of insulin resistance.

MATERIALS AND METHODS

Yeast Two-hybrid—Matchmaker LexA two-hybrid reagents were purchased from Clontech. *Saccharomyces cerevisiae* strain EGY48 (*Matα trp1*, *his3*, *ura3*, *6LexAop-LEU2*, *LYS2*), transformed with p8op-lacZ (kindly provided by T. A. Gustafson, Metabolex), was used as the host for interaction studies. EGY48/p8op-lacZ was sequentially transformed with plasmid constructs using polyethylene glycol/lithium acetate according to manufacturer's protocols. To determine protein-protein interactions as a function of leucine biosynthesis (LEU2), transformants were grown on synthetic dextrose agar plates for 3 days at 30 °C. Four independent colonies were streaked on synthetic dextrose glucose agar plates, grown overnight, replica-plated on synthetic galactose/raffinose agar plates, and regrown for 5 days at 30 °C to induce expression of B42 fusion proteins.

Disruptive Yeast Tri-hybrid—To create the disruptive yeast tri-hybrid assay, we modified the existing LexA yeast two-hybrid method. *S. cerevisiae* EGY48 cells were sequentially transformed as above with pLexA expressing human insulin receptor kinase (residues 940–1343) as bait, pB42AD expressing various human IRS-1 constructs as prey, and in most cases JNK1α1 in the third pDis plasmid. pDis was derived from the p426:Gal1 plasmid (ATCC) by replacing its multiple cloning region with another having a nuclear localization signal under Gal1 promoter control. In selected experiments alternative kinases were expressed in the pDis plasmid, including GSK3β(S9A), IKKα, IKKβ(S177E/S181E), p38 MAPK, PKA, PKCα, PKCβ2, PKCϵ, and PKCθ. Transformants were grown on the appropriate synthetic dextrose plates for 3 days at 30 °C. Four independent colonies were streaked on synthetic dextrose plates, incubated overnight, and replica-plated on GR plates. The plates were immediately replica-cleaned, incubated overnight, replica-cleaned, and incubated at 30 °C for 5 days to induce expression of pDis and B42 fusion proteins.

Cell Culture—Fao hepatoma cells were maintained in RPMI medium containing 25 mM glucose and 10% heat-inactivated fetal bovine serum (Sigma) under 5% CO_2. Before experiments, Fao cells were serum-starved for 16 h in RPMI containing 0.1% bovine serum albumin. After treatment, cells were washed (phosphate-buffered saline containing 1.0 mM phenylmethylsulfonyl fluoride, 3.0 μM aprotinin, 10 μM leupeptin, 5.0 μM pepstatin A, 25 mM benzamidine, 25 mM sodium vanadate, 5.0 mM glycerol phosphate, 100 mM NaF, 1.0 mM ammonium molybdate, 30 mM tetrasodium pyrophosphate, 5 mM EGTA) and lysed (in 30 mM HEPES, 150 mM NaCl, 1.0 mM phenylmethylsulfonyl fluoride, 3.0 μM aprotinin, 10 μM leupeptin, 5.0 μM pepstatin A, 25 mM benzamidine, 25 mM sodium vanadate, 5.0 mM glycerol phosphate, 100 mM NaF, 1.0 mM ammonium molybdate, 30 mM tetrasodium pyrophosphate, 5.0 mM EGTA, 10% glycerol, 1% Triton X-100, and 0.5% sodium deoxycholate, pH 7.4) for immunoprecipitation and Western-blotting experiments. CHO-IR cells were maintained in F-12 medium supplemented with 10% fetal bovine serum in the presence of 0.4 mg/ml G418 and 2 mM glutamine in 5% CO_2. Cells at 50–60% confluence were transfected using FuGENE 6 (Roche Applied Science) with pCMV(WT IRS-1), pCMV(IRS-1 S307A), pCMV(IRS-1 S312A), or pCMV(IRS-1 S307A/S3012A). Cells were incubated for 24 h, serum-starved overnight, treated for 30 min with 20 μM anisomycin or 20 nM calyculin A (Bio-Mol) and for 5 min with 1 nM insulin and lysed as describe above.

In Vivo Animal Studies—Fourteen week-old *ob/ob* ($Lep^{ob/ob}$) mice and congenic $Lep^{+/+}$ controls were sacrificed after an overnight fast. For the "diet-induced obesity" study, 8 week-old C57BL/6 (Jackson Laboratories) mice were fed a high fat diet (Research Diet D12451, 45% of calories from fat) for 8 weeks; controls were fed regular chow that derives 17% of calories from fat. After an overnight fast the 16-week-old mice were sacrificed. To determine the effects of acute, high dose insulin, chow-fed 12-week-old C57BL/6 mice were fasted overnight, injected intraperitoneally with 1 units/kg of insulin, and sacrificed after 10 min. Harvested livers, stored until use in liquid N_2, were pulverized and homogenized with a Polytron for 30 s in lysis buffer. Cleared lysates were used for immunoprecipitations and Western blotting.

Antibody Preparation and Use—Phosphospecific antibodies against IRS-1 Ser(P)-302 were generated in rabbits. Phosphopeptide RRSRTEpSITATSP (p indicates phosphorylated serine) was coupled to keyhole limpet hemocyanin for use as antigen. Rabbit antisera were passed first over immobilized RRSRTESITATSP to remove antibodies that recognized the unphosphorylated sequence. Phosphospecific antibodies were affinity-isolated by passing the precleared sera over immobilized RRSRTEpSITATSP followed by a low pH elution (62). Phosphospecific IRS-1 Ser(P)-307 antibody was either purchased from Cell Signaling or kindly provided by Morris White (Joslin Diabetes Center), anti-insulin receptor and IRS-1 antibodies were described previously (42, 43), anti-phosphotyrosine (4G10) antibody was from Upstate Biotechnology Inc., and horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Amersham Biosciences) was used for Western blotting.

Proteins were immunoprecipitated from cell or tissue lysates for 2 h at 4 °C using immobilized anti-IRS-1 and anti-IR antibodies (protein A-Sepharose beads, Amersham Biosciences). After extensive washing, proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). Blots were probed with the indicated antibodies, and proteins were detected by chemiluminescence (Pierce). For transfection experiments, FLAG-tagged IRS-1 was immunoprecipitated using anti-FLAG antibody (Stratagene).

RESULTS

Identification of Ser-302 Is an Inhibitory Phosphorylation Site in IRS-1—Gustafson (63) first used a conventional LexA yeast two-hybrid (Y2H) assay to identify direct interactions between IR and IRS-1. We modified this assay to develop a method to identify proteins that block IR-IRS interactions and potential molecular mechanisms for disruption. In our "disruptive yeast tri-hybrid" (Y3H) assay, bait and prey are expressed

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[2] Numbering of IRS-1 in this manuscript refers to rodent (r) sequences. Human (h) sequences are identical in the Ser-302/Ser-307 region, although the numbering is offset by 5 residues (rSer-307 = hSer-312; rSer-302 = hSer-307). hIRS-1, ^{295}SQVGLTRRSRTES^{307}ITATS^{312}PASMVGGKPGSFRVRAS^{334}; rIRS-1, ^{290}SQVGLTRRSRTES^{302}ITATS^{307}PASMVGGKPGSFRVRAS^{329}.

