Ca^{2+} Regulates Calmodulin Binding to IQ Motifs in IRS-1[†]

Hidayatullah G. Munshi,[‡] Deborah J. Burks,[§] John L. Joyal,[‡] Morris F. White,[§] and David B. Sacks*,[‡]

Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, and Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

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ABSTRACT: IRS-proteins couple the receptors for insulin and various cytokines to signalling proteins containing Src homology 2 (SH2) domains. Here we demonstrate that calmodulin, a mediator of Ca^{2+}-dependent physiological processes, associates with IRS-1 in a phosphotyrosine-independent manner. IRS-1 coimmunoprecipitated with calmodulin from lysates of Chinese hamster ovary cells expressing IRS-1. The interaction was modulated by Ca^{2+}, and calmodulin binding to IRS-1 was enhanced by increasing intracellular Ca^{2+} with A23187. In contrast, trifluoperazine, a cell-permeable calmodulin antagonist, decreased binding of calmodulin to IRS-1. Insulin stimulated tyrosine phosphorylation of IRS-1, but did not significantly alter the interaction between calmodulin and IRS-1. IQ-like motifs occur between residues 106−126 and 839−859 of IRS-1. Synthetic peptides based on these sequences inhibited the association between IRS-1 and calmodulin. These data demonstrate that calmodulin binds to IRS-1 in intact cells in a Ca^{2+}-regulated manner, providing a molecular link between the signalling pathways.

Agonist-stimulated elevations of cytoplasmic free Ca^{2+}, resulting from release of intracellular stores or through the opening of several Ca^{2+} channels, regulate many physiologic processes, including cell-cycle progression, differentiation, muscle contraction, and the activity of various cellular enzymes (Berridge, 1993). Biologic responses to this increase in Ca^{2+} are mediated by signalling proteins which undergo conformational changes and associate with specific target proteins during Ca^{2+} binding (Cohen & Klee, 1988). Calmodulin is a ubiquitous Ca^{2+}-binding protein implicated in a variety of signalling pathways, including the regulation of muscle glycogen metabolism (Cohen & Klee, 1988). The structure of Ca^{2+}-bound calmodulin has been solved in both the absence (Babu et al., 1985) and presence (Meador et al., 1992, 1993) of associated peptides. Ca^{2+}-induced conformational changes enable calmodulin to bind cytoskeletal proteins and to activate protein kinases, protein phosphatase 2B, and the plasma membrane Ca^{2+} pump (Cohen & Klee, 1988).

Ca^{2+} homeostasis is disrupted in diabetes mellitus; elevated cytosolic Ca^{2+} levels have been observed in several cell types obtained from both diabetic patients and animal models of insulin resistance (Levy et al., 1994). Moreover, Ca^{2+} has been implicated in the regulation of insulin sensitivity (Draznin et al., 1987) and is also important in glucose-stimulated insulin secretion (Turk et al., 1987). Insulin stimulates tyrosine phosphorylation of calmodulin in rat adipocytes (Colca et al., 1987) and hepatocytes (Joyal & Sacks, 1994; Sacks et al., 1992), and purified insulin receptors phosphorylate calmodulin *in vitro* (Graves et al., 1986; Sacks et al., 1989; Sacks & McDonald, 1988). However, the precise role of Ca^{2+} and calmodulin in modulating the insulin signal remains unresolved.

Insulin signalling is mediated through a small family of docking proteins, including IRS-1 (Sun et al., 1991, 1992; White et al., 1985; White & Kahn, 1994), IRS-2 (Sun et al., 1995), Gab-1 (Holgado-Madruga et al., 1996), and Shc (Pronk et al., 1993; Skolnik et al., 1993b). The IRS-proteins are the best characterized substrates for the insulin receptor (White et al., 1985; Sun et al., 1991, 1992, 1995; White & Kahn, 1994). These molecules are composed of two NH_2-terminal interaction modules: a pleckstrin homology domain (IH1PH) and a phosphotyrosine-binding domain (IH2PTB) (Sun et al., 1995). These domains provide a mechanism for the specific interaction of IRS-proteins with the insulin receptor (Myers et al., 1995; Sun et al., 1995). Phosphorylation of IRS-1 on tyrosine residues mediates its interaction with the Src homology 2 (SH2) domains of several proteins, including the $p55^{PIK}$ (Pons et al., 1995) and the 85 kDa (Backer et al., 1993) regulatory subunits of phosphatidylinositol 3-kinase, the protein tyrosine phosphatase SH-PTP2 (Kuhne et al., 1993), and the adaptor proteins Nck (Lee et al., 1993) and GRB2 (Baltensperger et al., 1993; Skolnik et al., 1993a). The association of SH2-proteins with IRS-1 couples the insulin receptor to downstream pathways (Backer et al., 1993; Baltensperger et al., 1993; Kuhne et al., 1993; Lee et al., 1993; Skolnik et al., 1993a; White & Kahn, 1994; Pons et al., 1995).

In addition to the multiple phosphorylation motifs, IRS-1 and IRS-2 contain IQ motifs. The complete IQ motif consists of approximately 23 residues with the consensus sequence IQXXXRGXXXR, and serves as a calmodulin-binding domain in certain proteins (Cheney & Mooseker, 1992). The presence of IQ motifs in IRS-proteins suggests that calmodulin or other related Ca^{2+}-binding proteins may interact with IRS-1 via this mechanism. Here we demonstrate the Ca^{2+}-mediated association of calmodulin with

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* Correspondence should be addressed to this author at Brigham and Women's Hospital, Thorn 430, 75 Francis St., Boston, MA 02115. Telephone: (617) 732-6627. Fax: (617) 278−6921.

[‡] Brigham and Woman's Hospital and Harvard Medical School.

[§] Joslin Diabetes Center and Department of Medicine, Harvard Medical School.

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IRS-1 and provide evidence for the involvement of IQ motifs in this interaction. Association of IRS-proteins with calmodulin provides a molecular link between tyrosine kinase signalling and Ca^{2+}/calmodulin signalling systems.

