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The Insulin Receptor Contains a Calmodulin-Binding Domain

Abstract. *Substantial evidence suggests that calcium has a pivotal role in regulating the initial events through which insulin alters plasma membrane metabolism. Because binding of insulin to its receptor represents the initial site of insulin action in the plasma membrane, studies were undertaken to determine whether the insulin receptor is a calmodulin-binding protein. Preparations enriched in the insulin receptor and calmodulin-binding proteins were isolated from detergent-solubilized rat adipocyte membranes by chromatography with wheat germ agglutinin agarose and calmodulin-conjugated Sepharose, respectively. Substantial purification of a manganese-dependent, insulin-sensitive phosphoprotein of 95K identified as the β subunit of the insulin receptor was accomplished. Binding and photocovalent cross-linking of iodine-125–labeled calmodulin to these affinity-purified preparations and to isolated plasma membranes, followed by immunoadsorption with insulin receptor antibodies bound to protein A Sepharose, resulted in significant purification of a binding complex of 110K to 140K. These results indicate that the adipocyte insulin receptor or a polypeptide closely associated with the receptor is a calmodulin-binding protein.*

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An association between Ca^{2+} and the action of insulin in the adipocyte plasmalemma is supported by observations that (i) insulin increases plasma membrane Ca^{2+} binding (*1*, *2*), (ii) the hormone decreases the activity (*3–6*) and phosphorylation (*7*) of a calmodulin-sensitive, high-affinity (Ca^{2+} and Mg^{2+}) adenosinetriphosphatase (ATPase), and (iii) extracellular Ca^{2+} is required for maximal insulin-stimulated glucose transport (*8*) and binding of insulin to (*9*) and phosphorylation of (*10*) the insulin receptor. The involvement of calmodulin is supported by the recent demonstration that insulin induces concentration-dependent increases (up to 75 percent) in binding of [^{125}I]calmodulin after addition of the hormone to adipocyte plasma membranes (*11*).

The insulin receptor is a heterologous molecule composed of two α (130K) and two β (95K) subunit glycoproteins linked by disulfide bonds (*12*). Binding studies with [^{125}I] insulin used in conjunction with cross-linking reagents or photoaffinity probes have suggested that the α subunit is the major insulin-binding domain (*13–16*). The β subunit is a tyrosine-specific protein kinase, and binding of insulin to its receptor stimulates this activity as well as phosphorylation of the β subunit (*17–20*). More importantly, the ability of insulin to stimulate this phosphorylation in detergent-solubilized membranes and purified receptor preparations provides a simple and convenient means to identify and monitor purification of the receptor.

Using insulin-stimulated phosphorylation as a marker, we determined that the β subunit of the insulin receptor was enriched in detergent-solubilized adipocyte membrane proteins isolated by affinity chromatography with calmodulin-conjugated Sepharose. As shown in lanes 3 and 4 in Fig. 1, an insulin-sensitive phosphoprotein of 95K was substantially purified by this approach. The specificity of this purification process is supported by the fact that the material applied to calmodulin Sepharose was characterized by several phosphoproteins (lanes 1 and 2 in Fig. 1). Moreover, similar results were observed after chromatography of solubilized membrane proteins with wheat germ agglutinin (WGA) agarose (lanes 5 and 6 in Fig. 1). The latter approach has been extensively used for enriching the insulin receptor (*17, 18, 21, 22*). Definitive identification of the insulin-sensitive 95K phosphoprotein illustrated in Fig. 1 as the β subunit was provided by its immunochemical isolation with antibody to the insulin receptor both before and after affinity chromatography. Thus, these results indicate that the insulin receptor binds to calmodulin in detergent-solubilized rat adipocyte membranes.

As evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, the profiles of proteins purified on calmodulin Sepharose and WGA agarose were markedly different. WGA-purified proteins were characterized by the presence of four major bands, of which the α and β subunits of the insulin receptor represented at least 50 percent of the stained protein. On the other hand, silver-stained gels of proteins purified on calmodulin Sepharose were characterized by a diverse array of proteins (12 major bands), of which the α and β subunits represented not more than 10 percent of the stained material. Thus, because the amounts of protein in the phosphorylation reactions depicted in lanes 3 and 4 [calmodulin-binding proteins (calmodulin BP's)] and lanes 5 and 6 (WGA BP's) in Fig. 1 were identical (0.5 μg per assay), the insulin receptor and hence the phosphorylated β subunit were present in greater amounts in the WGA BP preparation.

Using insulin-stimulated phosphorylation of the β subunit as a marker for the insulin receptor, we monitored the recovery of the receptor before adsorption to, and after elution from, WGA agarose and calmodulin Sepharose. Approximately 80 to 90 percent of the receptors present in detergent-solubilized plasma membranes were recovered from WGA agarose. When the WGA-purified receptor preparation was passed over calmodulin Sepharose, 25 to 50 percent of the receptors were recovered. These results suggest that only portions of the insulin receptor molecules bind to calmodulin Sepharose. Although it is tempting to speculate that the lack of quantitative binding reflects heterogeneity in the structure and calmodulin-binding properties of the receptor (for example, dissociation of α and β subunits), the reason for this apparent incongruity is unclear.

Photoaffinity labeling studies indicated that calmodulin binds to a protein in both calmodulin Sepharose– and WGA agarose–purified material which undergoes substantial purification after immunoadsorption with antibodies that recognize the insulin receptor. Calmodulin Sepha-

rose– and WGA agarose–purified proteins were incubated with [^{125}I]calmodulin and subjected to photocovalent cross-linking with the bifunctional cross-linking reagent *N*-hydrodxysuccinimidyl-4-azidobenzoate (HSAB). As shown in lanes 1 and 2 in Fig. 2, binding and cross-linking of [^{125}I]calmodulin to detergent-solubilized, calmodulin Sepharose–purified proteins revealed a broad band of radioactivity corresponding to a binding complex ranging from 110K to 140K which was specifically isolated by immunoadsorption with antibody to the insulin receptor. Moreover, identical studies with WGA agarose–purified material also resulted in immunospecific isolation of an [^{125}I]calmodulin-binding complex of 110K to 140K (lanes 3 and 4 in Fig. 2). The quantity of insulin receptor bound to and eluted from calmodulin Sepharose was less than that obtained by chromatography with WGA agarose. Yet the amount of ^{125}I-labeled cross-linked material adsorbed to and eluted from the insulin receptor antibody immunoadsorbent was roughly the same in the calmodulin BP (lane 2) and WGA BP (lane 4) preparations. This apparent anomaly is attributable to the fact that immunoadsorption was conducted under conditions of antigen excess.