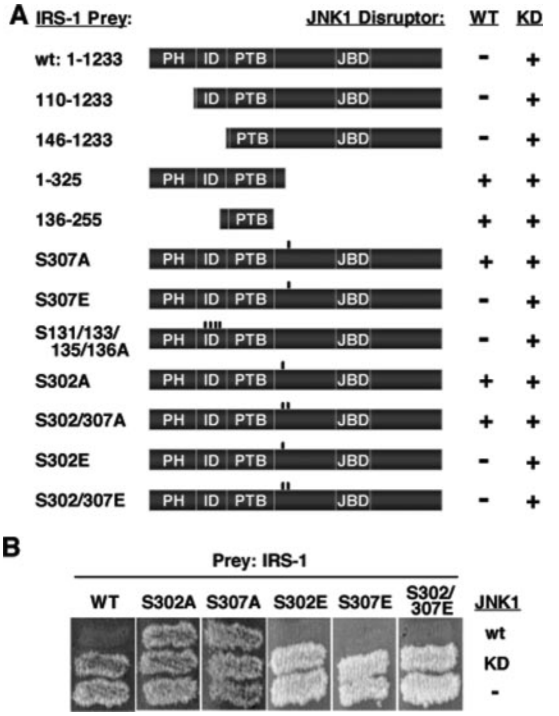

FIG. 1. **Disruptive Y3H identifies Ser-302 as a new inhibitory phosphorylation site for blocking IR/IRS-1 interactions.** Insulin receptor kinase (940–1343) was used as bait, the indicated wild-type or mutated or truncated versions of IRS-1 were used as prey, and either WT or kinase-deficient (*KD*) JNK1 was expressed from the pDis disruptive plasmid. *A*, interactions of the insulin receptor and IRS-1 were determined by growth of transformants on medium lacking leucine. Plus signs indicate an interaction (growth), whereas minus signs denote disruption (no growth). *B*, images are of yeast growing on selective media. Insulin receptor kinase is bait, the indicated IRS-1 proteins are prey, and either no kinase (−) or WT or kinase dead JNK1 were expressed from pDis.

as usual using pLexA and pB42AD plasmids, respectively, but we removed p8op-lacZ and replaced it with a distinct third plasmid (termed pDis or pDisruptor) for the expression of proteins that might modulate protein-protein interactions.

IR and IRS-1 bind one another under Y2H conditions and in the disruptive Y3H assay with an empty disruptive (pDis) plasmid (Fig. 1*B*). When JNK1 was expressed in the third plasmid the interaction was disrupted (Fig. 1, *A* and *B*) (18). Kinase activity was required for disruption, as kinase deficient (KD) JNK1 having T183A/Y185F substitutions within its kinase activation loop did not disrupt IR/IRS-1 binding. Binding of IR to IRS-1 was retained after removal of the IRS-1 PH domain (110–1242) or the PH domain plus interdomain (150–1242), and phosphorylation by JNK1 disrupted these interactions (Fig. 1*A*). These data are consistent with the suggestion that phosphorylation of Ser-307, a site C-terminal to the PTB domain, is involved in JNK1-mediated disruption (17, 18). Also consistent with these previous findings, a region in the C terminus of IRS-1 is required for JNK1-mediated disruption. In the disruptive Y3H assay shorter segments of IRS-1 such as one encompassing its PH and PTB domains (1–330) or its PTB domain alone (140–260) bound IR, but binding was not disrupted by JNK1 in either of these cases (Fig. 1*A*). Aguirre and White (17, 18) showed that JNK1 disruption requires binding at a C-terminal JNK binding domain (*JBD*) (residues 857–863) in addition to phosphorylation at Ser-307.

We next substituted Ser-307 with glutamic acid instead of alanine, as glutamate has been found to mimic phosphoserine in other proteins. Unlike S307A-substituted IRS-1, which was resistant to JNK1-mediated disruption, S307E-substituted IRS-1 behaved like the wild-type protein. IRS-1 S307E interacted with IR in the absence of disruptive protein, and binding was blocked by JNK1 phosphorylation. Because glutamate cannot be phosphorylated yet acts as though it were, these findings demonstrated that glutamate is indeed a phosphorylation mimic in this system. Much more importantly, however, these findings indicated that although Ser-307 phosphorylation is necessary for disruption, it is not sufficient. If phosphorylation at Ser-307 were sufficient, then binding would have been disrupted in the absence of kinase or the presence of catalytically inactive kinase, which clearly wasn't the case (Fig. 1, *A* and *B*). These findings prompted a search for additional phosphorylation site(s) potentially involved in JNK1 disruption.

We looked first at the interdomain region between the PH and PTB domains because it contains a discrete number of serines. We reasoned that phosphorylation(s) within the Ser-135, Ser-137, Ser-139, and Ser-140 cluster might inhibit IR/IRS-1 interactions by interacting with the PH or PTB domain. Each serine was substituted with alanine, independently and together. The substituted proteins behaved exactly like wild-type IRS-1 under Y3H conditions (Fig. 1*A*), clearly indicating that these sites are not involved in JNK1-mediated inhibition. We next hypothesized that the site(s) neighboring Ser-307 might be phosphorylated, and since Ser-302 is closest, this was mutated. The S302A substitution rendered IRS-1 resistant to JNK-1 inhibition (Fig. 1, *A* and *B*), exactly as had been seen previously with Ser-307. Perhaps not surprisingly, given that both of the singly substituted S302A or S307A proteins were resistant to JNK1-mediated inhibition, doubly substituted IRS-1 S302/307A was similarly resistant.

We proceeded to ask whether glutamate mimicked phosphoserine in this case as well. Analogous to the situation for IRS-1 S307E, the S302E-substituted protein behaved like wild-type IRS-1; it interacted with IR in the absence of disruptive protein, and binding was blocked by JNK1 phosphorylation (Fig. 1, *A* and *B*). These findings indicated that phosphorylation at both Ser-302 and Ser-307 is necessary for disruption by JNK1 but that phosphorylation at either site alone is insufficient. Doubly substituted IRS-1 S302E/S307E tested whether the two sites together are sufficient for disruption. If phosphorylation at Ser-302 and Ser-307 were sufficient, then binding would have been disrupted in the absence of kinase or the presence of catalytically inactive kinase. This was not the case (Fig. 1, *A* and *B*), suggesting that in addition to Ser-302 and Ser-307, JNK1-mediated disruption of IR/IRS-1 binding requires phosphorylation at additional site(s). We concluded that disruption by JNK1 requires a cluster of three or more phosphorylated serine residues. Preventing phosphorylation at one or both sites (S302A, S307A, or S302/307A) renders IRS-1 resistant to JNK1-mediated inhibition.