EXPERIMENTAL PROCEDURES

Materials. Chinese hamster ovary cell lines expressing human insulin receptors (CHO^{IR}), full-length rat IRS-1 (CHO/IRS-1), both human insulin receptors and full-length rat IRS-1 (CHO^{IR}/IRS-1) (Sun et al., 1992), or rat IRS-1 with deletion of the pleckstrin homology domain (residues 6−155) (CHO^{IR}/IRS-$1^{\Delta PH}$) (Myers et al., 1995) have been previously described. CHO^{IR}/IRS-2 cells were created by transfection of CHO^{IR} cells with mouse IRS-2 cDNA (Sun et al., 1995) using the calcium phosphate method (Sun et al., 1992). Rabbit polyclonal antisera containing antibodies against the C-terminal region of rat IRS-1 (Sun et al., 1992) or residues 619−746 of mouse IRS-2 (Sun et al., 1995) were used in these studies. Since the anti-IRS-1 antibody is directed against the COOH terminus of IRS-1, it recognizes IRS-1 and IRS-$1^{\Delta PH}$ equivalently (Myers et al., 1995). Anti-calmodulin monoclonal antibody has been previously described (Sacks et al., 1991). Anti-myoglobin monoclonal antibody was kindly provided by Dr. Jack Ladenson (Washington University School of Medicine, St. Louis, MO); anti-phosphotyrosine antibody was from Transduction Laboratories; horseradish peroxidase-conjugated antibodies were purchased from Amersham. Ham's F12 medium was from Gibco. Insulin was a gift from Eli Lilly Co. Affi-Gel, protein A−agarose, and protein G−agarose were purchased from Bio-Rad. Calmodulin−Sepharose was from Pharmacia. Poly(vinylidene difluoride) (PVDF) membranes were purchased from Millipore. A23187 was obtained from Calbiochem, and trifluoperazine was from Sigma. All other chemicals and reagents were of standard analytical grade.

Cell Culture. CHO cells were grown to 80% confluence in Ham's F-12 medium containing 10% fetal calf serum. The medium was replaced with serum-free Ham's F-12 medium for 3 h before each experiment. Where indicated, cells were treated for 5 min with either 100 nM insulin in 0.01% fatty acid-free bovine serum albumin (BSA) or 0.01% fatty acid-free BSA as control. The reaction was terminated by replacing the medium with 2 mL of either buffer A (100 mM Tris-base, pH 7.4, 30 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM sodium vanadate, 2 mM ammonium molybdate, 5 μM $ZnCl_2$, 1 mM phenylmethanesulfonyl fluoride, and 0.5% Triton X-100) or buffer B (190 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 1.25% Triton X-100) containing 1 mM $CaCl_2$ or 5 mM EDTA/1 mM EGTA. The lysates were collected and quick-frozen at −70 °C in methanol.

In selected experiments, trifluoperazine (TFP) at a final concentration of 100 μM was added to the serum-free Ham's F-12 medium for 30 min prior to lysis of cells with buffer B containing 1 mM $CaCl_2$. Where indicated, the serum-free Ham's F-12 medium was supplemented with 1 mM $CaCl_2$ and 5 μM A23187 or an equal volume of A23187-free dimethyl sulfoxide for the last 15 min of incubation. The cells were then lysed with buffer B containing 1 mM $CaCl_2$. Cell viability was assessed by trypan blue exclusion.

Immunoprecipitation. Equal amounts of cell lysate were diluted with buffer B containing either 1 mM $CaCl_2$ or 5 mM EDTA/1 mM EGTA. After preclearing with blocked Affi-Gel beads without antibody, samples were immunoprecipitated with Affi-Gel-linked anti-calmodulin monoclonal antibody as described previously (Joyal & Sacks, 1994). The immunoprecipitates were washed 3 times with 1 mL of wash buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, and 1 mM sodium vanadate) containing either 1 mM $CaCl_2$ or 1 mM EGTA, followed by three additional washes with wash buffer lacking Triton X-100 and containing either 1 mM $CaCl_2$ or 1 mM EGTA. Immunoprecipitation with anti-myoglobin monoclonal antibody was performed as described for anti-calmodulin monoclonal antibody. Immunoprecipitation with anti-IRS-1 antibody was performed as described above with the following modifications. Both the preclearing and the collection of immune complexes were performed with protein A−agarose (Sun et al., 1992). All immunoprecipitated samples were heated for 2 min at 100 °C in solubilization buffer, and proteins were resolved by SDS−PAGE.

In selected experiments, immediately prior to washing the immunoprecipitates an aliquot of the supernatant was removed and solubilized in buffer containing 10.5% (w/v) SDS, 0.94 M sucrose, 125 mM Tris, pH 7.2, 7.5 mM EDTA, 14% (v/v) β-mercaptoethanol, and 0.04% (w/v) bromophenol blue. The samples were heated for 2 min at 100 °C, and proteins were resolved by SDS−PAGE.

Peptides corresponding to the regions between 106−126 (designated IQ1) and 839−859 (designated IQ3) of IRS-1 were synthesized by the Biopolymer Laboratory at Harvard Medical School. CHO^{IR}/IRS-1 cells were lysed in buffer B containing 1 mM $CaCl_2$. Both IQ peptides were added to the cell lysate to a final concentration of 50 μM and incubated for 14 h at 4 °C. Following immunoprecipitation with anti-calmodulin monoclonal antibody, the antigen−antibody complexes were collected with protein G−agarose. The immunoprecipitated samples were washed and processed as described above.

Binding of Calmodulin to IRS-1 in Vitro. IRS-1 was purified as described previously from Sf9 cells infected with baculovirus containing rat IRS-1 cDNA (Tanasijevic at al., 1993). Assays were performed by incubating 60 nM purified calmodulin with 60 nM purified IRS-1 in 1 mL of buffer B containing either 1 mM $CaCl_2$ or 5 mM EDTA/1 mM EGTA for 3 h at 4 °C. Where indicated, TFP was included in the reaction mixture at a final concentration of 60 nM, 600 nM, or 6 μM. Samples were immunoprecipitated with anti-calmodulin monoclonal antibody linked to Affi-Gel. The immunoprecipitated samples were washed and processed as described for the *in vivo* samples above. In other experiments, 60 nM purified IRS-1 was incubated with 10 μL of 1 mg/mL calmodulin−Sepharose in 1 mL of buffer B containing either 1 mM $CaCl_2$ or 5 mM EDTA/1 mM EGTA for 3 h at 4 °C. Calmodulin−Sepharose beads were washed and processed in the same manner as the immunoprecipitated samples.

