Identification of Insulin-Stimulated Phosphorylation Sites on Calmodulin†

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ABSTRACT: Insulin enhances calmodulin phosphorylation *in vivo*. To determine the insulin-sensitive phosphorylation sites, phosphocalmodulin was immunoprecipitated from Chinese hamster ovary cells expressing human insulin receptors (CHO/IR). Calmodulin was constitutively phosphorylated on serine, threonine, and tyrosine residues, and insulin enhanced phosphate incorporation on serine and tyrosine residues. Phosphocalmodulin immunoprecipitated from control and insulin-treated CHO/IR cells, and calmodulin phosphorylated *in vitro* by the insulin receptor kinase and casein kinase II were resolved by two-dimensional phosphopeptide mapping. Several common phosphopeptides were detected. The phosphopeptides from the *in vitro* maps were eluted and phosphoamino acid analysis, manual sequencing, strong cation exchange chromatography, and additional proteolysis were performed. This strategy demonstrated that Tyr-99 and Tyr-138 were phosphorylated *in vitro* by the insulin receptor kinase and Thr-79, Ser-81, Ser-101 and Thr-117 were phosphorylated by casein kinase II. *In vivo* phosphorylation sites were identified by comigration of phosphopeptides on two-dimensional maps with phosphopeptides derived from calmodulin phosphorylated *in vitro* and by phosphoamino acid analysis. This approach revealed that Tyr-99 and Tyr-138 of calmodulin were phosphorylated in CHO/IR cells in response to insulin. Additional sites remain to be identified. The identification of the insulin-stimulated *in vivo* tyrosine phosphorylation sites should facilitate the elucidation of the physiological role of phosphocalmodulin.

The intracellular mechanism by which insulin controls glucose and amino acid uptake, glucose and fatty acid metabolism, gene expression, and protein synthesis is poorly understood. The insulin receptor is a heterotetramer composed of two extracellular α-subunits linked to two transmembrane β-subunits. Binding of insulin to the α-subunits activates the intrinsic tyrosine kinase which phosphorylates receptor β-subunits (White & Kahn, 1994). The activated receptor kinase catalyzes the phosphorylation of target proteins (Kasuga et al., 1990), but few endogenous substrates have been well characterized. Several substrates are thought to be intermediates in a signaling cascade that leads to the activation of downstream protein kinases, including phosphatidylinositol 3-kinase (White & Kahn, 1994), casein kinase II (Klarlund & Czech, 1988; Haystead et al., 1988), and MAP kinase (White & Kahn, 1994). For example, tyrosine-phosphorylated insulin receptor substrate-1 (IRS-1)[1] activates phosphatidylinositol 3-kinase through an interaction with the SH2 domain of its 85 kDa regulatory subunit (Myers & White, 1992). Although there has been considerable progress toward understanding the protein components involved in the insulin signaling pathway, there remains a lack of a clear comprehension of the function of several of the insulin receptor substrates.

Calmodulin, a ubiquitous Ca^{2+}-dependent effector protein, regulates multiple cellular processes including cyclic nucleotide metabolism, gene expression, protein phosphorylation, and cell division [reviewed in Cohen and Klee (1988)]. Calmodulin is an *in vitro* substrate for the insulin receptor kinase (Haring et al., 1985; Sacks & McDonald, 1988; Sacks et al., 1989) and the insulin-sensitive serine/threonine kinase, casein kinase II (Meggio et al., 1987; Sacks et al., 1992a). 1−2 mol of phosphate is incorporated per mol of calmodulin on Tyr-99 and Tyr-138 by the insulin receptor kinase (Joyal & Sacks, 1994; Williams et al., 1994) and on Thr-79, Ser-81, Ser-101, and Thr-117 by casein kinase II (Sacks et al., 1992a). In addition, Src kinase (Fukami et al., 1986), the epidermal growth factor receptor kinase (Benguria et al., 1994), a spleen tyrosine kinase (Meggio et al., 1987), and phosphorylase kinase (Plancke & Lazarides, 1983) phosphorylate calmodulin *in vitro*. Phosphorylation of calmodulin alters its ability to activate several calmodulin-dependent enzymes, including cyclic nucleotide phosphodiesterase (Williams et al., 1994; Sacks et al., 1992b), myosin-light-chain kinase (Sacks et al., 1992b), Ca^{2+}/calmodulin-dependent protein kinase II (Sacks et al., 1995), and the plasma membrane Ca^{2+}-ATPase (Quadroni et al., 1994).

Several lines of evidence indicate that calmodulin is phosphorylated *in vivo*. Phosphocalmodulin has been detected in chicken fibroblasts (Fukami et al., 1986), rat and chicken brain (Plancke & Lazarides, 1983; Nakajo et al., 1988), rat liver (Quadroni et al., 1994), and in ^{32}P-loaded

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[1] Abbreviations; IRS-1, insulin receptor substrate-1; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TCA, trichloroacetic acid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; CHO/IR, Chinese hamster ovary cells expressing the human insulin receptor.