Selectivity of the Ser-302 or Ser-307 Sites for JNK1-mediated Disruption—We have subcloned several additional kinases into pDis to ask (*a*) whether they disrupt IR/IRS-1 binding and (*b*), if they disrupt, whether Ser-302 or Ser-307 is involved. We examined the effects of GSK3β, IKKα, IKKβ, p38 MAPK, PKA, PKCα, PKCβI, PKCβ2, PKCϵ, and PKCθ, as each of these kinases has been proposed to be a potential mediator of insulin resistance (15, 17, 18, 20, 28–30, 34–42, 59). IKKα and IKKβ do not block IR/IRS-1 binding in the disruptive Y3H assay. Negative findings with the IKKs have been consistent in our hands under a variety of cellular and biochemical conditions (data not shown) (15), despite a claim to the contrary (59). By contrast, GSK3β, p38 MAPK, PKA, PKCα, PKCβI, PKCβ2, PKCϵ, and PKCθ all disrupt IR/IRS-1 binding like JNK1 (Table I). The substituted forms of IRS-1, S302A, S307A, or S302/307A, were used to determine whether these sites were in-

TABLE I

Disruptive Y3H assays with different kinases

IR/IRS-1 interactions are denoted by plus signs, and disrupted interactions are noted by minus signs. ND, not determined.

Even with active kinase	IRS-1:				
	WT Active	WT Dead	S302A Active	S307A Active	S302A/S307A Active
JNK1	−	+	+	+	+
GSK3β[a]	−	+	−	−	−
IKKα	+	ND	+	+	+
IKKβ[b]	+	+	+	+	+
p38 MAPK	−	+	−	−	−
PKA	−	ND	−	−	−
PKCα	−	ND	−	−	−
PKCβ1	−	ND	−	−	−
PKCβ2	−	ND	−	−	−
PKCϵ	−	ND	−	−	−
PKCθ	−	+	−	−	−

[a] GSK3β contains an activating S9A substitution.
[b] IKKβ contains activating S177E/S181E substitutions.

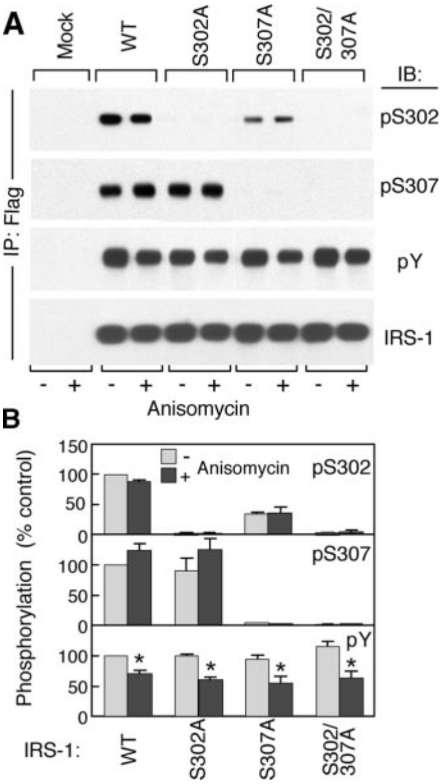

FIG. 2. **Antibody specificity and insulin/anisomycin-induced phosphorylation.** *A*, CHO-IR cells expressed FLAG-tagged forms of full-length IRS-1: WT, S307A, S312A, or S307A/S312A. The cells were treated sequentially with or without anisomycin (20 μM, 30 min) and insulin (all cells, 1 nM, 5 min). Cells were lysed, and IRS-1 proteins were immunoprecipitated (*IP*) with FLAG antibody and immunoblotted (*IB*) with anti-Ser(P)-302 (*pS302*), anti-Ser(P)-307 (*pS307*), anti-Tyr(P) (*pY*), or anti-IRS-1 antibodies. *B*, combined results for three separate experiments (mean ± S.E.; *, $p < 0.05$) were plotted relative to WT IRS-1 (no anisomycin, + insulin).

volved in disruption by each of the kinases. Each substitution was tested with every kinase, and each failed to rescue the interaction, with the exception of JNK1 as the kinase (Table I). These findings demonstrate that whereas Ser-302 and Ser-307 are involved in disruption by JNK1, neither site, alone or in combination, is involved in disruption by GSK3β, p38 MAPK, PKA, PKCα, PKCβI, PKCβ2, PKCϵ, and PKCθ.

Ser-302 Phosphorylation in Cultured Cells—Phosphospecific antibodies were prepared to determine whether Ser-302 is phosphorylated in cultured cells or *in vivo*. The antibodies were prepared against a phosphopeptide sequence corresponding to the region of IRS-1 surrounding Ser-302 (RRSRTEpSITATSP) that is identical in rats (Ser-302), mice (Ser-302), and humans (Ser-307) (45–47). The affinity-purified antibodies were used for Western blotting experiments. Chinese hamster ovary cells transfected with WT or mutated IRS-1 constructs were stimulated with insulin and anisomycin to test antibody efficacy and specificity. The FLAG-tagged IRS-1 proteins were immunoprecipitated with anti-FLAG antibodies and blotted with our new Ser(P)-302 antibodies or antibodies directed against p307, Tyr(P), or IRS-1 (Fig. 2). The Ser(P)-302 antibodies recognized WT IRS-1, indicating that IRS-1 is phosphorylated at Ser-302 in insulin-stimulated cells. The WT protein was recognized as well by anti-Ser(P)-307, anti-Tyr(P), and anti-IRS-1 antibodies. Expression of mutated forms of IRS-1 tested specificity. The anti-Ser(P)-302 antibodies did not recognize IRS-1 S302A, but anti-Ser(P)-307, anti-Tyr(P), and anti-IRS-1 antibodies did recognize IRS-1 S302A. In contrast, IRS-1 S307A was recognized by anti-Ser(P)-302(IRS-1) but not anti-Ser(P)-307(IRS-1) antibodies (Fig. 2). These findings verify that the two antibodies are equivalently specific for the sites they were raised against.

Interestingly, under the conditions used in this assay phosphorylation of IRS-1 S307A was reduced at Ser-302. Under more intensive phosphorylation conditions, for example in cells treated simultaneously with insulin and calyculin A, which promote Ser/Thr phosphorylation and inhibit Ser/Thr dephosphorylation, respectively, there were equivalent amounts of phosphorylation at Ser-302 in WT IRS-1 and IRS-1 S307A. These findings suggest that Ser-307 phosphorylation might be required for efficient Ser-302 phosphorylation. Ordered phosphorylation or "priming" mechanisms are well established in the kinase field but have not been described previously for IRS-1.

Anisomycin, an activator of stress kinases including JNK1, had little or no effect in stimulating phosphorylation further than insulin at either Ser-302 or Ser-307 (Fig. 2). Nevertheless, anisomycin led to a ~30% reduction of WT IRS-1 tyrosine phosphorylation. Anisomycin similarly decreased the tyrosine phosphorylation of singly substituted IRS-1 S302A and IRS-1 S307A and doubly substituted IRS-1 S302/307A by 30–50%. These findings suggested that anisomycin-mediated inhibition involves phosphorylation sites in addition to Ser-302 and Ser-307 and probably kinases other than JNK1, as JNK1-mediated inhibition of IR/IRS-1 binding was reversed by these identical mutations in disruptive Y3H assays (Fig. 1).