Peptide competition assays were performed by preincubating varying concentrations of IQ1, IQ3, or both peptides with 20 μL of 1 mg/mL calmodulin−Sepharose in 0.5 mL of incubation buffer (150 mM NaCl, 1% Triton X-100, 10% glycerol, 20 mM Tris, pH 7.5, 2 mM PMSF, and 2 mM leupeptin) containing 100 μM $CaCl_2$ for 30 min at 4 °C. Purified IRS-1 was added, and samples were incubated for

14 h at 4 °C. Calmodulin−Sepharose beads were washed and processed as described above.

Immunoblotting. Following SDS−PAGE, gels were equilibrated in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, and 0.02% (w/v) SDS] for 10 min and transferred to PVDF at 100 V (constant voltage) for 2 h. Nonspecific binding sites were blocked with 3% (w/v) BSA in TBS (25 mM Tris, pH 8.0, 140 mM NaCl, and 2.7 mM KCl) for 14−16 h at 4 °C. The membranes were probed with anti-IRS-1, anti-IRS-2, or anti-phosphotyrosine antibody in TBS containing 0.05% (v/v) Tween 20 (TTBS) for 2 h at 22 °C, followed by horseradish peroxidase-labeled IgG in TTBS for 1 h at 22 °C. The protein complexes were detected with Amersham ECL Kit, and blots were exposed to Reflection NEF film (DuPont). Where indicated, membranes were stripped according to the instructions in the ECL Kit and reprobed with an alternate antibody, and the protein complexes were detected with ECL. Densitometry was performed using a Molecular Dynamics Imaging System and analyzed with ImageQuant Software.

RESULTS

Coimmunoprecipitation of Calmodulin and IRS Proteins from Intact Cells. To examine the interaction between calmodulin and IRS-1 in intact cells, CHO^{IR}/IRS-1 lysates were immunoprecipitated with either anti-calmodulin monoclonal antibody, an irrelevant isotype-identical monoclonal antibody (anti-myoglobin IgG_1 κ antibody), or anti-IRS-1 antibody. The anti-calmodulin antibody is highly specific with no significant cross-reactivity with other cellular components (Sacks & McDonald, 1988; Sacks et al., 1992; Joyal & Sacks, 1994). IRS-1 was detected in anti-calmodulin and anti-IRS-1 immunoprecipitates by immunoblotting with anti-IRS-1 antibody (Figure 1A). No IRS-1 was immunoprecipitated by anti-myoglobin antibody, confirming the specificity of binding (Figure 1A). This interaction was relatively strong since IRS-1 also coimmunoprecipitated with calmodulin from rat hepatocytes and 3T3 cells which contain only endogenous IRS-1 (data not shown). Under the conditions used, the amount of IRS-1 that coimmunoprecipitated with calmodulin was approximately 25% of the amount immunoprecipitated with the anti-IRS-1 antibody (Figure 1A). The anti-calmodulin antibody does not recognize IRS-1 as it did not immunoprecipitate purified recombinant IRS-1 (data not shown). In addition, IRS-2 (Figure 1B) and IRS-$1^{\Delta PH}$ (Figure 2A) immunoprecipitated with anti-calmodulin antibody from CHO cells overexpressing these IRS proteins. Thus, both IRS-1 and IRS-2 bind calmodulin, as does IRS-$1^{\Delta PH}$, suggesting that the PH domain is not involved in mediating IRS association with calmodulin.

Effect of Insulin on Coimmunoprecipitation of Calmodulin and IRS-1. Insulin promotes the association of IRS-1 with a variety of signalling proteins (Backer et al., 1993; Baltensperger et al., 1993; Kuhne et al., 1993; Lee et al., 1993; Skolnik et al., 1993a; Sun et al., 1993; Pons et al., 1995). To evaluate whether insulin also affects association of IRS-1 with calmodulin, lysates were prepared from insulin-stimulated CHO^{IR}/IRS-1 cells. Although equivalent amounts of calmodulin were detected in both preparations (data not shown), less IRS-1 appeared to coimmunoprecipate with calmodulin from insulin-stimulated CHO^{IR}/IRS-1 cells than from untreated cells. Presumably this observation reflects insulin-stimulated degradation of IRS-1 rather than a release of calmodulin from IRS-1. When the total lysate is corrected

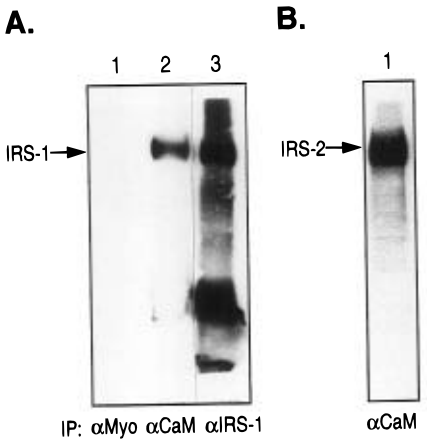

FIGURE 1: Coimmunoprecipitation of calmodulin and IRS-proteins from intact cells. (A) CHO^{IR}/IRS-1 cells were lysed in buffer A as described under Experimental Procedures. Equal amounts of cell lysate were immunoprecipitated (IP) with either anti-myoglobin monoclonal IgG1*k* antibody (αMyo), anti-calmodulin monoclonal IgG1κ antibody (αCaM), or anti-IRS-1 polyclonal antibody (αIRS-1). Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 antibody. Antigen−antibody complexes were detected by ECL. A representative experiment of two separate determinations is shown with the position of migration of IRS-1 indicated. (B) CHO^{IR}/IRS-2 cells were lysed as described under Experimental Procedures. Cell lysate was immunoprecipitated with anti-calmodulin monoclonal antibody. Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-2 antibody. Antigen−antibody complexes were detected by ECL. A representative experiment is shown with the position of migration of IRS-2 indicated.

