Binding of IRS Proteins to Calmodulin Is Enhanced in Insulin Resistance[†]

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ABSTRACT: The IRS proteins, major endogenous targets of the insulin receptor, bind to calmodulin in a Ca^{2+}-dependent manner. Here, we have examined the interaction between these proteins in animal and cultured cell models of insulin resistance. Both IRS-1 and IRS-2 co-immunoprecipitate with calmodulin from insulin target tissues in rats. The interaction between calmodulin and IRS proteins in rat soleus muscle was enhanced when insulin resistance was induced in rats by treatment with dexamethasone for 5 days. Moreover, injection of angiotensin II into the inferior vena cava enhanced the binding in rat cardiac muscle. Similarly, increased binding between calmodulin and IRS-1 was observed in isolated cells incubated with tumor necrosis factor-α. Overexpression of calmodulin in Chinese hamster ovary cells reduced the tyrosine phosphorylation of IRS-1 induced by insulin, with a concomitant decrease in insulin-stimulated association of IRS-1 with the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase. Insulin-stimulated phosphatidylinositol 3-kinase activity associated with IRS-1 was also reduced in cells overexpressing calmodulin, while this activity was increased in cells incubated with the cell-permeable calmodulin antagonist trifluoperazine. These data demonstrate an enhanced interaction between calmodulin and IRS proteins in models of insulin resistance and suggest a possible mechanism by which increased intracellular Ca^{2+} concentrations may contribute to impaired insulin sensitivity.

Insulin binds to the extracellular domain of specific transmembrane receptors, inducing tyrosine phosphorylation of the receptor cytoplasmic β-subunit and selected intracellular proteins. The IRS proteins are major endogenous targets of the insulin receptor kinase and function by transducing the insulin signal to downstream effectors (*1*, *2*). Following phosphorylation on tyrosine residues, IRS-1 and IRS-2 (and the more recently identified IRS-3 and IRS-4) (*3*, *4*) can associate with Src homology 2 (SH2) domains on target proteins. The targets for IRS-1 and IRS-2 include the 85-kDa (*5*) and 55-kDa (*6*) regulatory subunits of phosphatidylinositol 3-kinase (PI3-kinase),[1] the adaptor proteins GRB2 (*7*) and Nck, and the protein tyrosine phosphatase SHP-2 (previously called Syp) (*1*). These interactions propagate the insulin signal. For example, insulin-stimulated binding of PI3-kinase to phosphotyrosine residues in IRS-1 enhances the activity of the enzyme, ultimately culminating in many of the pleiotropic effects of insulin.

Insulin resistance, which is a decreased biological response to physiological insulin concentrations, is present early in type 2 diabetes mellitus (for review, see refs *8*, *9*). The molecular mechanisms that underlie insulin resistance are not understood, but evidence suggests that the IRS proteins may be involved. For example, altered amounts of IRS-1 protein have been described in cell and animal models of insulin resistance (*10*, *11*). Furthermore, IRS-1 is decreased in adipocytes obtained from subjects with type 2 diabetes (*12*) as well as in liver and muscle in fatty rats (*13*). An inhibitory form of IRS-1 has been observed in muscle and fat from obese rats (*14*). Important recent findings document that IRS-2 null mice have insulin resistance in the liver and skeletal muscle (*15*). These data, coupled with their prominence early in the insulin pathway, imply that an altered interaction between IRS proteins and other signaling molecules may contribute to insulin resistance.

Ca^{2+}, the most common signal transduction element in cells, controls many cellular processes, including cell growth, transformation, and neuronal signaling (*16*, *17*). Regulation of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is altered in diabetes mellitus; data obtained from both animal models and patients with diabetes indicate that $[Ca^{2+}]_i$ are increased (*18*). It has been proposed that abnormal $[Ca^{2+}]_i$ homeostasis causes insulin resistance and impairs insulin secretion (*19*). More recent evidence supports the postulate that the agouti protein promotes insulin resistance in mutant animals through its ability to increase $[Ca^{2+}]_i$ (*20*).

Numerous Ca^{2+}-regulated processes are mediated by the ubiquitous Ca^{2+}-modulating protein calmodulin (for review, see ref *21*). The effects of calmodulin are produced by direct interaction with targets or indirectly via protein kinases. The observation that IRS-1 and IRS-2 contain IQ motifs (*22*), which are calmodulin-binding domains (*23*), led to the premise that these regions couple IRS proteins to Ca^{2+}/calmodulin signaling. This hypothesis is supported by the demonstration that Ca^{2+} enhances the binding of IRS proteins to calmodulin in cultured cells in a phosphotyrosine-

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[1] Abbreviations: CHO, Chinese hamster ovary; PI3-kinase, phosphatidylinositol 3-kinase; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PVDF, poly(vinylidene difluoride); ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; TNF-α, tumor necrosis factor-α.

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independent manner (*22*) and that IRS proteins link insulin signaling to Ca^{2+} flux in rat skeletal and cardiac muscle (*24*). The association between calmodulin and IRS proteins couples tyrosine kinase and Ca^{2+}/calmodulin signaling. Therefore, we evaluated whether this interaction could underlie the mechanism by which increased $[Ca^{2+}]_i$ contributes to insulin resistance.