Calyculin A is an inhibitor of Ser/Thr phosphatases, which increases Ser/Thr phosphorylation in cells through direct mechanisms as well as through the activation of numerous kinases. Treatment of cells with calyculin A induces a large shift in the electrophoretic mobility of IRS-1, apparently due to the phosphorylation of many Ser/Thr residues (Fig. 3*A*) (15). Calyculin A stimulated phosphorylation at both Ser-302 and Ser-307, as evidenced by increased intensity of the bands recognized by the sequence-specific anti-Ser(P)-302(IRS-1) and anti-Ser(P)-307(IRS-1) antibodies. The S302A and S307A substitutions abrogated recognition by the anti- Ser(P)-302(IRS-1) and anti-Ser(P)-307(IRS-1) antibodies, respectively, although the substitutions either alone or in combination had no appreciable effect on the magnitude of the shift in electrophoretic mobility. These results demonstrate that although phosphorylation of Ser-302 and Ser-307 is promoted by calyculin A, many additional Ser/Thr sites in IRS-1 are similarly affected.

Cells were subsequently treated with insulin to assess the effects of calyculin A on insulin-stimulated IRS-1 Tyr-phosphorylation (Fig. 3*B*). Not surprisingly, given the large number of Ser/Thr phosphorylations involved, calyculin A effectively abolished Tyr phosphorylation of WT IRS-1. Neither S302A nor

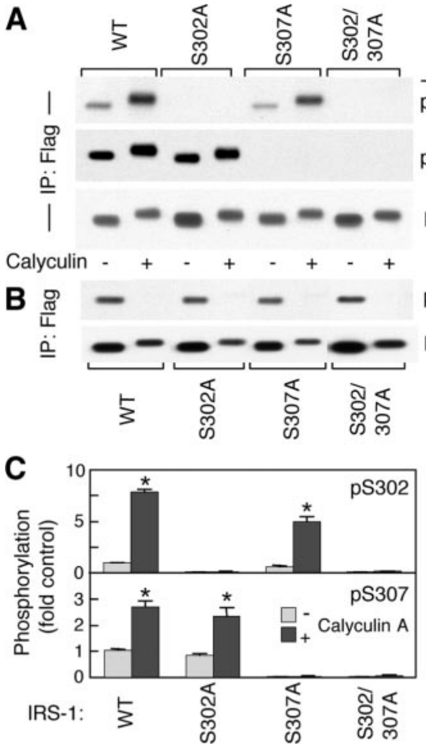

FIG. 3. **Calyculin A-induced phosphorylation.** CHO-IR cells expressing FLAG-tagged forms of full-length IRS-1 (WT, S307A, S312A, or S307A/S312A) were treated with calyculin A (20 nM, 30 min) alone (*A*) or sequentially with calyculin A (20 nM, 30 min) and insulin (1 nM, 5 min) (*B*). Cells were lysed, and IRS-1 proteins were immunoprecipitated (*IP*) with FLAG antibody and immunoblotted (*IB*) with anti-Ser(P)-302(IRS-1) (*pS302*), anti-Ser(P)-307(IRS-1) (*pS307*), anti-Tyr(P) (*pY*), or anti-IRS-1 antibodies. *C*, combined results for two separate experiments (mean ± S.E.; *, $p < 0.05$) were plotted relative to WT IRS-1 (no calyculin A).

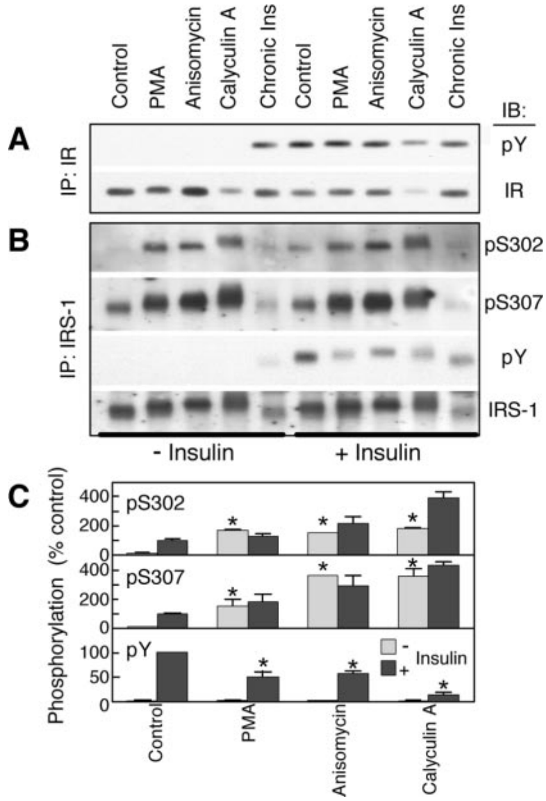

FIG. 4. **Conditions that affect Ser-302 and Ser-307 phosphorylation of endogenous IRS-1.** Fao hepatoma cells were pretreated with 10 μM PMA (30 min), 20 μM anisomycin (30 min), 20 nM calyculin A (30 min), or 10 nM insulin (16 h) and after these reagents were washed off and stimulated acutely with 10 nM insulin (5 min). Proteins were immunoprecipitated (*IP*) from cell lysates with anti-insulin receptor antibody (*A*) or anti-IRS-1 antibody (*B*) and immunoblotted (*IB*) as indicated with anti-Tyr(P) (*pY*), anti-IR, anti-Ser(P)-302(IRS-1) (*pS302*), anti-Ser(P)-307(IRS-1) (*pS307*), or anti-IRS-1 antibodies. *C*, combined results for four separate experiments (mean ± S.E.) were plotted relative to control IRS-1 (+ acute insulin, no other addition). Ser(P)-302 and Ser(P)-307: *, $p < 0.05$ *versus* control IRS-1 (minus acute insulin, no other addition); *pY*, (*Tyr(P)*): **, $p < 0.05$ *versus* control IRS-1 (plus acute insulin, no other addition).

S307A alone or in combination noticeably rescued insulin-stimulated Tyr phosphorylation. The findings demonstrate that calyculin A promotes inhibitory phosphorylations at a Ser/Thr site or sites in addition to IRS-1 Ser-302 and Ser-307.

Ser-302 Phosphorylation of Endogenous IRS-1 in Cultured Cells—Having established the specificity of the antibodies, we went on to ask whether endogenous IRS-1 in cultured cells was phosphorylated at Ser-302 under conditions known to promote Ser/Thr phosphorylation (33–37). Fao (rat) hepatoma cells were pretreated with PMA (a phorbol ester that activates PKC isoforms), anisomycin, calyculin A (an inhibitor of Ser/Thr phosphatases), or insulin (overnight). The reagents were removed, and cells were stimulated acutely with insulin. PMA and calyculin A induced Ser/Thr phosphorylation of the insulin receptor, as judged by small shifts in electrophoretic mobility (Fig. 4*A*). All four conditions produced shifts in the electrophoretic mobility of IRS-1, consistent with even greater increases in Ser/Thr phosphorylation (Fig. 4*B*). Insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased after each treatment (Fig. 4, *B* and *C*), consistent with Ser/Thr phosphorylation inhibiting tyrosine phosphorylation and downstream signaling. Calyculin A produced the largest shift in electrophoretic mobility of IRS-1, presumably due to the greatest increase in Ser/Thr phosphorylation, and essentially abolished tyrosine phosphorylation of IRS-1 (Fig. 4*C*).