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rat hepatocytes (Sacks et al., 1992a; Joyal & Sacks, 1994) and adipocytes (Colca et al., 1987). Importantly, insulin enhances calmodulin phosphorylation 3-fold in hepatocytes on serine, threonine, and tyrosine residues (Sacks et al., 1992a), to a stoichiometry of at least 0.5 mol of phosphate/mol of calmodulin (Joyal & Sacks, 1994). Although several of the constitutive phosphorylation sites on calmodulin have been identified in rat liver (Quadroni et al., 1994), the insulin-sensitive sites of phosphorylation have not been determined in intact cells.

In this study, phosphoamino acid analysis, manual Edman degradation, and HPLC analysis were performed on tryptic phosphopeptides of calmodulin phosphorylated *in vitro*. Comigration on two-dimensional maps and phosphoamino acid analysis of tryptic phosphopeptides of calmodulin immunoprecipitated from control and insulin-treated CHO/IR cells were employed to determine the insulin-sensitive *in vivo* phosphorylation sites.

MATERIALS AND METHODS

Materials. Phosphate-free RPMI 1640 was from Gibco/BRL. Radiochemicals were obtained from Du Pont-New England Nuclear. Affi-Gel was purchased from Bio-Rad. Ca^{2+}-free pig brain calmodulin was from Ocean Biologics. Immobilon P^{SQ} was obtained from Millipore. Sequencing grade TPCK-treated trypsin was purchased from Promega. Thin-layer chromatography cellulose plates were from Eastman Kodak. Materials and reagents for HPLC and manual Edman degradation were obtained from the suppliers indicated previously (Sacks et al., 1992a; Tanasijevic et al., 1993). Insulin was a gift from Eli Lilly Co. The baculovirus construct containing the coding sequence for the cytoplasmic domain of the insulin receptor was generously donated by Dr. R. Kallen (University of Pennsylvania, Philadelphia, PA). CHO/IR cells were kindly provided by Dr. M. White (Joslin Diabetes Center, Boston, MA). An engineered calmodulin with phenylalanine for Tyr-138 (Y138F)[2] was a gift from Dr. C.-L. A. Wang (Boston Biomedical Institute, Boston, MA). All other chemicals and reagents were of standard analytical grade.

Cell Culture and Calmodulin Labeling. CHO/IR cells were grown to 80% confluence in Ham's F-12 medium with 10% fetal bovine serum. The medium was replaced with serum-free, phosphate-free RPMI 1640 for 1 h, after which cells were labeled with 2 mCi of [^{32}P]orthophosphate/mL at 37 °C in the same medium containing 1 mM sodium orthovanadate. After 2 h, 100 nM insulin or an equal volume of 0.01% fatty acid-free BSA was added for 10 min. The medium was removed, and the reaction was terminated with 2 mL of stop buffer (100 mM Tris, pH 7.4, 30 mM $NaPP_i$, 100 mM NaF, 5 mM EDTA, 2 mM sodium orthovanadate, 5 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, 100 μM *N*-ethylmaleamide, 10 μM phenylarsine oxide, and 0.1% SDS). The cells were collected and lysed by quick-freezing with methanol/solid CO_2.

Immunoprecipitation of Calmodulin from CHO/IR Lysates. Cell lysates were thawed and diluted with immunoprecipitation buffer (190 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl pH 7.4, and 1.25% Triton X-100). The solution was precleared by incubating for 1 h at 4 °C with blocked Affi-Gel beads which did not have antibody linked. Calmodulin was then immunoprecipitated with anti-calmodulin monoclonal antibody (Sacks et al., 1991) linked to Affi-Gel for 3 h at 4 °C and washed as previously described (Sacks et al., 1992a). After immunoprecipitation, the Affi-Gel beads containing bound protein were boiled in SDS to elute the calmodulin.

In Vitro Phosphorylation Assays. Phosphorylation of calmodulin *in vitro* by casein kinase II (Sacks et al., 1992a) and the insulin receptor kinase (Joyal & Sacks, 1994) were carried out as previously described.

SDS−PAGE and Immunoblotting. Calmodulin phosphorylated *in vitro* and calmodulin immunoprecipitated from CHO/IR cells were resolved by SDS−PAGE and transferred to Immobilon P^{SQ} as detailed by Sacks *et al*. (1992a). Phosphocalmodulin was located by autoradiography. Following autoradiography, selected immunoblots were treated with 1 M KOH for 2 h at 55 °C [to hydrolyze phosphoserine and phosphothreonine residues (Boyle et al., 1991)], and re-exposed to X-ray film.

Proteolytic Digestion. Calmodulin was eluted from Immobilon P^{SQ} with 5% (v/v) trifluoroacetic acid in 70% (v/v) isopropyl alcohol for 1 h at 37 °C and lyophilized under vacuum. Recovery was >90%. Proteolytic digestion was carried out for 16 h at 37 °C with 4 μg of sequencing grade TPCK-treated trypsin in 100 mM NH_4CO_3/l mM EGTA, pH 7.8 (Joyal & Sacks, 1994).