EXPERIMENTAL PROCEDURES

Materials. Male Sprague-Dawley rats were obtained from Charles River. Radiochemicals were purchased from DuPont−NEN. Chinese hamster ovary (CHO) cell lines as well as anti-IRS-1 and anti-IRS-2 antibodies were generously provided by Dr. Morris White (Joslin Diabetes Center, Boston). Anti-IRS-1 and anti-p85 antibodies were from Upstate Biotechnology Inc. Anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories. Anti-calmodulin monoclonal antibody has been previously described (*25*). Horseradish peroxidase-conjugated antibodies were acquired from Amersham. Fao cells were a kind gift of Dr. C. Ronald Kahn (Joslin Diabetes Center). Poly(vinylidene difluoride) (PVDF) membranes were purchased from Millipore. Insulin, angiotensin II, and tumor necrosis factor-α (TNF-α) were from Gibco BRL, Sigma, and Biosource, respectively. Ham's F12 and RPMI media were from Gibco BRL.

Animals. Male Sprague-Dawley rats weighing ∼250 g were allowed free access to standard rat laboratory diet and water. All experimental procedures used in this study were approved by the Institutional Standing Committee on Animals. Animals had free access to water at all times, but food was withdrawn ∼14 h before experiments.

Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). After anesthesia was assured by loss of pedal reflexes (usually 10−15 min), the abdominal cavity was exposed and the inferior vena cava isolated. The inferior vena cava was injected with 200 μL of 0.9% NaCl with or without 10^{-6} M insulin. Soleus muscle was removed 5 min postinjection and immediately frozen in liquid nitrogen. Extracts were prepared by homogenizing tissues with three 30 s bursts in homogenization buffer at 4 °C [50 mM Tris, pH 7.4, 100 mM NaF, 30 mM sodium pyrophosphate, 5 mM EDTA, 0.5% (v/v) Triton X-100, 2 mM ammonium molybdate, 2 mM vanadate, 1 mM PMSF, and 1 μg/mL each of pepstatin, aprotinin, and leupeptin]. Samples were clarified by centrifugation at 15000*g* for 5 min at 4 °C.

In studies on the effects of insulin resistance, rats were injected daily with 1 mg/kg dexamethasone for 5 days as described (*11*). This regimen has been documented to induce insulin resistance (*11*). Control rats were injected with an equal volume of saline.

For the angiotensin II studies, rats were deprived of food for 14 h. Animals were anesthetized as described above, and then injected with 200 μL of saline or the indicated concentrations of angiotensin II via the inferior vena cava. Cardiac and soleus muscles were removed 5 min postinjection and processed as described above.

Cell Culture and Lysis. Fao hepatoma cells were grown to 80% confluence in RPMI containing 10% (v/v) fetal calf serum in a 37 °C humidified incubator. Cells were incubated with or without 5 nM TNF-α for 60 min at 37 °C, followed by treatment with or without 100 nM insulin for 1 min. The reaction was terminated by replacing the medium with 500 μL of lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, and 1 mM EGTA]. The lysates were collected and quick-frozen at −70 °C in methanol/solid CO_2.

CHO cell lines were grown in Ham's F12 medium containing 10% fetal calf serum. The medium was replaced 3 h before each experiment with serum-free Ham's F12. In selected experiments, trifluoperazine was added to the serum-free medium at a final concentration of 12.5 μM. After 30 min at 37 °C, the medium was removed, and cells were washed 3 times with phosphate-buffered saline and lysed as described above.

Immunoprecipitation and Immunoblotting. Samples were thawed and clarified by centrifugation at 15000*g* for 5 min at 4 °C. Equal amounts of protein lysate were immunoprecipitated with anti-calmodulin monoclonal antibody (*26*) or anti-IRS-1 polyclonal antibody (*22*) as described previously. The immunoprecipitates were washed 5 times and heated for 2 min at 100 °C in solubilization buffer. Proteins were resolved by SDS−PAGE and transferred to PVDF membranes. The membranes were probed with the antibodies indicated in the figure legends, and immune complexes were detected with the Amersham ECL Kit. Where indicated, membranes were stripped according to the manufacturer's instructions and reprobed with an alternate antibody.

Expression of Calmodulin cDNA in CHO Cells. The calmodulin-encoding cDNA from *Arbacia punctulata* (kindly provided by Dr. A. Persechini) was cloned into the *Nco*I−*Pst*I restriction sites of the bacterial expression vector pMEX7. A protein identical to vertebrate calmodulin is encoded (*27*). To obtain the cDNA flanked by restriction sites favorable for cloning into the LacSwitch expression vector, linkers were constructed and attached to the excised calmodulin cDNA. The *Pst*I site was changed to an *Afl*II site by insertion of a 16mer oligonucleotide (CGTCGACTTAAGTGCA). The *Nco*I site was made blunt-end by Klenow. The calmodulin cDNA was cloned into the Lac-operator-containing vector (pOPI3CAT) of the LacSwitch inducible mammalian expression system (Stratagene). Several modifications were made to the pOPI3CAT vector to allow insertion of both mammalian and *Drosophila* calmodulin cDNAs. pOPI3CAT was initially digested with *Not*I. The *Not*I sites were changed to *Xba*I and *Sma*I sites by replacing the excised 777 base pair *Not*I fragment with a synthesized 22mer oligonucleotide (GGCCTCTAGACCCGGGCTCAGC). About 500 base pairs of pMEX7 was inserted in pOPI3CAT at the *Sma*I−*Afl*II site. A gene for puromycin resistance was excised from pBABE-puromycin (generously provided by Dr. Jon Aster) with *Bam*HI and *Cla*I. Blunt ends were made with S1 nuclease. The 1055 base pair puromycin construct was inserted into pOPI3CAT at an *Aat*II site, which had also been made into a blunt end with S1 nuclease. The correct orientation of calmodulin cDNA in the pOPI3CAT vector was confirmed with restriction digests.