Blots with the phosphospecific antibodies showed that each of these conditions promoted phosphorylation at both Ser-302 and Ser-307. Levels of Ser/Thr phosphorylation promoted by PMA, anisomycin, or calyculin A were similar in the absence or presence of insulin stimulation. Insulin itself led to small but definite increases as well. These results indicated that phosphorylation at Ser-302 and Ser-307 occurs in parallel under a wide range of conditions.

Ser-302 Phosphorylation in Genetically Obese Mice—Following the goals of the laboratory to identify *in vivo* mechanisms of insulin resistance, we next asked whether Ser-302 was phosphorylated in animal models of insulin resistance. Livers were isolated from *ob*/*ob* ($Lep^{ob/ob}$) mice, a genetic model in which a defect in leptin structure leads to loss of function. $Lep^{ob/ob}$ mice are hyperphagic, profoundly obese, insulin-resistant, and hyperglycemic. Immunoprecipitated IRS-1 was analyzed by Western blotting with Ser(P)-302 and Ser(P)-307 antibodies. Both of the antibodies consistently identified increased IRS-1 Ser phosphorylation in $Lep^{ob/ob}$ mice relative to congenic $Lep^{+/+}$ (C57Bl/6) controls (Fig. 5). Levels of Ser(P)-302 were 5.80-fold (±1.50) greater in $Lep^{ob/ob}$ mice relative to $Lep^{+/+}$ controls (±0.41, $p = 0.036$); comparable levels of Ser(P)-307 were 3.06-fold (±0.47) greater (±0.31, $p = 0.022$). Similar increases suggest that in $Lep^{ob/ob}$ mice, phosphorylation at these two sites occurs in parallel.

Ser-302 Phosphorylation in Diet-induced Obesity—Diet-induced obesity offers a second model for insulin resistance in mice. Eight-week-old C57Bl/6 mice were fed either a high fat diet (45% of calories from fat) or control chow (17% of calories from fat) for 8 weeks. Consistent with appropriate dietary

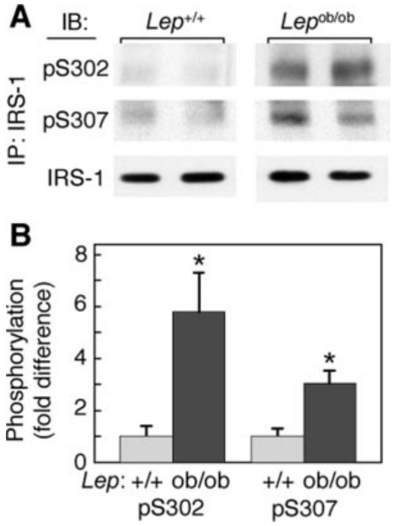

FIG. 5. **Obesity promotes Ser-302 and Ser-307 phosphorylation.** $Lep^{ob/ob}$ mice and control $Lep^{+/+}$ mice were fasted overnight and sacrificed ($n = 4$ of each). Proteins were immunoprecipitated (*IP*) from liver lysates with anti-IRS-1 antibody, and separated proteins were immunoblotted (*IB*) with anti-Ser(P)-302 (*pS302*), anti-Ser(P)-307 (*pS302*), or anti-IRS-1 antibodies. *B*, combined results for three separate experiments (mean ± S.E.; *, $p < 0.04$) were plotted relative to $Lep^{+/+}$ controls.

responses, mice fed the high fat diet weighed 17% more than controls (39.8 *versus* 34.0 g; $p = 0.016$), due to >250% increases in fat mass as assessed by Dexa scanning. Fasting glucose (5.33 ± 0.23 mM *versus* 4.65 ± 0.29 mM, $p = 0.034$) and insulin levels (148.0 ± 10.3 microunits/ml *versus* 67.1 ± 12.2 microunits /ml, $p = 0.003$) were used for HOMA-IR (homeostatic model assessment) calculations, which demonstrated significantly increased insulin resistance in mice fed the high fat diet (34.8 ± 1.5) relative to chow-fed controls (14.05 ± 3.04; $p = 0.003$). Under these conditions HOMA-IR correlates well with more complex measurements, including sensitivity indices (S_I) determined using the hyperinsulinemic-euglycemic clamp method (64, 65). IRS-1 isolated from the livers of these mice was analyzed for Ser-302 and Ser-307 phosphorylation. Both of the phosphospecific antibodies (Ser(P)-302 and Ser(P)-307) identified consistent increases in serine phosphorylation of IRS-1 from mice fed the high fat diet compared with control chow (Fig. 6). High fat diet increased Ser(P)-302 by 3.3-fold (±0.47) relative to chow controls (±0.001, $p = 0.035$) and similarly increased Ser(P)-307 by 2.2-fold (±0.19) relative to chow controls (±0.01, $p = 0.01$).

Ser-302 Phosphorylation in Hyperinsulinemia—Hyperinsulinemia represents a third model in which Ser/Thr phosphorylation might mediate insulin resistance. To test whether Ser-302 and Ser-307 are phosphorylated during acute hyperinsulinemia, chow-fed C57Bl/6 mice were injected intraperitoneally with a single, large 1.0-unit/kg dose of insulin. Tissues were harvested within 10 min before the development of severe hypoglycemia, and liver IRS-1 was analyzed for Ser-302 and Ser-307 phosphorylation. Acute hyperinsulinemia increased Ser(P)-302 by 2.3-fold (±0.47) relative to saline-injected controls (±0.19, $p = 0.04$). This maneuver similarly increased Ser(P)-307 by 1.8-fold (±0.31) relative to saline-injected controls (±0.03, $p = 0.09$) (Fig. 7). We conclude that *in vivo* Ser/Thr phosphorylation is promoted at both sites by acute hyperinsulinemia. We were, thus, able to see consistent increases in Ser-302 and Ser-307 phosphorylation in three diverse models of murine insulin resistance, obesity, high fat diet, and hyperinsulinemia, suggesting that Ser-302 and Ser-307 might play important roles in related human conditions.

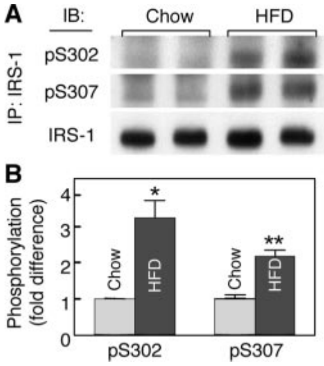

FIG. 6. **High fat diet promotes Ser-302 and Ser-307 phosphorylation.** *A*, C57BL/6 mice fed normal chow ($n = 4$) or high fat diet (*HFD*) ($n = 6$) were fasted overnight and sacrificed. Proteins were immunoprecipitated (*IP*) from liver lysates with anti-IRS-1 antibody, and separated proteins were immunoblotted (*IB*) with anti-Ser(P)-302 (*pS302*), anti-Ser(P)-307 (*pS307*), or anti-IRS-1 antibodies. *B*, combined results for two separate experiments (mean ± S.E.; *, $p < 0.04$; **, $p < 0.02$) were plotted relative to chow-fed controls.