Two-Dimensional Phosphopeptide Mapping. Tryptic phosphopeptides of calmodulin were separated on phosphocellulose plates using 2.2% (v/v) formic acid/7.8% (v/v) acetic acid (pH 1.9) for the electrophoretic dimension and isobutanol:pyridine:acetic acid:water (1.5:1:0.3:1.2) for ascending chromatography as described (Joyal & Sacks, 1994; Boyle et al., 1991). Radiolabeled phosphopeptides were detected by autoradiography, and recovered from the plates by scraping the resin from the plastic support with a razor blade. Peptides were eluted from the phosphocellulose by rinsing three times with 7% (v/v) formic acid and lyophilized under vacuum. ^{32}P incorporation was determined by Cerenkov counting.

Phosphoamino Acid Analysis. Aliquots of radiolabeled phosphopeptide eluted from the two-dimensional maps were lyophilized and hydrolyzed in 6 M HCl at 110 °C for 1 and 4 h. Phosphoamino acids were separated by anion exchange on a Vydac oligonucleotide column (McCroskey et al., 1988). Alternatively, acid hydrolysis of the eluted radiolabeled phosphopeptides or the intact phosphocalmodulin bound to PVDF was performed for 2 h and phosphoamino acids were resolved by either thin-layer electrophoresis in 7% (v/v) formic acid or two-dimensional thin-layer electrophoresis using 2.2% (v/v) formic acid/7.8% (v/v) acetic acid (pH 1.9) for the first dimension and 5% (v/v) acetic acid/0.5% (v/v) pyridine (pH 3.5) for the second dimension (Boyle et al., 1991). After ninhydrin staining, the thin-layer plates were analyzed on a Molecular Dynamics Phosphorimager.

Determination of Phosphorylation Sites. Radiolabeled tryptic phosphopeptides were covalently coupled to Sequelon-AA membranes (Milligen/Biosearch) and analyzed by manual Edman degradation as previously described (Sullivan & Wong, 1991). The temperature of the coupling reaction and trifluoroacetic acid cleavage reaction was 55 °C. For some phosphopeptides, additional rounds of proteolysis, e.g., with Asp-N (Williams et al., 1994), or chromatography on

[2] S. Zhuang, D. B. Sacks, and C.-L. A. Wang, manuscript submitted.

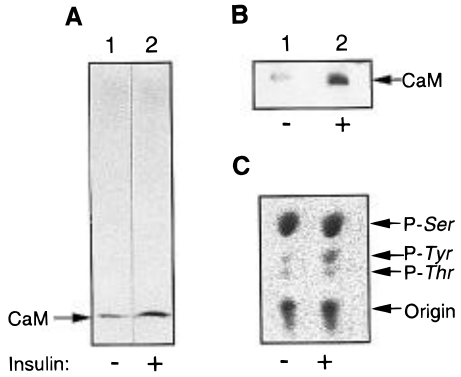

FIGURE 1: Insulin-stimulated phosphorylation of calmodulin. (A) ^{32}P-loaded control CHO/IR cells (lane 1) and CHO/IR cells treated with insulin for 10 min (lane 2) were lysed, and equal amounts of protein were immunoprecipitated with a specific anti-calmodulin monoclonal antibody as described in Materials and Methods. Proteins were separated by SDS−PAGE, transferred to PVDF, and autoradiography was performed. A representative autoradiograph of three separate experimental determinations is shown. The position of migration of calmodulin (CaM) is indicated. (B) In a separate experiment, an immunoblot processed as described in A above was treated with 1 M KOH prior to autoradiography. The region around calmodulin (CaM) is depicted. The exposure times for panels A and B were 2 and 36 h, respectively. (C) Radiolabeled phosphocalmodulin, immunoprecipitated from control and insulin-treated CHO/IR cells, was hydrolyzed with 6 M HCl. Phosphoamino acids were resolved by thin-layer electrophoresis and visualized by phosphorimaging. The origin and positions of migration of phosphoserine (P-Ser), phosphotyrosine (P-Tyr), and phosphothreonine (P-Thr) are depicted.

a strong cation exchange matrix (Tanasijevic et al., 1993) were required to unambiguously assign phosphosequence (see details in text).

Other Materials. Casein kinase II was purified from calf brain by sequential chromatography as described (Tanasijevic et al., 1993). Y138F calmodulin was prepared by site-directed mutagenesis as described.[2] The cytoplasmic domain of the insulin receptor kinase was expressed in baculovirus and purified from insect Sf9 cells by chromatography (Joyal & Sacks, 1994). The protein concentration of the hepatocyte lysate was determined by the Bradford method (Bradford, 1976), using BSA as standard. The concentration of calmodulin was determined on a Gilford spectrophotometer with $\epsilon_{1mg/mL;276nm} = 0.20$ (Wolff et al., 1977). Phosphorimaging was carried out on the Molecular Dynamics Imaging System and analyzed by Image Quant software. Densitometry was performed using NIH-Image.