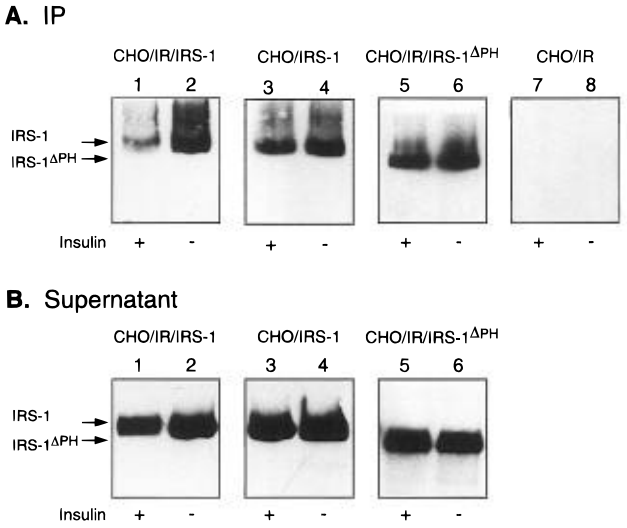

FIGURE 2: Effect of insulin on coimmunoprecipitation of calmodulin and IRS-1. (A) CHO^{IR}/IRS-1, CHO/IRS-1, CHO^{IR}/IRS-$1^{\Delta PH}$, and CHO^{IR} cells were treated with (+) or without (−) insulin and lysed in buffer B as described under Experimental Procedures. For each cell type, equal amounts of cell lysate were immunoprecipitated (IP) with anti-calmodulin monoclonal antibody. Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 antibody. Antigen−antibody complexes were detected by ECL. (B) An aliquot of the post-immunoprecipitation supernatant from CHO^{IR}/IRS-1, CHO/IRS-1, and CHO^{IR}/IRS-$1^{\Delta PH}$ cells was solubilized as described under Experimental Procedures. Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 antibody. Antigen−antibody complexes were detected by ECL. A representative experiment of four separate determinations is shown with the positions of migration of IRS-1 and IRS-$1^{\Delta PH}$ indicated.

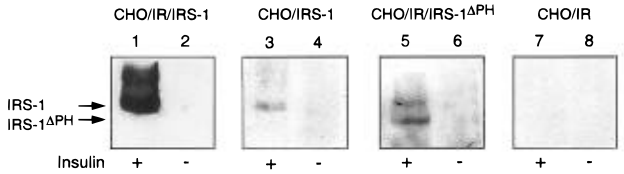

FIGURE 3: Tyrosine phosphorylation of IRS-1 in anti-calmodulin immunoprecipitates. The immunoblots depicted in Figure 2A were stripped and reprobed with anti-phosphotyrosine antibody as described under Experimental Procedures. Antigen−antibody complexes were detected by ECL. A representative experiment of three separate determinations is shown with the positions of migration of IRS-1 and IRS-1$^{\Delta PH}$ indicated. See legend to Figure 2 for experimental details.

for this insulin-stimulated reduction in the total amount of IRS-1 (Figure 2B), insulin has no apparent stimulatory effect on the association of IRS-1 with calmodulin. Furthermore, CHO/IRS-1 cells have low levels of endogenous insulin receptors, and insulin had no significant effect on the amount of IRS-1 that coimmunoprecipitated with calmodulin (Figure 2A). Similarly, the amount of IRS-1$^{\Delta PH}$ that coimmunoprecipitated with calmodulin was not significantly altered by insulin (Figure 2A). No IRS-1 was detected in anti-calmodulin immunoprecipitates from CHO cells that did not overexpress IRS-1 (Figure 2A).

To examine whether the IRS-1 present in the anti-calmodulin immunoprecipitates was tyrosine-phosphorylated in response to insulin, the immunoblots depicted in Figure 2A were stripped and reprobed with anti-phosphotyrosine antibody (Figure 3). Insulin promoted tyrosine phosphorylation of IRS-1 in all three cell types that overexpress IRS-1 proteins (Figure 3). The magnitude of the insulin effect was greatest in CHO^{IR}/IRS-1 cells. Although a low level of phosphotyrosine was detected in IRS-1$^{\Delta PH}$, this represents a very small fraction of the protein in the immunoprecipitate (Figure 2A). No band was detected in the region of IRS-1 or IRS-1$^{\Delta PH}$ in anti-calmodulin immunoprecipitates of CHO^{IR} cell lysates (Figure 3), confirming that the phosphoproteins detected with anti-phosphotyrosine antibody were IRS-1 or IRS-1$^{\Delta PH}$.

Effect of Ca^{2+} on the Binding of Calmodulin to IRS-1. Since calmodulin is an intracellular regulator of Ca^{2+} signals (Cohen & Klee, 1988), the effect of Ca^{2+} on the interaction between calmodulin and IRS-1 was evaluated. Purified recombinant IRS-1 was incubated *in vitro* with purified calmodulin in the presence or absence of Ca^{2+}, and samples were immunoprecipitated with anti-calmodulin monoclonal antibody. IRS-1 bound directly to calmodulin, and chelation of Ca^{2+} with EGTA/EDTA significantly decreased their association (Figure 4A). Similarly, IRS-1 bound in the presence of 1 mM Ca^{2+} to calmodulin that was immobilized on Sepharose beads, and minimal binding was observed in the absence of Ca^{2+} (Figure 4B).

The effect of Ca^{2+} on the binding of calmodulin to IRS-1 was examined with intact cells. The Ca^{2+}-ionophore A23187 increases intracellular Ca^{2+} concentrations by mediating the influx of extracellular Ca^{2+}. Incubation with A23187 significantly increased the association of calmodulin with IRS-1 in CHO^{IR}/IRS-1 cells (Figure 4C). A23187 had no effect on the short-term viability of CHO^{IR}/IRS-1 cells as determined by trypan blue exclusion (data not shown). This result is consistent with the finding that Ca^{2+} mediates the interaction of IRS-1 with calmodulin *in vitro*.