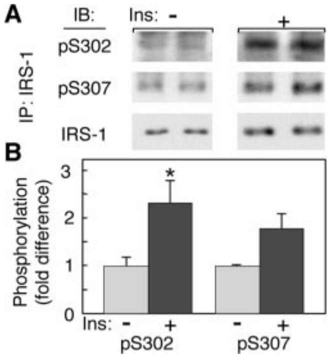

FIG. 7. **Hyperinsulinemia promotes Ser-302 and Ser-307 phosphorylation.** *A*, fasting C57BL/6 mice ($n = 4$, each group) were injected intraperitoneally with insulin (*Ins*, 1 units/kg) or carrier, and after 10 min, the mice were sacrificed, and tissues were harvested. Proteins were immunoprecipitated (*IP*) from liver lysates with anti-IRS-1 antibody, and separated proteins were immunoblotted (*IB*) with anti-Ser(P)-302 (*pS302*), anti-Ser(P)-307 (*pS307*), or anti-IRS-1 antibodies. *B*, combined results for four separate experiments (mean ± S.E.; *, $p < 0.05$) were plotted relative to controls.

DISCUSSION

Deletion of IRS-1 in mice causes insulin resistance and diabetes, consistent with a relevant role for this adapter protein in insulin signaling (66–69). These findings clearly demonstrate that alterations in IRS-1 amount can have serious consequences. Although controversial, the G972R polymorphism in the human *irs1* gene has been correlated with both insulin resistance and insulin secretory defects. Most relevant to this study, although patients with type 2 diabetes have near normal amounts of the protein, functional deficiencies in IRS-1 have been identified (70). In particular, tyrosine phosphorylation appears to be reduced (71–74). Ser/Thr phosphorylation has been increasingly recognized as a negative counterbalance to positive IRS signaling through tyrosine phosphorylation (23). Thus Ser/Thr phosphorylation could be a generalized mechanism for insulin resistance. From a molecular perspective it has been difficult, however, to identify discrete sites that are both phosphorylated *in vivo* and when phosphorylated, have relevant functional consequences.

Most investigations in this area have relied on the overexpression of candidate kinases in cultured cells and analyses of sites of Ser/Thr phosphorylation through protein sequencing or mass spectrometry. However, overexpression carries with it the potential for spurious phosphorylations outside of either usual sites or stoichiometries found physiologically. This could be due to greater than normal concentrations of the transfected candidate kinase or even more insidious in its ability to mislead, the transfected kinase may activate other kinases or inhibit phosphatases that are responsible for the detected phosphorylation. Virtually all of the candidate "insulin resistance" kinases that have been identified function in signaling cascades that involve other kinases and phosphatases. As an alternative approach that circumvents some of these problems, we developed the disruptive Y3H method.

Ser-307 has attracted significant attention as a targeted site for phosphorylation in cultured cells and *in vivo* (20, 42, 51, 54, 54–56, 59, 61). In disruptive Y3H experiments phosphorylation of Ser-307 blocks IR/IRS-1 binding (Fig. 1) (18), providing a consistent and coherent mechanism that spans the biological and biochemical findings. We have now used disruptive Y3H to identify a second site in IRS-1 that is phosphorylated in cells and *in vivo* under equivalent conditions of insulin resistance. Both of these sites were identified as JNK1 targets. Previous studies had shown that Ser-307 was a necessary JNK1-mediated disruption (18), and we now show that Ser-302 is similarly necessary. A host of other kinases disrupt IR/IRS-1 binding in Y3H assays (Table I), including GSK3β, p38 MAPK, PKA, PKCα, PKCβ2, PKCϵ, and PKCθ, yet their molecular mechanisms are distinct as disruption by these other kinases definitely does not involve either Ser-302 or Ser-307. Several kinases associated with inflammatory processes like JNK1 do not disrupt IR/IRS-1 interactions, including the NF-κB-activating kinases IKKα and IKKβ.

The strengths of our findings lie in showing unequivocally that JNK-mediated phosphorylation at either Ser-302 or Ser-307 (*a*) abrogates IR/IRS-1 binding and (*b*) that this occurs *in vivo* in three distinct animal models of insulin resistance; genetically obese $Lep^{ob/ob}$ mice, diet-induced obesity, and hyperinsulinemia. Our findings are consistent with a role for JNK1 in insulin resistance, as JNK1 phosphorylates Ser-302 and Ser-307 under Y3H conditions, and these sites are phosphorylated *in vivo* in models of insulin resistance. Nine other Ser/Thr kinases that are potentially associated with insulin resistance do not target Ser-302 and Ser-307 in Y3H experiments (Table I), making it highly unlikely that these kinases target Ser-302 and Ser-307 *in vivo*. Yet, with ~395 Ser/Thr kinases in the human and mouse genomes (75, 76), it should not be concluded that JNK1 alone is responsible for either the *in vivo* phosphorylation of Ser-302 or Ser-307 or insulin resistance.

During the review of our manuscript, two additional studies have implicated IRS-1 Ser-302 as a potential phosphorylation site. Greene *et al.* (77) used PKCδ to radioactively label IRS-1 *in vitro* and a phosphopeptide-mapping approach to identify phosphorylations at Ser-302, Ser-319, and Ser-570 (in their manuscript they used the equivalent human IRS-1 numbering: Ser-307, Ser-324, and Ser-575). Under the conditions of their assays, PKCδ did not appreciably phosphorylate Ser-307. Giraud *et al.* (78) used a phosphospecific antibody/candidate phosphorylation site approach to identify Ser-302. They reported that activation of mTOR signaling induced Ser-302 phosphorylation and that this positively influenced signaling through the IR/IRS-1 axis. The three studies identified Ser-302 using independent methods. Our data and the complementary findings of Greene *et al.* (77) clearly show that Ser-302 phosphorylation disrupts IR/IRS-1 signaling. Although we cannot speak to reasons that Giraud *et al.* (78) came to the opposite conclusion, this suggestion is clearly inconsistent with our findings. Phosphorylation at Ser-302 and Ser-307 is equivalently inhibitory in our studies, and it occurs at these two sites in parallel in yeast, in cultured cells, and *in vivo* under conditions of insulin resistance.

One of our more interesting and provocative findings relates to potential mechanisms of IR/IRS-1 disruption. Although we clearly show that JNK1 phosphorylates IRS-1 at Ser-302 and Ser-307 and both are necessary for disruption, these two phosphorylations either alone or in combination are not sufficient for disruption (Fig. 1). This means that phosphorylation of Ser-302 and Ser-307 and additional site or sites are required. In other words, a cluster of phosphorylated serines and possibly threonines mediates disruption. Although we continue to identify additional sites, the notion of "clustering" lends itself to considering new and testable hypotheses for potential mechanisms of disruption and, by extension, potential mechanisms for insulin resistance.