The Lac-repressor-expressing vector (p3′SS) and the pOPI3CAT with the cDNA for mammalian calmodulin were transfected into CHO cells. CHO cells were incubated for 3 min with 10% glycerol and transfected with calmodulin cDNA by the calcium phosphate precipitation method. The pOPI3CAT vector without calmodulin cDNA was used as a

control. Approximately 48 h after transfection, cells were incubated in Ham's F12 medium with 10% fetal calf serum containing 1.5 mg/mL puromycin. Eleven positive clones were screened for calmodulin expression by Western blotting. Induction was performed by incubating cells with 3 mM IPTG for 18−24 h. Two clones with the highest level of calmodulin expression were used in experiments. In all cases, controls of CHO cells transfected with empty vector (CHO/I3) were induced in parallel with IPTG.

Phosphoamino Acid Analysis. CHO/I3 cells and CHO cells overexpressing calmodulin (CHO/CaM), induced with 3 mM IPTG for 24 h, were washed in phosphate-free RPMI medium and loaded with [^{32}P]P_i as described (*26*, *28*). After incubation with or without 100 nM insulin for 5 min, the reaction was terminated by quick-freezing with methanol/solid CO_2. Equal amounts of protein lysate were immunoprecipitated with anti-IRS-1 antibody. Samples were processed by SDS−PAGE and transferred to PVDF. IRS-1 was localized by autoradiography, and pieces of the membrane containing IRS-1 were excised. After hydrolysis of phosphorylated IRS-1 on PVDF in 6 M HCl for 2 h at 110 °C, phosphoamino acids were separated by thin-layer electrophoresis at pH 1.8 as described previously (*29*). Radiolabel incorporation was quantified after autoradiography by scraping the phosphoamino acids from the chromatography plate and performing Cerenkov counting.

Phosphatidylinositol 3-Kinase Activity. PI3-kinase activity was measured in immunoprecipitates as previously described (*30*). Briefly, CHO cells transfected with empty vector (CHO/I3) or with calmodulin cDNA (CHO/CaM) were induced with 3 mM IPTG for 18−24 h. Equal numbers of cells were incubated with or without 100 nM insulin for 5 min at 37 °C. Equal amounts of protein lysate were immunoprecipitated with anti-IRS-1 antibody, and immune compexes were collected with protein A−Sepharose as described (*22*). Immunoprecipitates were washed 5 times, and phosphorylation of phosphatidylinositol was carried out as previously described (*30*). Samples were resolved by thin-layer chromatography, and phosphatidylinositol phosphate was located by autoradiography.

Northern Blotting. RNA was extracted from CHO cells with RNAzol B according to the manufacturer's instructions (Tel-Test, Inc.). Thirty micrograms of RNA was resolved by electrophoresis in agarose gels and transferred to nitrocellulose. Blots were hybridized with calmodulin cDNA radiolabeled with a random primer labeling kit (Gibco BRL). RNA was detected by autoradiography.

Miscellaneous. Protein concentrations were determined with the DC Protein Assay (Bio-Rad) using bovine serum albumin as standard. Densitometry was analyzed with NIH Image. Cell viability was determined by trypan blue dye exclusion. Phosphorimaging was performed with the Molecular Dynamics Imaging System, and analysis was conducted with Image Quant software.

RESULTS

Co-immunoprecipitation of Calmodulin with IRS-1 and IRS-2 from Intact Cells. Our previous analysis revealed an interaction between calmodulin and IRS proteins in CHO cells expressing IRS-1 or IRS-2 (*22*). To determine whether these proteins associate in rat tissues that are normally responsive to insulin, several rat tissues were isolated and lysates were immunoprecipitated with anti-calmodulin monoclonal antibody (Figure 1). Both IRS-1 and IRS-2 co-immunoprecipitated with calmodulin from rat liver, soleus muscle, cardiac muscle, and adipose tissue (Figure 1). The amounts of lysate subjected to immunoprecipitation were 60, 10, 30, and 10 mg for liver, soleus muscle, cardiac muscle, and adipose tissue, respectively. Binding was specific for calmodulin as no IRS proteins coprecipitated with anti-myoglobin antibody, an irrelevant isotype-identical monoclonal antibody (data not shown).

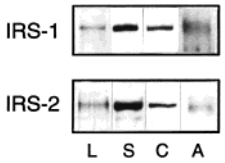

FIGURE 1: Co-immunoprecipitation of calmodulin and IRS proteins from rat tissues. Tissues were removed from male Sprague-Dawley rats and immediately frozen in liquid nitrogen. Extracts were prepared by homogenizing tissues in homogenization buffer, as described under Experimental Procedures. Lysates were immunoprecipitated with anti-calmodulin monoclonal antibody. Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 antibody (upper panel). Antigen−antibody complexes were detected by ECL. After stripping according to the manufacturer's instructions, membranes were reprobed with anti-IRS-2 antibody (lower panel). A representative experiment of three separate determinations is shown. L, liver; S, soleus muscle; C, cardiac muscle; and A, adipose tissue.