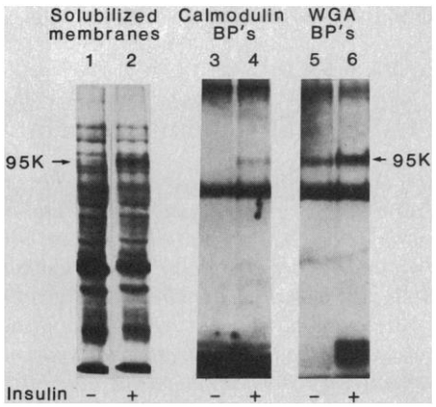

Fig. 1. Purification of the insulin receptor after affinity chromatography of detergent-solubilized adipocyte membranes with calmodulin Sepharose. Rat adipocytes were isolated from epididymal fat pads (*33*) and processed to yield the P3 membrane fraction, which contains predominantly plasma membranes and mitochondria (*34*). The membrane pellets were suspended in 50 m*M* Hepes (*p*H 7.4) containing 1 percent (by volume) Triton X-100 and 1 m*M* phenylmethylsulfonyl fluoride and incubated for 60 minutes at 4°C. The resultant detergent-solubilized membrane preparation (solubilizate) was used for in vitro phosphorylation assays or subjected to affinity chromatography on calmodulin Sepharose or WGA agarose. Calmodulin was purified from rat brain (*35*) and conjugated to CNBr-activated Sepharose 4B (*36*). Triton solubilizate (15 mg of protein; 1.0 ml) was adjusted to contain 100 μ*M* $CaCl_2$ and applied to a calmodulin-Sepharose column (1.5 by 5 cm) equilibrated with 20 m*M* tris-HCl (*p*H 7.4), 0.1 percent (by volume) Triton X-100, 1.0 m*M* $MgCl_2$, 2.0 m*M* $CaCl_2$, 1.0 m*M* EDTA, and 0.5 m*M* EGTA. Protein in the effluent was monitored by absorbance at 254 nm and the column was washed with equilibration buffer until no further protein was eluted. Calmodulin BP's were then eluted with 20 m*M* tris-HCl (*p*H 7.4), 0.1 percent (by volume) Triton X-100, 1.0 m*M* EDTA, and 4.0 m*M* EGTA. Affinity chromatography with WGA agarose and isolation of WGA BP's was accomplished as described by Kasuga *et al.* (*37*). Phosphorylation assays were conducted in a final volume of 250 μl containing 0.2 percent (by volume) Triton X-100, 25 m*M* Pipes (*p*H 7.4), 5.0 m*M* $MnCl_2$, 0.5 m*M* EGTA, and 25 μ*M* [γ-^{32}P]adenosine triphosphate in the absence (−) or presence (+) of 67 n*M* insulin. Reactions were initiated by adding 50 μg of solubilized membrane protein (lanes 1 and 2), 0.5 μg of calmodulin BP's (lanes 3 and 4), or 0.5 μg of WGA BP's (lanes 5 and 6), followed by incubation for 30 minutes at 25°C. Phosphorylation was initiated by adding [^{32}P]ATP, followed by incubation for 5 minutes at 30°C. Labeling was terminated by adding 250 μl of ice-cold 10 percent (weight to volume) trichloroacetic acid. Precipitates were collected by microcentrifugation and prepared for electrophoresis by solubilization in 100 μl of solution containing 25 m*M* tris-HCl (*p*H 7.2), 2.5 percent (weight to volume) SDS, 2 percent (by volume) β-mercaptoethanol, 2.0 m*M* EDTA, 0.25*M* sucrose, and 0.01 percent (weight to volume) bromphenol blue. SDS-PAGE was conducted in 9 percent gels (*38*). Autoradiography of dried gels was accomplished with Kodak XAR-5 film and DuPont Cronex Lightning-Plus intensifying screens. Migration of the 95K β subunit of the insulin receptor is designated.