RESULTS

Phosphorylation of Calmodulin in Vivo. Lysates from ^{32}P-loaded control CHO/IR cells and CHO/IR cells treated with insulin for 10 min were immunoprecipitated with a specific anti-calmodulin monoclonal antibody (Sacks et al., 1991). This demonstrated that calmodulin was constitutively phosphorylated and that insulin produced a 2-fold enhancement of phosphorylation (Figure 1A, compare lanes 1 and 2). Hydrolysis of phosphoproteins with KOH selectively enriches for phosphotyrosine by reducing the content of phosphoserine and phosphothreonine (Boyle et al., 1991). Base hydrolysis of the immunoblot revealed that calmodulin was constitutively phosphorylated at a low level on tyrosine, with a 5-fold increase in insulin-treated cells (Figure 1B, compares lanes 1 and 2). Direct phosphoamino acid analysis verified both the low level of phosphotyrosine and the 5-fold enhancement of tyrosine phosphorylation mediated by insulin (Figure 1C). Insulin enhanced serine and threonine phosphorylation by 1.7- and 1.3-fold, respectively. Phosphate incorporation was predominantly on serine, with phosphotyrosine comprising 18% of the total phosphate incorporated into calmodulin in insulin-treated cells.

Two-Dimensional Phosphopeptide Mapping of Calmodulin Phosphorylated in Vivo and in Vitro. To identify the *in vivo* insulin-sensitive phosphorylation sites, calmodulin phosphorylated *in vitro* by either the insulin receptor kinase or casein kinase II was compared with calmodulin immunoprecipitated from CHO/IR cells by two-dimensional phosphopeptide mapping. A two-dimensional tryptic phosphopeptide map of calmodulin phosphorylated *in vitro* by the insulin receptor (Figure 2A) revealed six radioactive spots, 1−4, 12, and 13, while eleven spots, 1−11, were detected on the two-dimensional map of calmodulin phosphorylated *in vitro* by casein kinase II (Figure 2B). Phosphopeptides 3 and 4 were not always clearly resolved from one another (for example, see Figure 2A). A schematic diagram illustrating the location of the spots is shown in Figure 2C, and the data are summarized in Table 1.

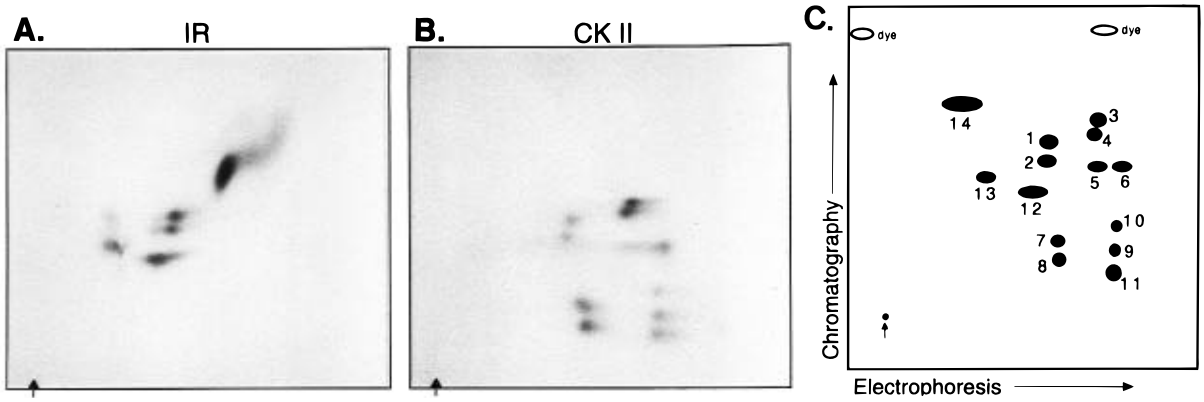

FIGURE 2: Two-dimensional tryptic phosphopeptide maps of calmodulin phosphorylated *in vitro* by casein kinase II or the insulin receptor kinase. Calmodulin was phosphorylated *in vitro* by (A) the insulin receptor kinase (IR) or (B) casein kinase II (CKII), resolved by SDS−PAGE, and digested with trypsin as described in Materials and Methods. Peptides were applied to the phosphocellulose plates and separated by thin-layer electrophoresis at pH 1.9 in the first dimension and ascending chromatography in the second dimension, followed by autoradiography. A representative experiment of five separate determinations is depicted. (C) Schematic diagram identifying the numbering of phosphopeptides. The direction of electrophoresis and chromatography are indicated. Maps were aligned by the point of sample application (arrow) and the migration of two running dyes, DNP-lysine and xylene cyanol (dye).