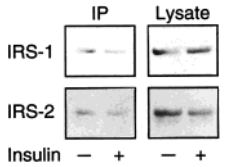

FIGURE 2: Effect of insulin on the binding of IRS-1 and IRS-2 to calmodulin in rat soleus muscle. Male Sprague-Dawley rats were fasted for 14 h prior to the injection of 200 μL of saline or 1 μM insulin via the inferior vena cava. Soleus muscle was removed 5 min postinjection and immediately frozen in liquid nitrogen. Extracts were prepared by homogenizing tissues as described under Experimental Procedures. Equal amounts of protein lysate were separated directly (Lysate) or immunoprecipitated (IP) with anti-calmodulin monoclonal antibody. Proteins were resolved by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 antibody. Antigen−antibody complexes were detected by ECL. The blots were stripped according to the manufacturer's instructions and reprobed with anti-IRS-2 antibody. A representative experiment of two separate determinations is shown.

Effect of Insulin on the Binding of IRS-1 and IRS-2 to Calmodulin in Rat Soleus Muscle. Although insulin promotes the association of IRS-1 with a variety of signaling proteins (*31*), it did not substantially alter the interaction between calmodulin and IRS-1 in CHO cells expressing human insulin receptors and rat IRS-1 (*22*). Overexpression may disrupt normal intracellular homeostatic mechanisms and alter interactions among signaling proteins. Therefore, we examined a more physiologically relevant system by injecting rats with insulin. Intravenous injection of insulin into rats did not significantly change the amount of IRS-1 or IRS-2 that co-immunoprecipitated with calmodulin from soleus muscle (Figure 2). Analysis of a range of concentrations of insulin (between 10^{-10} and 10^{-5} M) and different times of exposure to insulin (1, 3, 5, 10, or 20 min) revealed no significant difference in the amount of IRS proteins that co-immuno-

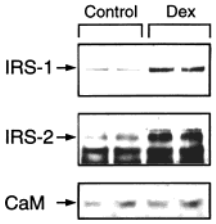

FIGURE 3: Effect of dexamethasone on the binding of IRS-1 and IRS-2 to calmodulin in rat soleus muscle. Male Sprague-Dawley rats were injected for 5 days with vehicle (Control) or 1 mg/kg per day of dexamethasone (Dex). Soleus muscle was removed and immediately frozen in liquid nitrogen. Equal amounts of muscle lysate were immunoprecipitated with anti-calmodulin antibody. Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 (top panel) or anti-calmodulin (CaM) (bottom panel) antibodies. Antigen−antibody complexes were detected by ECL. After stripping, membranes were reprobed with anti-IRS-2 antibody (middle panel). Data are from two rats under each condition and are representative of three separate experimental determinations.

precipitated with calmodulin (data not shown). The lack of effect was not due to translocation of the IRS to an insoluble fraction as insulin did not appreciably change the amount of IRS-1 or IRS-2 in the lysate (Figure 2). Thus, the insulin-independent—but Ca^{2+}-regulated—binding of calmodulin to IRS is different from the interaction of IRS with other target proteins. This observation raised the question of whether agents that disrupt insulin signaling would enhance calmodulin−IRS binding. Such an effect could provide a potential molecular mechanism by which increased $[Ca^{2+}]_i$ contributes to insulin resistance.

Effects of Glucocorticoids on the Binding of IRS-1 and IRS-2 to Calmodulin in Rat Soleus Muscle. Initial evaluation was performed with hormonal models of insulin resistance in rats. Glucocorticoid excess is a well-known cause of insulin resistance, and dexamethasone-treated rats have reduced insulin-stimulated IRS-1 associated PI3-kinase (*11*). Rats were rendered insulin-resistant by daily dexamethasone injections as described (*11*), and the interaction between calmodulin and IRS proteins was analyzed. Treatment of rats with dexamethasone significantly enhanced the amount of IRS-1 that co-immunoprecipitated with calmodulin from soleus muscle (Figure 3). Similarly, dexamethasone treatment increased the association between IRS-2 and calmodulin. The total amount of IRS-1 and IRS-2 in soleus muscle was not increased by dexamethasone (data not shown). The amount of calmodulin immunoprecipitated was not altered by treatment (Figure 3).

Effect of Angiotensin II on the Binding of IRS-1 and IRS-2 to Calmodulin in Rat Cardiac and Soleus Muscles. Angiotensin II, an important regulator of cardiovascular and renal function, induces insulin resistance in rats (*32*) and decreases insulin-stimulated IRS-associated PI3-kinase activity (*33*). Because angiotensin II increases $[Ca^{2+}]_i$ (*16*) and may be of pathophysiologic significance in insulin resistance (*33*, *34*), we examined its effect on the interaction between calmodulin and IRS proteins in rat muscle. Intravenous injection of angiotensin II into rats enhanced in a dose-dependent manner the binding of IRS-1 and IRS-2 to calmodulin in cardiac muscle (Figure 4A, top panels). Maximum increases were 5.29 ± 1.41- and 2.66 ± 0.07-fold (mean ± range, $n = 2$) for IRS-1 and IRS-2, respectively. By contrast, the calmodulin−IRS interaction was not significantly altered in