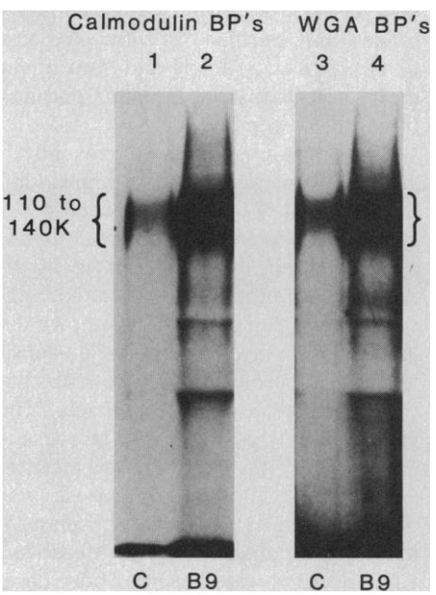

Fig. 2. Immunospecific purification with insulin receptor antibodies of a calmodulin-protein complex of 110K to 140K from calmodulin Sepharose– and WGA agarose–purified adipocyte membrane proteins. Purified rat brain calmodulin was labeled with ^{125}I by using chloramine-T–derivatized polystyrene beads (IODO-Beads, Pierce) (*39*). Calmodulin Sepharose BP's and WGA agarose BP's prepared as described in the legend to Fig. 1 were incubated with [^{125}I]-labeled calmodulin (final concentration 1 μ*M*; 2.0 × 10^6 cpm/μg) for 30 minutes at 25°C in 200 μl of buffer mixture containing 25 m*M* Pipes (*p*H 7.4), 0.1 percent (weight to volume) Triton X-100, 1.0 m*M* EGTA, and 1.1 m*M* $CaCl_2$. Samples were then adjusted to contain 50 μ*M* HSAB, incubated for 5 minutes at 4°C, and photolyzed (*23*). The resultant photocovalent cross-linked preparations were incubated with protein A Sepharose–bound antibody [immunoglobulin G (IgG)] to the insulin receptor (B9) or with nonimmune control IgG (C) for 16 hours at 4°C with gentle rotation. Unbound contaminants were removed by washing the immunoadsorbents with 50 m*M* tris-HCl (*p*H 7.4), 150 m*M* NaCl, and 2 percent (weight to volume) bovine serum albumin by repeated suspension and low-speed centrifugation. Immunospecifically retained material was eluted with 20 m*M* tris-HCl (*p*H 7.2) containing 7*M* urea and 2 percent SDS. Electrophoretic characterization of the eluted material was carried out as described in the legend to Fig. 1.

Identical experiments with isolated plasma membranes confirmed that [^{125}I]calmodulin binds to a protein having immunologic identity with the insulin receptor. Binding and photocovalent cross-linking of [^{125}I]calmodulin to intact adipocyte plasma membranes resulted in formation of four major binding complexes of 140K, 125K, 112K, and 102K (lane 3 in Fig. 3). The 125K binding complex was specifically isolated after detergent solubilization of these membranes and immunoadsorption with insulin receptor antibodies (lane 2 in Fig. 3). Importantly, binding and cross-linking of [^{125}I]calmodulin to proteins in calmodulin Sepharose– and WGA agarose–purified material and plasma membranes were dramatically reduced in experiments conducted in the presence of a 100-fold excess of unlabeled calmodulin. Thus, binding of calmodulin to proteins having immunologic reactivity with insulin receptor antibodies in these preparations was specific.

The broadness of the radiolabeled material illustrated in Fig. 2 makes comparison with the discrete bands in Fig. 3 somewhat difficult. It is, however, important to point out that the molecular weight range 110K to 140K determined for the material represented in Fig. 2 corresponds to an average molecular weight of 125K, which in turn corresponds to the 125K species purified by immunoadsorption (lane 2 in Fig. 3). These differences in electrophoretic migratory behavior (broad versus discrete bands) may be attributable to the fact that binding and cross-linking of [^{125}I]calmodulin was conducted in the presence of a detergent (0.1 percent Tri-

ton X-100) in the experiments represented in Fig. 2 but in the absence of detergent in the experiments depicted in Fig. 3. Indeed, cross-linking of [^{125}I]calmodulin to detergent-solubilized membranes resulted in the formation of broad-binding complexes that overlap to such an extent that separate binding species cannot be identified. This is in contrast to the discrete bands observed when [^{125}I]calmodulin is cross-linked to intact membranes (*23*).