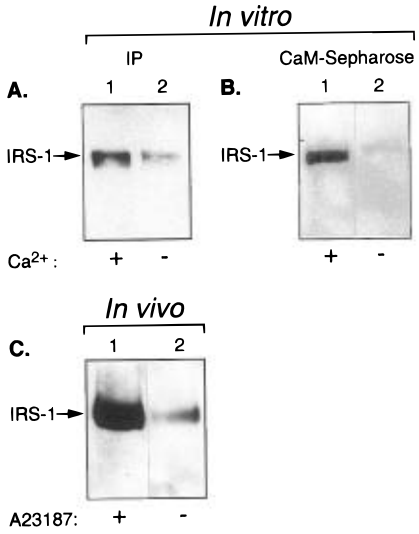

FIGURE 4: Effect of Ca^{2+} on binding of calmodulin to IRS-1. (A) Purified IRS-1 and calmodulin were incubated *in vitro* with 1 mM $CaCl_2$ ($+Ca^{2+}$) or 1 mM EGTA/5 mM EDTA ($-Ca^{2+}$) as described under Experimental Procedures. Calmodulin was immunoprecipitated (IP) with anti-calmodulin monoclonal antibody. (B) IRS-1 was incubated with calmodulin (CaM)−Sepharose in the presence of 1 mM $CaCl_2$ ($+Ca^{2+}$) or 1 mM EGTA/5 mM EDTA ($-Ca^{2+}$) as described under Experimental Procedures. (C) CHO^{IR}/IRS-1 cells were incubated with (+) or without (−) A23187 as described under Experimental Procedures, and cell lysates were immunoprecipitated with anti-calmodulin antibody. Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 antibody. Antigen−antibody complexes were detected by ECL. Representative experiments of three separate determinations are shown with the position of migration of IRS-1 indicated.

Effect of a Calmodulin Antagonist on Calmodulin−IRS-1 Interaction. Trifluoperazine, a widely accepted calmodulin antagonist (Motohashi, 1991), binds with high affinity to calmodulin (Levin & Weiss, 1977) by making extensive contacts with the C-terminal domain of calmodulin (Cook et al., 1994). Purified calmodulin and IRS-1 were incubated *in vitro* with 1 mM Ca^{2+} and varying concentrations of TFP. TFP significantly decreased the Ca^{2+}-dependent interaction between calmodulin and IRS-1 in a dose-dependent manner (Figure 5A). Since TFP enters cells (Kolesnick & Hemer, 1989), the effect of the calmodulin antagonist on the interaction between the two proteins was examined *in vivo*. No IRS-1 coimmunoprecipitated with calmodulin from cells incubated with TFP (Figure 5B), supporting the conclusion that the association between calmodulin and IRS-1 is specific. Importantly, TFP was removed from the outside of the cells immediately prior to cell lysis, indicating that it prevented the interaction between calmodulin and IRS-1 intracellularly.

Identification of Calmodulin-Binding Sites on IRS-1. Calmodulin binds to basic amphiphilic α-helices and to IQ motifs on proteins (Andreasen et al., 1983; Alexander et al., 1987; O'Neil & DeGrado, 1990; Baudier et al., 1991; Cheney & Mooseker, 1992; Brockerhoff et al., 1994). The IQ motif comprises a sequence of approximately 23 amino acids with the core fitting the consensus IQXXXRGXXXR (Cheney & Mooseker, 1992). IRS-1 contains at least three potential IQ motifs, and IRS-2 contains two (Figure 6A). Peptides corresponding to the IQ1 and IQ3 motifs in IRS-1 were used in peptide-competition assays. IQ1, IQ3, or both peptides were incubated *in vitro* with calmodulin−Sepharose and IRS-1 in the presence of 100 μM Ca^{2+}. IQ1 decreased

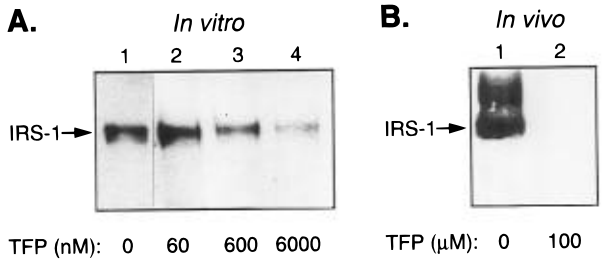

FIGURE 5: Effect of trifluoperazine on calmodulin−IRS-1 interaction. (A) Calmodulin and IRS-1 were incubated *in vitro* in the presence of 1 mM $CaCl_2$ and different concentrations of TFP as described under Experimental Procedures, and samples were immunoprecipitated with anti-calmodulin monoclonal antibody. (B) CHO^{IR}/IRS-1 cells were incubated with 100 μM TFP for 30 min. After removing the medium containing TFP, cells were lysed with buffer B containing 1 mM $CaCl_2$ as described under Experimental Procedures. Calmodulin was immunoprecipitated with anti-calmodulin monoclonal antibody, and immunoblots were probed with anti-IRS-1 antibody. Antigen−antibody complexes were detected by ECL. Representative experiments of three separate determinations for panel B and a single determination for panel A are shown with the position of migration of IRS-1 indicated.

binding of IRS-1 to calmodulin−Sepharose in a dose-dependent manner, with 55% inhibition occurring at 50 μM peptide (Figure 6B). A dose-dependent decrease in binding was also produced by IQ3, with 75% inhibition occurring at 50 μM peptide (Figure 6B). When IQ1 and IQ3 peptides were added together, the inhibition of binding of calmodulin to IRS-1 was not significantly different from that produced by IQ3 alone (Figure 6B). By contrast, a peptide from a non-IQ region of IRS-1, containing the region around tyrosine 608 where phosphatidylinositol 3-kinase binds (DDGPYPIMPMSPGNGDPYMPMSPKS), did not inhibit the association of IRS-1 with calmodulin (data not shown).

The effect of the IQ peptides on binding of calmodulin to IRS-1 was also examined using cell lysates. IQ1 and IQ3 peptides were added together to CHO^{IR}/IRS-1 cell lysate containing 1 mM Ca^{2+}, and calmodulin was immunoprecipitated with anti-calmodulin antibody. The IQ peptides markedly decreased the binding of calmodulin to IRS-1 (Figure 6C), suggesting that calmodulin binds to IRS-1 *in vivo* at least in part via IQ motifs.

DISCUSSION

We demonstrate a specific interaction between IRS-proteins and calmodulin, a ubiquitous mediator of Ca^{2+}-dependent physiologic processes. Coimmunoprecipitation revealed that the association between calmodulin and IRS-proteins in CHO cells is modulated by Ca^{2+}. Moreover, the association is mediated, at least in part, via conserved IQ motifs in IRS-1.