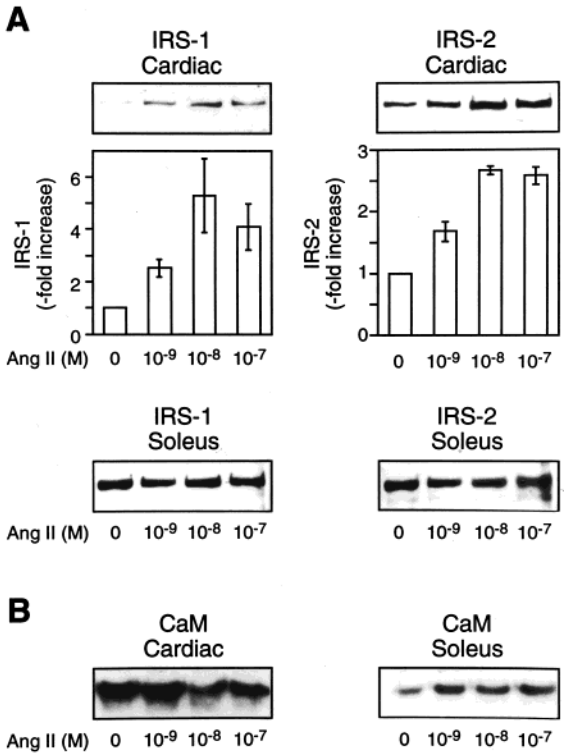

FIGURE 4: Effect of angiotensin II on the binding of IRS-1 and IRS-2 to calmodulin in rat cardiac and soleus muscle. Male Sprague−Dawley rats were fasted for 14 h prior to the injection of 200 μL of saline (0) or the indicated concentrations of angiotensin II (Ang II) via the inferior vena cava. Cardiac and soleus muscles were removed 5 min postinjection and immediately frozen in liquid nitrogen. Tissues were homogenized, and equal amounts of protein lysates were immunoprecipitated with anti-calmodulin antibody. Proteins were separated by SDS−PAGE and transferred to PVDF. (A) Blots were probed with anti-IRS-1 antibody (left panel), and antigen−antibody complexes were detected by ECL. The blots were stripped and reprobed with anti-IRS-2 antibody (right panel). Representative experiments of two separate determinations are shown. The relative amounts of IRS-1 and IRS-2 protein precipitated from each cardiac muscle sample were quantified. The data, presented in the middle panels, are expressed as percentages of the control (no angiotensin II) and represent the mean ± range, $n = 2$. (B) The lower halves of the blots from cardiac muscle (left panel) and soleus muscle (right panel) were probed with anti-calmodulin (CaM) antibody and developed with ECL.

soleus muscle in the same rats (Figure 4A, bottom panels). The latter observation is not surprising as skeletal muscle does not contain angiotensin II receptors and is not a direct target organ for angiotensin II (*35*). These data imply that the increased association between calmodulin and IRS proteins is a direct effect of angiotensin II and is not secondary to other physiological alterations (e.g., a change in blood pressure) produced by the hormone. Angiotensin II treatment altered neither the amount of IRS-1 or IRS-2 in the lysates of muscle tissue (data not shown) nor the amount of calmodulin in the immunoprecipitates (Figure 4B). Thus, two independent models of insulin resistance in rats—namely, glucocorticoid- and angiotensin II-induced—revealed enhanced association between calmodulin and IRS proteins.

Enhanced Association of IRS-1 with Calmodulin in Cultured Mammalian Cells. Several lines of evidence indicate that tumor necrosis factor-α (TNF-α) plays a role in insulin resistance in diabetes and obesity (*36*). For example, adipocytes from obese rodents and humans overexpress TNF-α (*36*). At the molecular level, TNF-α inhibits

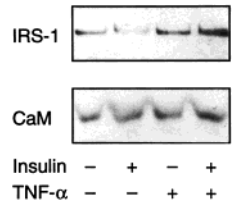

FIGURE 5: Effect of TNF-α on the binding of IRS-1 to calmodulin. Fao hepatoma cells were incubated with or without 5 nM TNF-α for 1 h. Cells were then incubated with or without 100 nM insulin for 1 min and lysed, and equal amounts of protein were immunoprecipitated with anti-calmodulin antibody. Samples were resolved by SDS−PAGE and transferred to PVDF, and membranes were probed with anti-IRS-1 (upper panel) or anti-calmodulin (CaM) (lower panel) antibodies. Data are representative of two separate experimental determinations.

insulin-stimulated tyrosine phosphorylation of IRS-1 and induces serine phosphorylation of IRS-1; this modified IRS-1 inhibits insulin receptor signaling (*14*). Although contradictory evidence exists (*37*, *38*), additional support for the TNF-α hypothesis has been obtained from TNF-α knockout mice. The absence of TNF-α resulted in improved insulin sensitivity in diet-induced obesity and that resulting from the *ob/ob* model of obesity (*39*). Thus, it seems likely that TNF-α has a role in at least some types of insulin resistance. Importantly, TNF-α induces a rise in $[Ca^{2+}]_i$ (*40*).

Insulin-sensitive Fao cells were incubated with TNF-α under conditions that led to a 65% decrease in insulin-induced tyrosine phosphorylation of IRS-1 (*41*). Incubation of Fao cells with TNF-α significantly enhanced the amount of IRS-1 that coimmunoprecipitated with calmodulin (Figure 5). This increase was observed in both basal and insulin-treated cells. The amount of calmodulin precipitated was not changed by insulin or TNF-α (Figure 5). Thus, TNF-α, which increases $[Ca^{2+}]_i$ (*40*) and induces insulin resistance (*36*), promotes the association of IRS-1 with calmodulin. Therefore, two distinct models—whole animals and cultured cells—support the hypothesis that the interaction of calmodulin and IRS is enhanced in insulin resistance.