Table 1: Phosphopeptides of Calmodulin Identified by Two-Dimensional Phosphopeptide Mapping[a]

	CaM phosphorylated by		αCaM immunoprecipitate	
spot[b]	IR	CKII	control	insulin
1	+	+	−	+
2	+	+	−	+
3	+	+	+	+
4	+	+	+	+
5	−	+	−	−
6	−	+	−	−
7	−	+	−	−
8	−	+	−	−
0	−	+	−	−
10	−	+	−	−
11	−	+	−	−
12	+	−	−	+
13	+	−	−	−
14	−	−	+	+

[a] Tryptic phosphopeptides of calmodulin phosphorylated *in vitro* by casein kinase II or the insulin receptor or immunoprecipitated from control and insulin-treated CHO/IR cells were resolved by two-dimensional mapping as described in Materials and Methods. +, phosphopeptide present; −, phosphopeptide absent; CaM, calmodulin; αCaM, anti-calmodulin monoclonal antibody; IR, insulin receptor kinase; CKII, casein kinase II. [b] Numbers correspond to the scheme in Figure 2C.

Calmodulin was immunoprecipitated from lysates obtained from radiolabeled control and insulin-treated CHO/IR cells, digested with trypsin, and resolved by two-dimensional phosphopeptide mapping. Calmodulin from control cells yielded one major and three minor phosphopeptides (Figure 3A). Insulin enhanced by 1.8-fold the phosphorylation of the major phosphopeptide (number 14) in 10 min (Figure 3B), as determined by Cerenkov counting of the eluted phosphopeptide. With exposure of cells to insulin for 30 min (Figure 3C), the phosphorylation of phosphopeptide 14 returned to the basal level. Two of the minor phosphopeptides comigrated with phosphopeptides 3 and 4 from the *in vitro* two-dimensional maps. Insulin significantly enhanced the phosphorylation of these phosphopeptides (compare Figure 3A with B and C), but the weak signal in control cells prevented accurate determination of the magnitude of the stimulation by insulin. Phosphopeptides 1−4 and 12 were detected on the two-dimensional maps of calmodulin immunoprecipitated from insulin-treated CHO/IR cells (Figure 3B,C), and were more prominent after 30 min of insulin treatment than after 10 min (compare Figure 3A with B and C). With a longer time of exposure, a number of additional spots were detected on the two-dimensional maps of calmodulin isolated from insulin-treated cells (data not shown). The major spots identified in individual maps are summarized in Table 1.

Determination of the in Vitro Serine/Threonine Sites of Phosphorylation. Tryptic phosphopeptides were eluted from the map of calmodulin phosphorylated *in vitro* by casein kinase II, and phosphoamino acid analysis was performed on an aliquot of each peptide. Spots 1−4, 7, 9, and 10 contained only phosphoserine, spots 5 and 6 contained only phosphothreonine, and spots 8 and 11 contained both phosphoserine and phosphothreonine.

The specific sites of phosphorylation in each phosphopeptide were determined by manual Edman degradation. An example is shown in Figure 4 for spot 1. HPLC anlysis demonstrated phosphoserine exclusively (Figure 4A,B). The additional peak in fractions 18−24 in Figure 4A was nonhydrolyzed phosphopeptide that disappeared with longer hydrolysis (Figure 4B). Manual Edman degradation revealed the release of radioactivity at cycle 7 (Figure 4C). As illustrated in Figure 5, Ser-81 and Ser-101 are the only serine residues in calmodulin located seven amino acid residues distal to a trypsin digestion site (following Arg-74 and Lys-94, respectively) (Watterson et al., 1980). Phosphopeptide 1 was barely retained on a strong cation exchange column (Tanasijevic et al., 1993) and eluted at fractions 8−13 (4−6.5 min) (data not shown), indicating that it contained few basic residues. Since the peptide (R^{74})K^{75}MKDTDSEEEIR^{86}, containing Ser-81, has three basic residues plus a free N-terminus, it would be expected to elute at about 20 min or greater on this column (Tanasijevic et al., 1993). Thus, Ser-101 is the amino acid phosphorylated in phosphopeptide 1 (Table 2). This assignment strategy is reinforced by the tyrosine phosphorylation sites described below.

Similar analysis was performed on all of the other phosphopeptides. The assignment of phosphorylation sites for these spots is shown in Table 2. Manual sequencing revealed that a single tryptic phosphopeptide containing phosphoserine-101 migrated to spots 1 and 2. Spot 2 likely contained an isoaspartyl linkage derived from Asn-97 and Gly-98 (Potter et al., 1993), which altered its migration relative to spot 1. Spots 3 and 4 also contained phospho-