Incubation of CHO^{IR}/IRS-1 cells with insulin reduced the amount of IRS-1 that bound to calmodulin; however, this decrease corresponded to the reduction in the levels of IRS-1. These data are consistent with previous observations that insulin promotes proteolysis of IRS-1 (Rice et al., 1993), suggesting that the decreased levels of IRS-1 in the anti-calmodulin immunoprecipitates were due to insulin-mediated degradation of IRS-1. Note that there were no protease inhibitors in either the lysis or the immunoprecipitation buffers. Moreover, insulin had no significant effect on the amount of IRS-1 that coimmunoprecipitated with calmodulin from CHO/IRS-1 or CHO^{IR}/IRS-$1^{\Delta PH}$ cells, consistent with the stability of IRS-1 owing presumably to the low levels of insulin signalling in these cells.

Although insulin did not regulate the interaction between calmodulin and IRS-1, Ca^{2+} significantly altered binding. The affinity of IRS-1 for Ca^{2+}/calmodulin *in vitro* was markedly greater than for Ca^{2+}-free calmodulin. Similarly, increasing the intracellular free Ca^{2+} concentration with the Ca^{2+}-ionophore A23187 enhanced the binding of calmodulin to IRS-1. These results are consistent with the effect of Ca^{2+} on the interaction between calmodulin and its target proteins (Cohen & Klee, 1988). Although Ca^{2+} enhances the association of calmodulin with IRS-1, it should be noted that an IRS-1−calmodulin complex occurs without Ca^{2+}, albeit at a much lower level (data not shown). Thus, analogous to certain unconventional myosins (Bähler et al., 1994; Brockerhoff et al., 1994), IRS-1 may contain domains that allow it to interact with calmodulin both in the absence and in the presence of Ca^{2+}.

It is difficult to exclude interaction after cell lysis when employing the technique of coimmunoprecipitation. However, A23187 selectively increases the permeability of plasma membranes to Ca^{2+} (Borle & Snowdowne, 1982) and has no effect on Ca^{2+} concentrations after cell lysis. Therefore, the data indicate that both binding of calmodulin to IRS-1 and the modulation produced by Ca^{2+} occur inside the cell. Since approximately equivalent amounts of IRS-1 co-immunoprecipitated with calmodulin from CHO^{IR}/IRS-1 and CHO/IRS-1 cells, the *in vivo* interaction of calmodulin and IRS-1 does not occur via a ternary complex with the insulin receptor. Furthermore, *in vitro* studies using purified components confirm a direct interaction between calmodulin and IRS-1.

Proteins that bind to calmodulin either form basic amphiphilic α-helices (O'Neil & DeGrado, 1990) or contain IQ motifs (Andreasen et al., 1983; Alexander et al., 1987; Baudier et al., 1991; Cheney & Mooseker, 1992; Brockerhoff et al., 1994). Although most proteins with IQ motifs bind to Ca^{2+}-free calmodulin (Andreasen et al., 1983; Alexander et al., 1987; Baudier et al., 1991; Cheney & Mooseker, 1992; Brockerhoff et al., 1994), certain IQ motif-containing proteins bind Ca^{2+}/calmodulin. For example, the IQ motif of Ras-GRF (Farnsworth et al., 1995) and one of the IQ motifs of rat myr4 (Bähler et al., 1994) bind with higher affinity to Ca^{2+}/calmodulin than to Ca^{2+}-free calmodulin. The IQ motif comprises a sequence of approximately 23 amino acids with the core fitting the consensus IQXXXRGXXXR (Cheney & Mooseker, 1992). The conserved portion of the IQ motif (IQXXXR; the first residue need not be isoleucine, but is usually hydrophobic) is the most critical region and determines both the conformation and the positioning of the C-terminal lobe of calmodulin (Houdusse & Cohen, 1995). IRS-1 has at least three potential IQ motifs. These IQ motifs are "incomplete" as they have only the conserved NH_2-terminal region (Houdusse & Cohen, 1995). We hypothesized that one or more of the IQ motifs of IRS-1 may be responsible for binding to calmodulin. This was supported by the demonstration that the IQ1 and IQ3 peptides inhibited binding of purified calmodulin to IRS-1. Similar observations were made in peptide competition assays which revealed disruption of the association between calmodulin and IRS-1 in CHO^{IR}/IRS-1 cell lysates. IRS-$1^{\Delta PH}$ mutant, which does not have IQ1, coimmunoprecipitated with calmodulin, indicating that calmodulin binds to more than