Development of Cell Lines That Overexpress Calmodulin. If the increased association between calmodulin and IRS proteins is a component of insulin resistance, one would anticipate that enhancing this interaction could decrease the binding of IRS to downstream components in the insulin signaling pathway. To test this hypothesis, we developed CHO cell lines that overexpress calmodulin under the control of an inducible promoter. The cDNA for mammalian calmodulin was cloned in the pOPI3CAT vector of the LacSwitch inducible mammalian expression system. Expression was induced by incubation of the transfected cells with IPTG, a lactose analogue that binds and inhibits the Lac repressor. Overexpression of calmodulin protein was confirmed by immunoblotting (Figure 6A, lanes 1 and 2). In induced cells, calmodulin reached concentrations 2−3-fold greater than endogenous concentrations (Figure 6A). Northern blotting verified the inducible expression of calmodulin mRNA (Figure 6B). Note that the increase in calmodulin mRNA in induced cells is much greater than the increase in calmodulin protein. These observations are consistent with published data obtained with mouse C127 cells where calmodulin mRNA concentrations were increased 20−50-fold while protein was only 2−4-fold higher than control cells (*42*). The reason for this finding is not known, but

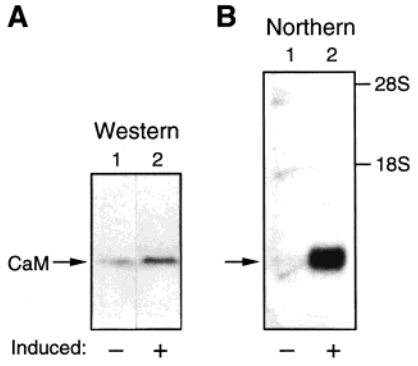

FIGURE 6: Overexpression of calmodulin in CHO cells. CHO cells transfected with calmodulin cDNA in the LacSwitch system were induced (+) or not induced (−) with IPTG and lysed. (A) Equal amounts of protein were resolved by SDS−PAGE and transferred to PVDF. Membranes were probed with anti-calmodulin antibody, and antigen−antibody complexes were detected by ECL. (B) 30 μg of RNA was separated on agarose gels, transferred to nitrocellulose, and hybridized with calmodulin cDNA labeled with random primers with a DNA labeling system. The positions of migration of calmodulin (CaM) protein and RNA are indicated. Data are representative of at least three separate experimental determinations.

presumably reflects tight control of the concentration of calmodulin protein in cells. Because calmodulin regulates multiple fundamental cellular processes, including DNA synthesis (*43*), it is not surprising that intracellular calmodulin concentrations would be maintained within fairly narrow limits.

Effect of Overexpression of Calmodulin on Insulin-Stimulated Phosphorylation of IRS-1. The effect of calmodulin on insulin-stimulated IRS-1 phosphorylation was examined in CHO cells overexpressing calmodulin. Insulin stimulated the tyrosine phosphorylation of IRS-1 in both cell lines, but the magnitude of the stimulation was substantially attenuated by calmodulin overexpression (Figure 7A). Probing the blots with anti-IRS-1 antibody revealed that the decreased extent of tyrosine phosphorylation in CHO/CaM cells was not caused by a reduction in the amount of IRS-1 protein (Figure 7A). Direct phosphoamino acid analysis demonstrated that serine was the major amino acid phosphorylated in IRS-1 (Figure 7B). Insulin did not significantly increase (<10%) phosphate incorporation into serine or threonine residues of IRS-1 in CHO/I3 or CHO/CaM cells. Longer exposure of the autoradiogram confirmed the presence of insulin-stimulated tyrosine phosphorylation (data not shown). By contrast, calmodulin enhanced serine and threonine phosphorylation of IRS-1. Quantification of radioactivity by phosphorimaging of each phosphoamino acid showed that calmodulin overexpression increased $[^{32}P]P_i$ incorporation into serine and threonine residues by 1.8- and 1.7-fold, respectively. The relative amounts of the phosphoamino acids in IRS-1 we observed in CHO/I3 cells are virtually identical to the data of Sun et al. (*2*), who documented in CHO cells that phosphoserine was the predominant phosphoamino acid, with substantially less phosphothreonine and a small amount of phosphotyrosine.

Effect of Overexpression and Antagonism of Calmodulin on Insulin-Stimulated Interaction of IRS-1 with PI3-Kinase. We examined the effect of overexpressing calmodulin on the interaction between IRS-1 and PI3-kinase. Calmodulin overexpression in CHO cells significantly decreased insulin-stimulated binding of the p85 subunit of PI3-kinase to IRS-1