Calmodulin binds and can be photocovalently cross-linked to a protein with a resultant complex molecular weight of 125K which has antigenic determinants recognizable by insulin receptor antibodies. Binding of calmodulin (17K) to the α (130K) or β (95K) subunit would theoretically result in complexes with aggregate molecular weights of 147K and 112K, respectively. While the 125K species does not correspond to these theoretical complexes, photoaffinity cross-linking of calmodulin to a protein could alter the electrophoretic migratory properties of the resultant binding complex, yielding anomalously higher or lower molecular weights than those predicted by simply adding the molecular weights of the free unbound proteins. On the other hand, the enrichment of the β subunit of the insulin receptor by calmodulin-Sepharose chromatography indicates that there is at least a calmodulin-binding domain associated with the β subunit. Along these lines, binding of two calmodulin molecules (2 × 17K) to one β subunit (95K) would yield a binding complex of 129K.

It is also possible that calmodulin binds to a protein that is closely associated with the insulin receptor. Indeed, in addition to the α and β subunits, there appear to be other proteins that bind to and have been proposed to regulate the receptor (*24–26*). A calmodulin-sensitive, high-affinity (Ca^{2+} and Mg^{2+})-ATPase copurifies with the insulin receptor after chromatography of detergent-solubilized adipocyte plasma membranes with wheat germ lectin Sepharose (*27*). It has been proposed that the ATPase and the insulin receptor are functionally and spatially coupled and that the interaction of calmodulin with these entities plays a role in the mechanism by which insulin alters plasma membrane Ca^{2+} homeostasis (*5, 28*).

In conclusion, it appears that the insulin receptor of rat adipocytes has a calmodulin-binding domain. Although the functional significance of this interaction is unclear, we have evidence that Ca^{2+} and calmodulin increase the affinity of the insulin receptor for insulin in isolated adipocyte plasma membranes (*29*). Moreover, Ca^{2+} and calmodulin enhance insulin-stimulated phosphorylation of the β subunit of the insulin receptor in affinity-purified receptor preparations (*30*). Finally, we (*31*) and others (*32*) recently demonstrated that insulin stimulates phosphorylation of calmodulin in enriched preparations of the insulin receptor. Thus, it appears that the interaction of calmodulin with the insulin receptor is linked to at least two early events in insulin action, insulin binding and stimulation of insulin receptor phosphorylation. Collectively, these observations provide additional evidence that Ca^{2+} and calmodulin occupy an important niche in the molecular mechanism of insulin action.

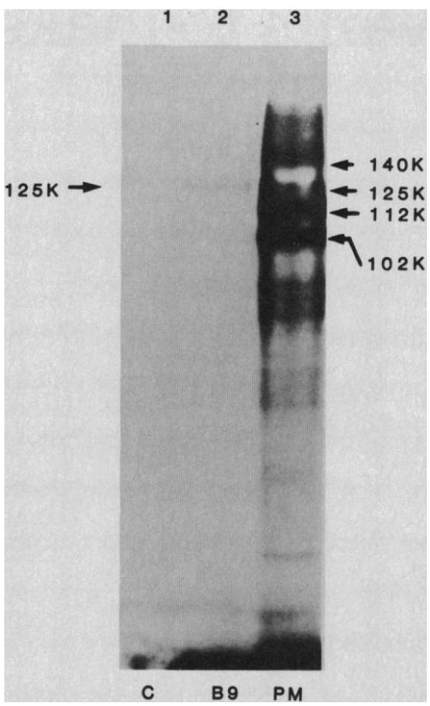

Fig. 3. Immunospecific purification with insulin receptor antibodies of a calmodulin-protein complex of 125K from adiopocyte plasma membranes. Rat adipocyte plasma membranes were isolated as described by Jarett (*34*). Membranes (50 μg of protein per assay) were incubated with [^{125}I]calmodulin, treated with HSAB, and photolyzed. After photolysis, membranes were pelleted by microcentrifugation and solubilized with Triton X-100 or characterized by SDS-PAGE (PM, lane 3). Triton-solubilized membranes were adsorbed to and eluted from protein A Sepharose–bound control IgG (C, lane 1) and antibody (IgG) to insulin receptors (B9, lane 2). The molecular weights of [^{125}I]calmodulin cross-linked protein complexes are designated.

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