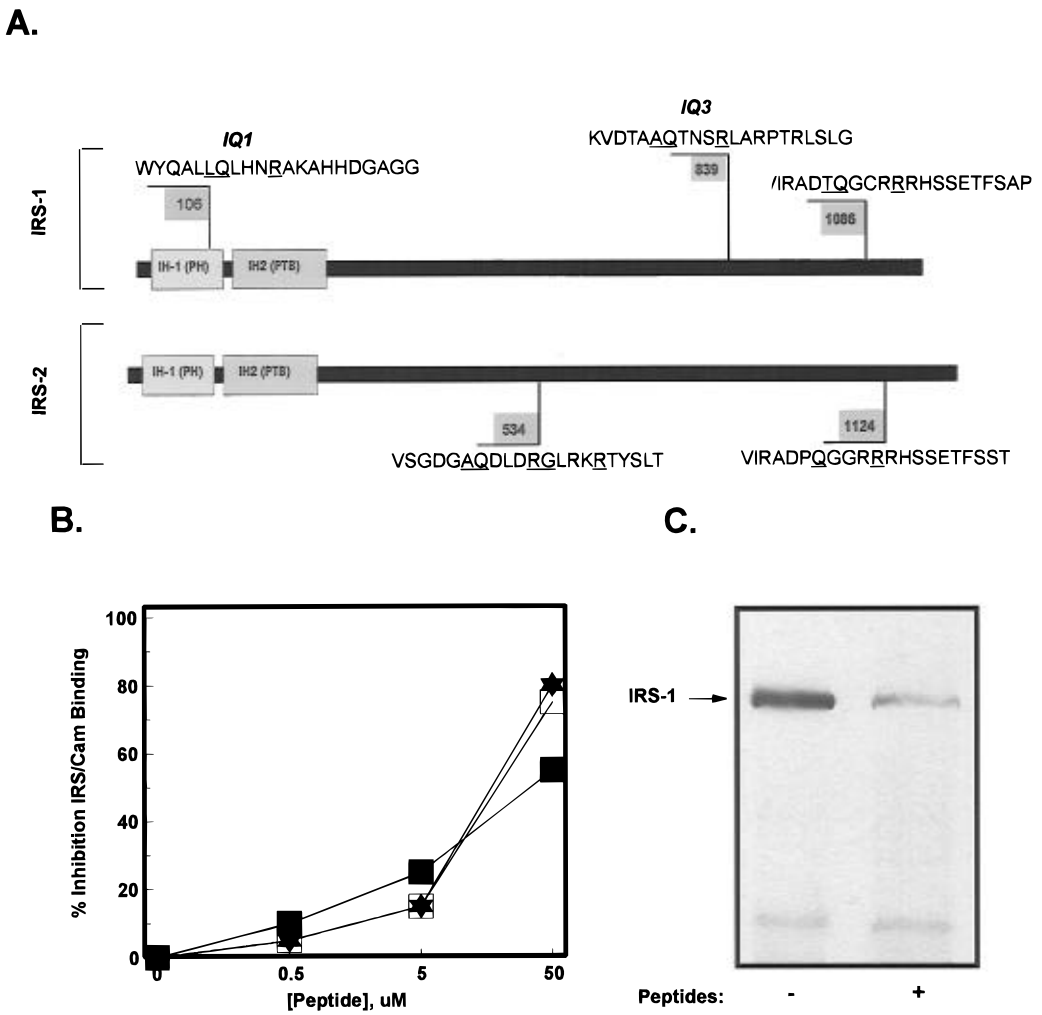

FIGURE 6: Identification of calmodulin-binding sites on IRS-1. (A) A linear model of rat IRS-1 and mouse IRS-2, with the location and sequence of potential IQ motifs indicated. Also shown are the two IRS-homology (IH) domains: IH1, which contains a pleckstrin homology (PH) domain, and IH2, which contains a phosphotyrosine binding (PTB) domain. (B) Synthetic peptides corresponding to IQ1 (■), IQ3 (□), or both IQ1 and IQ3 (★) were incubated *in vitro* with calmodulin−Sepharose and purified IRS-1 protein in the presence of 100 μM $CaCl_2$ as described under Experimental Procedures. Calmodulin−Sepharose beads were washed and processed by SDS−PAGE, and immunoblots were probed with anti-IRS-1 antibody. For quantification, densitometric analysis of the anti-IRS-1 Western blots was performed. Data are expressed as percentage inhibition of IRS-1/calmodulin (Cam) binding. (C) CHO^{IR}/IRS-1 cell lysates were incubated with (+) or without (−) 50 μM of both IQ1 and IQ3 in the presence of 1 mM $CaCl_2$ as described under Experimental Procedures. Calmodulin was immunoprecipitated with anti-calmodulin monoclonal antibody. Proteins were separated by SDS−PAGE, transferred to PVDF, and probed with anti-IRS-1 antibody. Antigen−antibody complexes were detected by ECL, and IRS-1 was quantified by densitometry. The data in panel B are representative of three separate experiments. A representative experiment of two separate determinations is shown for panel C.

one region of IRS-1. IQ3 may be the preferred binding site since the corresponding peptide displaced IRS-1 more efficiently than the IQ1 peptide. Although the motifs corresponding to IQ1 and IQ3 of IRS-1 are not present in IRS-2, the latter protein contains at least two other potential IQ motifs (see Figure 6A). Note that the IQ2 motif of IRS-2 (residues 534−554) is "complete" as it has both the highly conserved region and the second part (GXXXR) of the IQ motif (Houdusse & Cohen, 1995). This distinction is important because the binding of calmodulin to complete IQ motifs does not require Ca^{2+} (Houdusse & Cohen, 1995). Therefore, the IQ2 motif in IRS-2 may bind calmodulin at a lower Ca^{2+} concentration than the IQ motifs in IRS-1.

The interaction of calmodulin with its target proteins is altered by TFP, which makes extensive contacts with residues in the C-terminal domain of calmodulin, but few contacts at the N-terminus (Cook et al., 1994). IQ motifs also bind to the C-terminal lobe of calmodulin (Houdusse & Cohen, 1995), and both TFP (Cook et al., 1994) and IQ-peptides (Urbauer et al., 1995) alter the conformation of the C-terminus of calmodulin. Therefore, the inhibition of the association of calmodulin/IRS-1 by TFP is consistent with the involvement of an IQ motif in the binding. Furthermore, TFP prevented binding of IRS-1 to calmodulin in intact cells, suggesting that the association with IRS-1 is specific. The correlation between the *in vitro* and *in vivo* data supports direct and specific binding of calmodulin to IRS-1 inside the cells.

In conclusion, we have demonstrated that calmodulin binds to IRS-1 in intact cells in a Ca^{2+}-regulated manner, modulated, at least in part, via IQ motifs. The binding of calmodulin to IRS-1 is unique since it does not occur via SH2 domains nor does tyrosine phosphorylation of IRS-1 appear to be important for binding. The findings presented here suggest that Ca^{2+} may modulate insulin and other IRS-mediated signals by regulating the interaction between calmodulin and IRS-1. In this model, calmodulin may function as an adaptor to recruit Ca^{2+}-regulated molecules to the IRS−protein complex, thus establishing a mechanism for integrating Ca^{2+}-mediated events and IRS signalling.

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REFERENCES

Alexander, K. A., Cimler, B. M., Meier, K. E., & Storm, D. R. (1987) *J. Biol. Chem. 262*, 6108−6113.

Andreasen, T. J., Luetje, C. W., Heideman, W., & Storm, D. R. (1983) *Biochemistry 22*, 4615−4618.

Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., & Cook, W. J. (1985) *Nature 315*, 37−40.

Backer, J. M., Myers, M. G., Jr., Sun, X. J., Chin, D. J., Shoelson, S. E., Miralpeix, M., & White, M. F. (1993) *J. Biol. Chem. 268*, 8204−8212.