The disruptive Y3H approach provides a general method for analyzing macromolecular complexes in addition to identifying potential mechanisms of insulin resistance. Related methods have been published, including Vidal's forward and reverse Y*N*H approaches (79), but we were unable to apply these methods to the insulin resistance problem. In this study we specifically used our disruptive Y3H to investigate mechanisms for disruption of a protein-protein interaction. A similar Y3H approach is equally useful for studying the formation of ternary and higher order complexes. As one example, we have used a variant of the approach, termed "bridging" Y3H, to analyze quaternary complexes between JAKs and APS or SH2-B proteins (80).[3] These methods appear to be both robust and of general utility.

In summary, we have developed a new approach for disrupting protein-protein interactions and used the method to analyze mechanisms for inhibiting IR/IRS-1 interactions. We identified Ser-302 as a new phosphorylation site in IRS-1 and showed that Ser-302 phosphorylation blocks IR/IRS-1 binding. Disruptive Y3H was further used to investigate and identify clustering as a potential mechanism for JNK1 mediated disruption. Phosphospecific antibodies were developed and used to show that Ser-302 is phosphorylated in cells and *in vivo* in a variety of models of obesity, insulin resistance, and type 2 diabetes. These approaches should have general utility in analyzing potential mechanisms of insulin resistance by identifying other disruptive kinases and additional, functionally relevant phosphorylation sites. This should help to provide a more complete understanding of the molecular basis of insulin resistance.

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REFERENCES

1. National Diabetes Data Group (1994) *Diabetes in America*, 2nd Ed., NIDDK, National Institutes of Health, Bethesda, MD
2. Mokdad, A. H., Ford, E. S., Bowman, B. A., Nelson, D. E., Engelgau, M. M., Vinicor, F., and Marks, J. S. (2000) *Diabetes Care* **23,** 1278–1283
3. Mokdad, A. H., Serdula, M. K., Dietz, W. H., Bowman, B. A., Marks, J. S., and Koplan, J. P. (2000) *J. Am. Med. Assoc.* **284,** 1650–1651
4. Mokdad, A. H., Bowman, B. A., Ford, E. S., Vinicor, F., Marks, J. S., and Koplan, J. P. (2001) *J. Am. Med. Assoc.* **286,** 1195–1200
5. Mokdad, A. H., Ford, E. S., Bowman, B. A., Dietz, W. H., Vinicor, F., Bales, V. S., and Marks, J. S. (2003) *J. Am. Med. Assoc.* **289,** 76–79
6. Ford, E. S., Giles, W. H., and Dietz, W. H. (2002) *J. Am. Med. Assoc.* **287,** 356–359
7. Ridker, P. M., Buring, J. E., Cook, N. R., and Rifai, N. (2003) *Circulation* **107,** 391–397
8. Fried, S. K., Bunkin, D. A., and Greenberg, A. S. (1998) *J. Clin. Endocrinol. Metab.* **83,** 847–850
9. Pickup, J. C., Mattock, M. B., Chusney, G. D., and Burt, D. (1997) *Diabetologia* **40,** 1286–1292
10. Kern, P. A., Saghizadeh, M., Ong, J. M., Bosch, R. J., Deem, R., and Simsolo, R. B. (1995) *J. Clin. Investig.* **95,** 2111–2119
11. Hotamisligil, G. S. (2000) *Int. J. Obes. Relat. Metab. Disord.* **24,** Suppl. 4, 23–27

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[3] E. Werner and S. Shoelson, unpublished data.