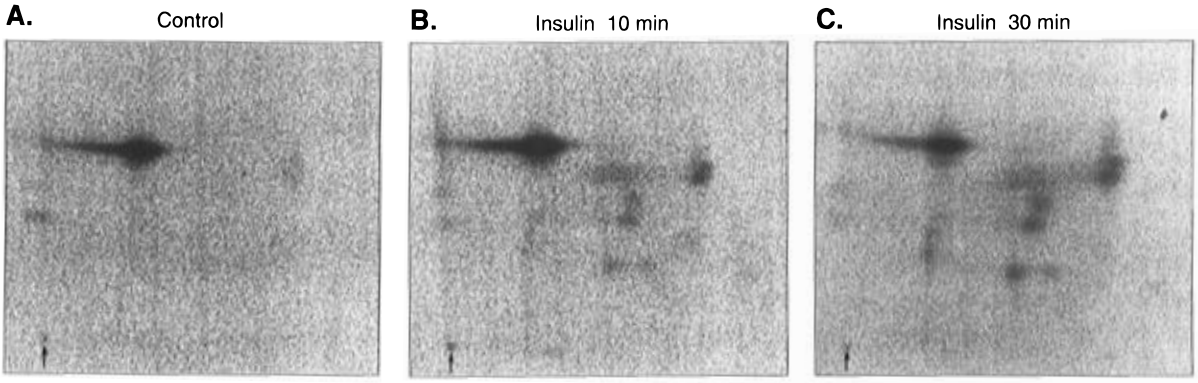

FIGURE 3: Two-dimensional tryptic phosphopeptide maps of calmodulin immunoprecipitated from CHO/IR cells. Phosphocalmodulin immunoprecipitated from control CHO/IR cells (A) or CHO/IR cells treated with insulin for either 10 min (B) or 30 min (C) was resolved by SDS−PAGE and digested with trypsin as described in Materials and Methods. Equal amounts of peptides were applied to the phosphocellulose plates, and tryptic phosphopeptide mapping was performed. Peptides were separated by thin-layer electrophoresis at pH 1.9 in the first dimension and ascending chromatography in the second dimension, followed by autoradiography. The key to the numbering of the phosphopeptides is shown in Figure 2C.

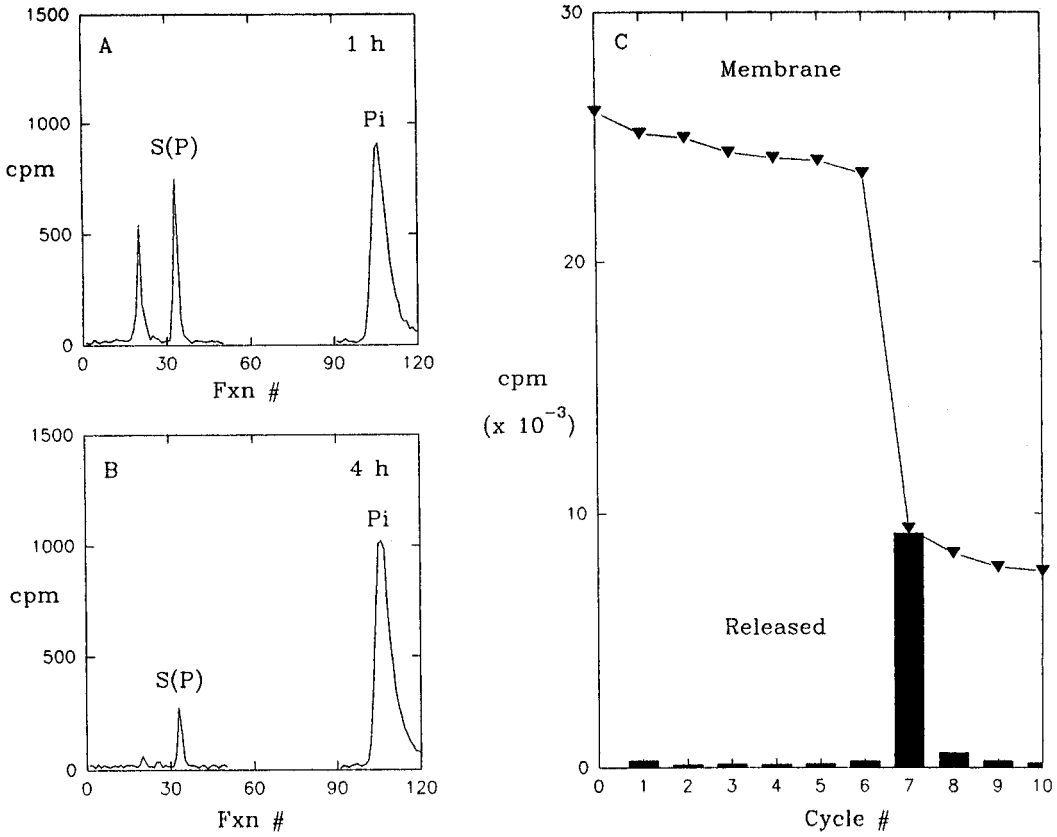

FIGURE 4: Determination of the site of phosphorylation of tryptic phosphopeptide 1 of calmodulin. An aliquot of spot 1, isolated from the two-dimensional phosphopeptide map displayed in Figure 2B, was subjected to limited acid hydrolysis and to manual sequencing as described in Materials and Methods. Radiochromatogram panels A and B represent acid hydrolysis at 110 °C for 1 and 4 h, respectively. The elution positions of authentic, cold phosphoamino acid standards were: phosphothreonine at fractions 24−26, phosphoserine (S(P)) at fractions 32−34, and phosphotyrosine at fractions 38−40. The elution position of inorganic phosphate (P_i) was determined from acid hydrolysis of [γ-^{32}P]ATP. Panel C shows the manual radiosequencing analysis of spot 1. Inverted triangles (▼) indicate radioactivity remaining on the membrane at the end of each cycle, and shaded bars represent radioactivity released from the membrane.