Bähler, M., Kroschewski, R., Stöffler, H.-E., & Behrmann, T. (1994) *J. Cell Biol. 126*, 375−389.

Baltensperger, K., Kozma, L. M., Cherniack, A. D., Klarlund, J. K., Chawla, A., Banerjee, U., & Czech, M. P. (1993) *Science 260*, 1950−1952.

Baudier, J., Deloulme, J. C., Van Dorsselaer, A., Black, D., & Matthes, H. W. D. (1991) *J. Biol. Chem. 266*, 229−237.

Berridge, M. J. (1993) *Nature 361*, 315−325.

Borle, A. B., & Snowdowne, K. W. (1982) *Science 217*, 252−254.

Brockerhoff, S. E., Stevens, R. C., & Davis, T. N. (1994) *J. Cell Biol. 124*, 315−324.

Cheney, R. E., & Mooseker, M. S. (1992) *Curr. Opin. Cell Biol. 4*, 27−35.

Cohen, P., & Klee, C. B. (1988) *Calmodulin*, Elsevier, New York.

Colca, J. R., DeWald, D. B., Pearson, J. D., Palazuk, B. J., Laurino, J. P., & McDonald, J. M. (1987) *J. Biol. Chem. 262*, 11399−11402.

Cook, W. J., Walter, L. J., & Walter, M. R. (1994) *Biochemistry 33*, 15259−15265.

Draznin, B., Sussman, K., Kao, M., Lewis, D., & Sherman, N. (1987) *J. Biol. Chem. 262*, 14385−14388.

Farnsworth, C. L., Freshney, N. W., Rosen, L. B., Ghosh, A., Greenberg, M. E., & Feig, L. A. (1995) *Nature 376*, 524−527.

Graves, C. B., Gale, R. D., Laurino, J. P., & McDonald, J. M. (1986) *J. Biol. Chem. 261*, 10429−10438.

Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., & Wong, A. J. (1996) *Nature 379*, 560−564.

Houdusse, A., & Cohen, C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10644−10647.

Joyal, J. L., & Sacks, D. B. (1994) *J. Biol. Chem. 269*, 30039−30048.

Kolesnick, R. N., & Hemer, M. R. (1989) *J. Biol. Chem. 264*, 14057−14061.

Kuhne, M. R., Pawson, T., Lienhard, G. E., & Feng, G.-S. (1993) *J. Biol. Chem. 268*, 11479−11481.

Lee, C.-H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G., Jr., White, M. F., Schlessinger, J., & Skolnik, E. Y. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 11713−11717.

Levin, R. M., & Weiss B. (1977) *Mol. Pharmacol. 13*, 690−697.

Levy, J., Gavin, J. R., III, & Sowers, J. R. (1994) *Am. J. Med. 96*, 260−273.

Meador, W. E., Means, A. R., & Quiocho, F. A. (1992) *Science 257*, 1251−1255.

Meador, W. E., Means, A. R., & Quiocho, F. A. (1993) *Science 262*, 1718−1721.

Motohashi, N. (1991) *Anticancer Res. 11*, 1125−1164.

Myers, M. G., Jr., Grammer, T. C., Brooks, J., Glasheen, E. M., Wang, L.-M., Sun, X. J., Blenis, J., Pierce, J. H., & White, M. F. (1995) *J. Biol. Chem. 270*, 11715−11718.

O'Neil, K. T., & DeGrado, W. F. (1990) *Trends Biochem. Sci. 15*, 59−64.

Pons, S., Asano, T., Glasheen, E., Miralpeix, M., Zhang, Y., Fisher, T. L., Myers, M. G., Jr., Sun, X. J., & White, M. F. (1995) *Mol. Cell. Biol. 15*, 4453−4465.

Pronk, G. J., McGlade, J., Pelicci, G., Pawson, T., & Bos, J. L. (1993) *J. Biol. Chem. 268*, 5748−5753.

Rice, K. M., Turnbow, M. A., & Garner, C. W. (1993) *Biochem. Biophys. Res. Commun. 190*, 961−967.

Sacks, D. B., & McDonald, J. M. (1988) *J. Biol. Chem. 263*, 2377−2383.

Sacks, D. B., Fujita-Yamaguchi, Y., Gale, R. D., & McDonald, J. M. (1989) *Biochem. J. 263*, 803−812.

Sacks, D. B., Porter, S. E., Ladenson, J. H., & McDonald, J. M. (1991) *Anal. Biochem. 194*, 369−377.

Sacks, D. B., Davis, H. W., Crimmins, D. L., & McDonald, J. M. (1992) *Biochem. J. 286*, 211−216.

Skolnik, E. Y., Batzer, A., Li, N., Lee, C.-H., Lowenstein, E., Mohammadi, M., Margolis, B., & Schlessinger, J. (1993a) *Science 260*, 1953−1955.

Skolnik, E. Y., Lee, C.-H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ullrich, A., White, M. F., & Schlessinger, J. (1993b) *EMBO J. 12*, 1929−1936.

Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., & White, M. F. (1991) *Nature 352*, 73−77.

Sun, X. J., Miralpeix, M., Myers, M. G., Jr., Glasheen, E. M., Backer, J. M., Kahn, C. R., & White, M. F. (1992) *J. Biol. Chem. 267*, 22662−22672.

Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., & White, M. F. (1993) *Mol. Cell. Biol. 13*, 7418−7428.

Sun, X. J., Wang, L.-M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glasheen, E., Lane, W. S., Pierce, J. H., & White, M. F. (1995) *Nature 377*, 173−177.

Tanasijevic, M. J., Myers, M. G., Jr., Thoma, R. S., Crimmins, D. L., White, M. F., & Sacks, D. B. (1993) *J. Biol. Chem. 268*, 18157−18166.

Turk, J., Wolf, B. A., & McDaniel, M. L. (1987) *Prog. Lipid Res. 26*, 125−181.

Urbauer, J. L., Short, J. H., Dow, L. K., & Wand, A. J. (1995) *Biochemistry 34*, 8099−8109.

White, M. F., & Kahn, C. R. (1994) *J. Biol. Chem. 269*, 1−4.

White, M. F., Maron, R., & Kahn, C. R. (1985) *Nature 318*, 183−186.

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