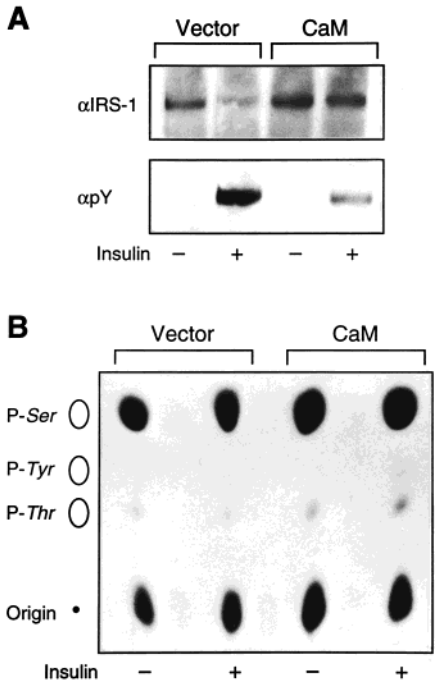

FIGURE 7: Effect of overexpression of calmodulin on insulin-stimulated phosphorylation of IRS-1. (A) CHO cells expressing vector alone (Vector) or calmodulin (CaM) were induced with IPTG for 24 h. Where indicated, cells were incubated with 100 nM insulin for 5 min prior to cell lysis. Equal amounts of protein lysate were immunoprecipitated with anti-IRS-1 antibody, resolved by SDS−PAGE, and transferred to PVDF. Blots were probed with anti-phosphotyrosine antibody (αpY) (lower panel), and immune complexes were visualized by ECL. After stripping according to the manufacturer's instructions, membranes were reprobed with anti-IRS-1 antibody (αIRS-1) (upper panel). Data are representative of two separate experimental determinations performed with different CHO/CaM clones. (B) CHO cells containing vector alone or calmodulin (CaM) were induced with IPTG and loaded with [^{32}P]-P_i. Cells were incubated with or without insulin, and equal amounts of protein lysate were immunoprecipitated with anti-IRS-1 antibody. Following electrophoresis and immunoblotting, the IRS-1 band was excised and hydrolyzed in 6 M HCl. Phosphoamino acids were separated by thin-layer electrophoresis at pH 1.8 on phosphocellulose plates. The origin and the positions of migration of phosphoamino acid standards, phosphoserine (P-*Ser*), phosphotyrosine (P-*Tyr*), and phosphothreonine (P-*Thr*), visualized with ninhydrin, are indicated.

(Figure 8A). Moreover, insulin-stimulated PI3-kinase activity in anti-IRS-1 immunoprecipitates from CHO/CaM cells was 55% lower than that from CHO/I3 cells (Figure 8B). The identity of PI3-kinase was confirmed by HPLC analysis of the phosphatidylinositol phosphate spot, which revealed phosphatidylinositol 3-phosphate (data not shown). The amount of IRS-1 immunoprecipitated was not changed by overexpression of calmodulin (Figure 8A).

Thus, increased intracellular calmodulin concentrations impair the ability of IRS-1 to bind to and activate PI3-kinase in response to insulin. We therefore examined whether decreasing the interaction between calmodulin and IRS-1 would enhance its association with PI3-kinase. This question was addressed with trifluoperazine, a cell-permeable calmodulin antagonist, that attenuates the binding of calmodulin to IRS-1 (*22*). Incubation of cells with trifluoperazine increased by 1.7-fold insulin-stimulated PI3-kinase activity associated with IRS-1 (Figure 8C). By contrast, basal IRS-1-associated PI3-kinase activity was not altered by trifluoperazine

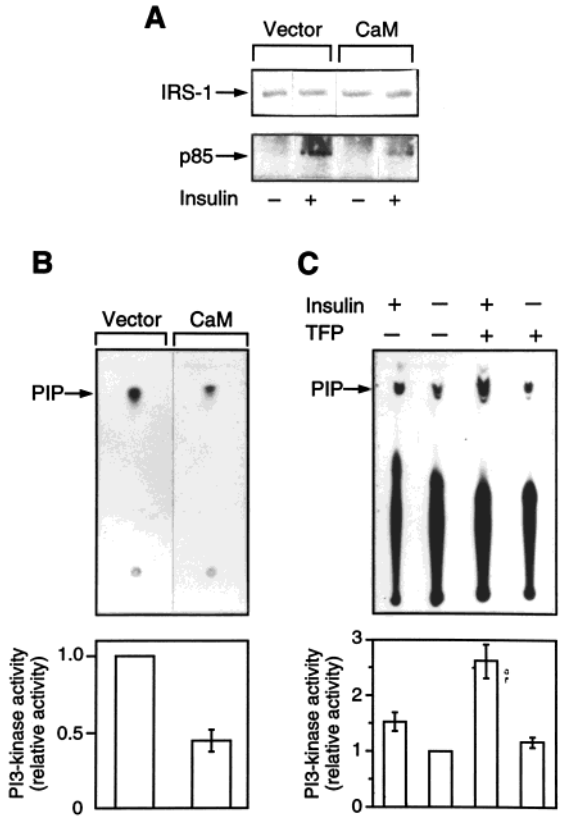

FIGURE 8: Effect of overexpressing calmodulin and calmodulin antagonism on insulin-stimulated PI3-kinase association with IRS-1. (A) CHO cells expressing vector alone (Vector) or calmodulin (CaM) were induced with IPTG for 24 h. Where indicated, cells were incubated with 100 nM insulin for 5 min. Equal amounts of protein lysate were immunoprecipitated with anti-IRS-1 antibody. Samples were resolved by SDS−PAGE and transferred to PVDF, and blots were probed with anti-IRS-1 (upper panel) or anti-p85 antibodies (lower panel). The positions of migration of IRS-1 and the p85 subunit of PI3-kinase (p85) are indicated. The data are representative of two independent experimental determinations performed with different CHO/CaM clones. (B) CHO cells expressing vector alone or calmodulin (CaM) were induced with IPTG and incubated with insulin as described in (A) above. Equal amounts of protein lysate were immunoprecipitated with anti-IRS-1 antibody. PI3-kinase activity was determined by incubating immunoprecipitates with [γ-^{32}P]ATP and phosphatidylinositol. Samples were resolved by thin-layer chromatography and phosphatidylinositol 3-phosphate (PIP) was identified by autoradiography (upper panel). PI3-kinase activity was quantified by determining the relative amount of PIP in the autoradiograms. Results, presented in the lower panel, are expressed relative to vector and represent the mean ± range from two independent experiments. (C) CHO cells were incubated with vehicle or 12.5 μM trifluoperazine (TFP) for 30 min, followed by stimulation with or without insulin for 5 min. After washing, cells were lysed, and equal amounts of protein were immunoprecipitated with anti-IRS-1 antibody. PI3-kinase activity was determined as described under (B) above. A representative experiment is shown with the position of migration of PIP indicated (upper panel). PI3-kinase activity in anti-IRS-1 immunoprecipitates was quantified by determining the relative amount of PIP in the autoradiograms. Results, expressed relative to no treatment, represent the mean ± range from two independent experimental determinations (lower panel).