Table 2: Phosphorylation Sites of Tryptic Phosphopeptides of Calmodulin Phosphorylated *in Vitro*[a]

spot[b]	charge	sequence[c]	phosphorylation site
1, 2	+1	(K^{94})D^{95}GNGYISAAELR^{106}	Tyr-99 or Ser-101
3, 4	+2	(R^{90})V^{91}FDKDGRGYISAAELR^{106}	Tyr-99 or Ser-101
5	+2	(K^{75})M[O]^{76}KDTDSEEEIR^{86}	Thr-79
6	+3	(R^{106})H^{107}VM[O]TNLGEK(CH$_3$)$_3$LTDEEVDEM[O]IR^{126}	Thr-117
7	+1	(K^{77})D^{78}TDSEEEIR^{86}	Ser-81
8	+1	(K^{75})M^{76}KDTDSEEEIR^{86}	Thr-79 and Ser-81
9	+2	(K^{75})M^{76}KDTDSEEEIR^{86}	Ser-81
10	+2	(K^{77})D^{78}TDSEEEIREAFR^{90}	Ser-81
11	+2	(K^{75})M^{76}KDTDSEEEIREAFR^{90}	Thr-79 and Ser-81
12	+1	(R^{126})E^{127}ADIDGDGQVNYEEFVQM[O]MTAK^{148}	Tyr-138
13	+1	(R^{126})E^{127}ADIDGDGQVNYEEFVQM[O]M[O]TAK^{148}	Tyr-138

[a] Tryptic phosphopeptides of calmodulin phosphorylated *in vitro* by casein kinase II or the insulin receptor were isolated from two-dimensional maps, and both phosphoamino acid analysis and manual sequencing were performed. The first residue in each peptide is adjacent to a trypsin digestion site (shown in parentheses). Phosphorylated residues are underlined. $K(CH_3)_3$ indicates a trimethyllysine, which does not provide a trypsin digestion site. [b] Numbers correspond to the scheme in Figure 2C. Spots 2 and 4 likely contain isoaspartyl linkages derived from Asn-97 and Gly-98. [c] M[O] refers to an oxidized methionine residue.

serine-101 but migrated differently from phosphopeptides 1 and 2 because the trypsin digestion site was at Arg-90, while spots 1 and 2 were cleaved at Lys-94 (Table 2). Analogous to spot 2, spot 4 likely contained the isoaspartyl linkage derived from Asn-97 and Gly-98.

Spots 5, 7−10, and 11 contained either phosphothreonine-79, phosphoserine-81, or both (Table 2). The large number of phosphopeptides detected was due to differences in the sites of trypsin cleavage and/or the number of phosphates incorporated. Spot 6 contained phosphothreonine-117. All of these sites are identical to the sites previously identified using HPLC by Sacks et al. (1992a) in calmodulin phosphorylated *in vitro* by casein kinase II. The distance of migration of peptides in the electrophoretic and chromatographic dimensions should be proportional to the net charge on the peptide and the relative hydrophobicity of the peptide, respectively (Boyle et al., 1991). The apparent net charge at pH 1.9 of the identified phosphopeptides are listed in Table 2 and the majority are consistent with the observed two-dimensional migration pattern (Figure 2B). The aberrant

^{1}ADQLTEEQIAEFK↓EAFSLFD^{20}

^{21}K↓DGDQTITTK↓ELGTVMR↓SLG^{40}

^{41}QNPTEAELQDMINEVDADGN^{60}

^{61}GTIDFPEFLTMMAR↓K↓MK↓DTD^{80}

^{81}SEEEIR↓EAFR↓VFDK↓DGNGYI^{100}

^{101}SAAELR↓HVMTNLGEK*LTDEE^{120}

^{121}VDEMIR↓EANIDGDGQVNYEE^{140}

^{141}FVQMMTAK^{148}

FIGURE 5: Sites of trypsin cleavage in calmodulin. The amino acid sequence of mammalian calmodulin (Watterson et al., 1980) is shown. Arrows denote trypsin cleavage sites. The "*" after amino acid 115 indicates a trimethyllysine, which does not provide a trypsin digestion site.

migration of phosphopeptide 6 is probably due to oxidized methionine residues that retard electrophoretic mobility.