12. Moller, D. E. (2000) *Trends Endocrinol. Metab.* **11,** 212–217
13. Pradhan, A. D., Manson, J. E., Rifai, N., Buring, J. E., and Ridker, P. M. (2001) *J. Am. Med. Assoc.* **286,** 327–334
14. Spranger, J., Kroke, A., Mohlig, M., Hoffmann, K., Bergmann, M. M., Ristow, M., Boeing, H., and Pfeiffer, A. F. (2003) *Diabetes* **52,** 812–817
15. Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z. W., Karin, M., and Shoelson, S. E. (2001) *Science* **293,** 1673–1677
16. Kim, J. K., Kim, Y. J., Fillmore, J. J., Chen, Y., Moore, I., Lee, J., Yuan, M., Li, Z. W., Karin, M., Perret, P., Shoelson, S. E., and Shulman, G. I. (2001) *J. Clin. Investig.* **108,** 437–446
17. Aguirre, V., Uchida, T., Yenush, L., Davis, R., and White, M. F. (2000) *J. Biol. Chem.* **275,** 9047–9054
18. Aguirre, V., Werner, E. D., Giraud, J., Lee, Y. H., Shoelson, S. E., and White, M. F. (2002) *J. Biol. Chem.* **277,** 1531–1537
19. Hotamisligil, G. S., Budavari, A., Murray, D., and Spiegelman, B. M. (1994) *J. Clin. Investig.* **94,** 1543–1549
20. Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M., and Hotamisligil, G. S. (2002) *Nature* **420,** 333–336
21. Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P., and Lewis, R. E. (1988) *J. Biol. Chem.* **263,** 11017–11020
22. Saltiel, A. R. (1994) *FASEB J.* **8,** 1034–1040
23. Zick, Y. (2001) *Trends Cell Biol.* **11,** 437–441
24. Mothe, I., and Van Obberghen, E. (1996) *J. Biol. Chem.* **271,** 11222–11227
25. Tanti, J. F., Gremeaux, T., Van Obberghen, E., and Marchand-Brustel, Y. (1994) *J. Biol. Chem.* **269,** 6051–6057
26. Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R., and Karasik, A. (1995) *J. Biol. Chem.* **270,** 23780–23784
27. Paz, K., Hemi, R., LeRoith, D., Karasik, A., Elhanany, E., Kanety, H., and Zick, Y. (1997) *J. Biol. Chem.* **272,** 29911–29918
28. Stadtmauer, L., and Rosen, O. M. (1986) *J. Biol. Chem.* **261,** 3402–3407
29. Miele, C., Formisano, P., Sohn, K. J., Caruso, M., Pianese, M., Palumbo, G., Beguino, L., and Beguinot, F. (1995) *J. Biol. Chem.* **270,** 15844–15852
30. Roth, R. A., and Beaudoin, J. (1987) *Diabetes* **36,** 123–126
31. Paz, K., Liu, Y. F., Shorer, H., Hemi, R., LeRoith, D., Quan, M., Kanety, H., Seger, R., and Zick, Y. (1999) *J. Biol. Chem.* **274,** 28816–28822
32. Li, J., DeFea, K., and Roth, R. A. (1999) *J. Biol. Chem.* **274,** 9351–9356
33. Jakobsen, S. N., Hardie, D. G., Morrice, N., and Tornqvist, H. E. (2001) *J. Biol. Chem.* **276,** 46912–46916
34. Liu, Y. F., Paz, K., Herschkovitz, A., Alt, A., Tennenbaum, T., Sampson, S. R., Ohba, M., Kuroki, T., LeRoith, D., and Zick, Y. (2001) *J. Biol. Chem.* **276,** 14459–14465
35. Takayama, S., White, M. F., and Kahn, C. R. (1988) *J. Biol. Chem.* **263,** 3440–3447
36. Ravichandran, L. V., Esposito, D. L., Chen, J., and Quon, M. J. (2001) *J. Biol. Chem.* **276,** 3543–3549
37. De Fea, K., and Roth, R. A. (1997) *Biochemistry* **36,** 12939–12947
38. Griffin, M. E., Marcucci, M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., Goodyear, L. J., Kraegen, E. W., White, M. F., and Shulman, G. I. (1999) *Diabetes* **48,** 1270–1274
39. De Fea, K., and Roth, R. A. (1997) *J. Biol. Chem.* **272,** 31400–31406
40. Eldar-Finkelman, H., and Krebs, E. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9660–9664
41. Greene, M. W., and Garofalo, R. S. (2002) *Biochemistry* **41,** 7082–7091
42. Lee, Y. H., Giraud, J., Davis, R. J., and White, M. F. (2003) *J. Biol. Chem.* **278,** 2896–2902
43. Hartman, M. E., Villela-Bach, M., Chen, J., and Freund, G. G. (2001) *Biochem. Biophys. Res. Commun.* **280,** 776–781
44. Ozes, O. N., Akca, H., Mayo, L. D., Gustin, J. A., Maehama, T., Dixon, J. E., and Donner, D. B. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4640–4645
45. Lam, K., Carpenter, C. L., Ruderman, N. B., Friel, J. C., and Kelly, K. L. (1994) *J. Biol. Chem.* **269,** 20648–20652
46. Pirola, L., Bonnafous, S., Johnston, A. M., Chaussade, C., Portis, F., and Van Obberghen, E. (2003) *J. Biol. Chem.* **278,** 15641–15651
47. Lee, A. V., Gooch, J. L., Oesterreich, S., Guler, R. L., and Yee, D. (2000) *Mol. Cell. Biol.* **20,** 1489–1496
48. Hemi, R., Paz, K., Wertheim, N., Karasik, A., Zick, Y., and Kanety, H. (2002) *J. Biol. Chem.* **277,** 8961–8969
49. Hartley, D., and Cooper, G. M. (2002) *J. Cell. Biochem.* **85,** 304–314
50. Freund, G. G., Wittig, J. G., and Mooney, R. A. (1995) *Biochem. Biophys. Res. Commun.* **206,** 272–278
51. Greene, M. W., Sakaue, H., Wang, L., Alessi, D. R., and Roth, R. A. (2003) *J. Biol. Chem.* **278,** 8199–8211
52. Begum, N., Sandu, O. A., Ito, M., Lohmann, S. M., and Smolenski, A. (2002) *J. Biol. Chem.* **277,** 6214–6222
53. Horike, N., Takemori, H., Katoh, Y., Doi, J., Min, L., Asano, T., Sun, X. J., Yamamoto, H., Kasayama, S., Muraoka, M., Nonaka, Y., and Okamoto, M. (2003) *J. Biol. Chem.* **278,** 18440–18447
54. Rui, L., Aguirre, V., Kim, J. K., Shulman, G. I., Lee, A., Corbould, A., Dunaif, A., and White, M. F. (2001) *J. Clin. Investig.* **107,** 181–189
55. Jiang, G., Dallas-Yang, Q., Liu, F., Moller, D. E., and Zhang, B. B. (2003) *J. Biol. Chem.* **278,** 180–186
56. Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J. K., Cushman, S. W., Cooney, G. J., Atcheson, B., White, M. F., Kraegen, E. W., and Shulman, G. I. (2002) *J. Biol. Chem.* **277,** 50230–50236
57. Delahaye, L., Mothe-Satney, I., Myers, M. G., White, M. F., and Van Obberghen, E. (1998) *Endocrinology* **139,** 4911–4919
58. Qiao, L. Y., Zhande, R., Jetton, T. L., Zhou, G., and Sun, X. J. (2002) *J. Biol. Chem.* **277,** 26530–26539
59. Gao, Z., Hwang, D., Bataille, F., Lefevre, M., York, D., Quon, M. J., and Ye, J. (2002) *J. Biol. Chem.* **277,** 48115–48121
60. Gao, Z., Zuberi, A., Quon, M. J., Dong, Z., and Ye, J. (2003) *J. Biol. Chem.* **278,** 24944–24950
61. Gual, P., Gonzalez, T., Gremeaux, T., Barres, R., Marchand-Brustel, Y., and Tanti, J. F. (2003) *J. Biol. Chem.* **278,** 26550–26557
62. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory
63. O'Neill, T. J., Craparo, A., and Gustafson, T. A. (1994) *Mol. Cell. Biol.* **14,** 6433–6442
64. Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., and Turner, R. C. (1985) *Diabetologia* **28,** 412–419
65. Radziuk, J. (2000) *J. Clin. Endocrinol. Metab.* **85,** 4426–4433
66. Araki, E., Lipes, M. A., Patti, M.-E., Bruning, J. C., Haag, B. I., Johnson, R. S., and Kahn, C. R. (1994) *Nature* **372,** 186–190
67. Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, H., Yoshioka, S., Horikoshi, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki, Y., and Aizawa, S. (1994) *Nature* **372,** 182–186
68. Kido, Y., Burks, D. J., Withers, D., Bruning, J. C., Kahn, C. R., White, M. F., and Accili, D. (2000) *J. Clin. Investig.* **105,** 199–205
69. Yamauchi, T., Tobe, K., Tamemoto, H., Ueki, K., Kaburagi, Y., Yamamoto-Honda, R., Takahashi, Y., Yoshizawa, F., Aizawa, S., Akanuma, Y., Sonenberg, N., Yazaki, Y., and Kadowaki, T. (1996) *Mol. Cell. Biol.* **16,** 3074–3084
70. Virkam, Ueki, K., and Kahn, C. R. (1999) *J. Clin. Investig.* **103,** 931–943
71. Freidenberg, G. R., Reichart, D., Olefsky, J. M., and Henry, R. R. (1988) *J. Clin. Investig.* **82,** 1398–1406
72. Thies, R. S., Molina, J. M., Ciaraldi, T. P., Freidenberg, G. R., and Olefsky, J. M. (1990) *Diabetes* **39,** 250–259
73. Goodyear, L. J., Giorgino, F., Sherman, L. A., Carey, J., Smith, R. J., and Dohm, G. L. (1995) *J. Clin. Investig.* **95,** 2195–2204
74. Bjornholm, M., Kawano, Y., Lehtihet, M., and Zierath, J. R. (1997) *Diabetes* **46,** 524–527
75. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., *et al.* (2001) *Science* **291,** 1304–1351
76. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) *Science* **298,** 1912–1934
77. Greene, M. W., Morrice, N., Garofalo, R. S., and Roth, R. A. (2004) *Biochem. J.* **378,** 105–116
78. Giraud, J., Leshan, R., Lee, Y. H., and White, M. F. (2004) *J. Biol. Chem.* **279,** 3447–3454
79. Vidal, M., and Legrain, P. (1999) *Nucleic Acids Res.* **27,** 919–929
80. Dhe-Paganon, S., Werner, E. D., Nishi, M., Hansen, L., Chi, Y.-I., and Shoelson, S. E. (2004) *Nat. Struct. Mol. Biol.*, in press

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