DISCUSSION

IRS-1 and IRS-2, important components in the initial phase of the insulin signaling pathway, are likely to be involved in the pathogenesis of insulin resistance. Impaired insulin-stimulated interaction of IRS-1 and IRS-2 with downstream

targets, including PI3-kinase, has been observed in insulin resistance (*11*, *13*). Moreover, IRS-2 null mice exhibit insulin resistance and reduced β-cell mass, suggesting that functional abnormalities in IRS-2 could be involved in the pathogenesis of type 2 diabetes (*15*). The evidence linking increased $[Ca^{2+}]_i$ to insulin resistance (*18*), coupled with the Ca^{2+}-mediated binding of calmodulin to IRS-1 (*22*), prompted this work which examined the interaction between calmodulin and IRS proteins in models of insulin resistance.

Initial analysis established that both IRS-1 and IRS-2 co-immunoprecipitated with calmodulin from insulin-sensitive rat tissues. The phosphotyrosine-independent interaction between calmodulin and IRS proteins in rat muscle was enhanced by both glucocorticoid excess and angiotensin II, agents known to induce insulin resistance in rats (*11*, *32*). Similarly, a model of insulin resistance in cultured cells revealed an increased association between calmodulin and IRS-1. These data raised the important question of whether the binding of calmodulin modulated IRS function. This hypothesis was evaluated by two complementary strategies. In the first, we developed cell lines in which calmodulin could be inducibly overexpressed. Analysis of the insulin-stimulated interaction between IRS-1 and PI3-kinase revealed that when calmodulin concentrations were increased both the amount of p85 and the PI3-kinase activity associated with IRS-1 in response to insulin were reduced. Analogous findings have been previously described with 14-3-3β protein, which binds to IRS-1 and inhibited insulin-stimulated PI3-kinase activity in 3T3L1 adipocytes (*44*).

In the second approach, the widely accepted calmodulin antagonist trifluoperazine was used. Previously we documented that trifluoperazine blocked the association of IRS-1 with calmodulin (*22*). Consistent with the data obtained by overexpressing calmodulin, inhibiting the binding of calmodulin to IRS-1 enhanced the association between PI3-kinase and IRS-1 in insulin-stimulated cells. Although frequently used to determine the participation of calmodulin in cell metabolism (*45*−*48*), calmodulin antagonists have been reported to affect other proteins. However, these nonspecific interactions occur at relatively high concentrations of the antagonists. To minimize possible nonspecific effects, we used a low concentration (12.5 μM) of trifluoperazine. Analysis with purified proteins in an in vitro system showed that trifluoperazine had an IC_{50} value of 12.7 μM for the inhibition of calmodulin-stimulated cAMP phosphodiesterase activity, but had no effect on purified protein kinase C at this concentration (*49*).

In this paper we have demonstrated an enhanced interaction between calmodulin and IRS proteins in selected models of insulin resistance. Moreover, we observed that calmodulin modulates the insulin-stimulated association between IRS-1 and PI3-kinase. The molecular mechanism by which calmodulin negatively regulates the IRS-1/PI3-kinase interaction is not known, but several possibilities exist. First, calmodulin may alter the tertiary conformation of IRS-1, changing its ability to bind other targets. Allosteric inhibition by calmodulin has been reported for other proteins, including IQGAP1 (*50*, *51*) and caldesmon (*52*). Second, calmodulin may sterically hinder access of PI3-kinase to the phosphotyrosine motifs of IRS-1. For example, $p59^{fyn}$ and GRB2 compete for IRS-1 binding (*53*), while calmodulin displaces actin from G protein-coupled receptor kinase 5 (*54*) and displaces α-actinin-2 from *N*-methyl-D-aspartate receptors (*55*). Third, calmodulin may regulate the subcellular location of IRS-1. The association of IRS-1 with PI3-kinase occurs predominantly in the low-density membranes and plasma membranes, with maximum activation of PI3-kinase seen in the low-density membranes (*56*). Failure of IRS-1 to translocate in response to insulin (*57*) could impair its ability to activate PI3-kinase. Calmodulin is known to participate in protein trafficking. Several proteins, including Rad (*58*), a protein associated with insulin resistance, and cyclin-dependent kinase 4 (*47*), exhibit aberrant subcellular location when calmodulin binding is altered. Fourth, IRS-1 may be phosphorylated on serine/threonine residues by (a) calmodulin-dependent kinase(s). Serine/threonine phosphorylation of IRS-1 decreases its ability to be tyrosine-phosphorylated in response to insulin and to interact with PI3-kinase (*14*, *59*−*61*). The last model is most consistent with our data, but may not be the sole mechanism. Regardless of the mode by which calmodulin attenuates the interaction between IRS-1 and PI3-kinase, the findings presented here suggest a possible mechanism by which increased $[Ca^{2+}]_i$ could contribute to insulin resistance.

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