Determination of the in Vitro Tyrosine Sites of Phosphorylation. Mammalian calmodulin contains two tyrosine residues, both of which are phosphorylated by the insulin receptor kinase (Joyal & Sacks, 1994; Williams et al., 1994). Each tryptic phosphopeptide obtained from calmodulin phosphorylated *in vitro* by the insulin receptor (Figure 2A) was analyzed as described above. Phosphorylation was exclusively on tyrosine residues (data not shown). Spots 1−4 contained phosphotyrosine-99, and spots 12 and 13 contained phosphotyrosine-138 (Table 2). Although the phosphopeptide corresponding to spots 12 and 13 has a net charge of +1, the oxidation of one methionine residue in spot 12 and two methionine residues in spot 13 (Williams et al., 1994) probably accounts for the altered mobility and the presence of more than one spot. The presence of one or two oxidized methionine residues in this phosphopeptide was confirmed by mass spectrometry.[3] The relative amounts of phosphopeptides 1−4, 12, and 13 varied among preparations. When phosphorylation stoichiometry of calmodulin was low, phosphopeptides 1−4 were more prevalent than phosphopeptides 12 and 13. In some experiments, phosphopeptide 13 was absent and the intensity of spot 12 was enhanced (data not shown). In addition, the ratio of phosphopeptides 1 and 2 to 3 and 4 varied among experiments.

To verify the accuracy of assignment of the tyrosine phosphorylation sites described above, two variant calmodulins were used. Wheat germ calmodulin, which contains phenylalanine instead of tyrosine at position 99 (Cohen & Klee, 1988), and a mutant calmodulin with phenylalanine substituted for Tyr-138 (Y138F) were phosphorylated *in vitro* by the insulin receptor. Both calmodulin variants were phosphorylated exclusively on tyrosine residues (data not shown). Y138F calmodulin was digested with trypsin and resolved by two-dimensional phosphopeptide mapping (Figure 6). Only spots 1−4, which contain Tyr-99, were present on the autoradiograph. A two-dimensional tryptic map of

[3] R. Annan, D. B. Sacks, and S. Carr, manuscript in preparation.

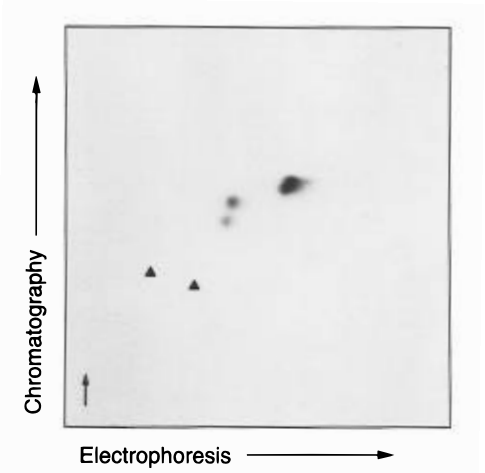

FIGURE 6: Two-dimensional phosphopeptide map of Y138F calmodulin phosphorylated *in vitro*. A mutated calmodulin, with phenylalanine substituted for Tyr-138 (Y138F), was phosphorylated *in vitro* by the insulin receptor kinase, digested with trypsin and resolved by two-dimensional mapping as described in Materials and Methods. Peptides were separated by thin-layer electrophoresis at pH 1.9 in the first dimension and ascending chromatography in the second dimension, followed by autoradiography. The point of sample application (arrow) is indicated. The locations of Y138-containing phosphopeptides are identified by arrowheads. The key to the numbering of the phosphopeptides is shown in Figure 2C.

wheat germ calmodulin lacked spots 1−4 (data not shown). Together, these data provide independent verification that the assignment of phosphorylation sites is correct.

Determination of the Sites of Phosphorylation in Vivo. To determine the *in vivo* insulin-sensitive phosphorylation sites of calmodulin, phosphopeptides were eluted from the two-dimensional maps of calmodulin immunoprecipitated from control and insulin-treated CHO/IR cells (see Figure 3). Two-dimensional phosphoamino acid analysis demonstrated that phosphopeptide 14 from the map of both control (Figure 7A) and insulin-treated (Figure 7B) cells contained exclusively phosphoserine. There was no counterpart for spot 14 on the *in vitro* maps (see Figure 2), and attempts to identify the phosphorylation site(s) by manual sequencing were unsuccessful. Thus, the phosphorylation site(s) remains unidentified. An additional phosphoserine-containing phosphopeptide was detected on the two-dimensional map of calmodulin isolated from insulin-treated cells (data not shown), but the phosphorylation site was not identified.

Manual sequencing of spots 1−4 from the *in vitro* maps demonstrated that the amino acid sequence of spot 1 is identical to that of spot 2 and the amino acid sequence of spot 3 is identical to that of spot 4 (Table 2). Therefore, following isolation from the two-dimensional map of calmodulin immunoprecipitated from CHO/IR cells treated with insulin for 30 min, phosphopeptides 1 and 2 were pooled, and phosphopeptide 3 was combined with phosphopeptide 4. All of these phosphopeptides contained exclusively phosphotyrosine (Figure 7C,D). These spots comigrated with spots 1−4 on the map of calmodulin phosphorylated *in vitro* by the insulin receptor, identifying Tyr-99 as a residue phosphorylated on calmodulin in intact cells in response to insulin. Similarly, spot 12 from the two-dimensional map of calmodulin immunoprecipitated from insulin-treated CHO/IR cells also contained exclusively phosphotyrosine (Figure 7E). On the basis of its comigration with calmodulin phosphorylated *in vitro* (Figure 2A), Tyr-138 is the insulin-sensitive phosphorylation site in phosphopeptide 12. Analy-

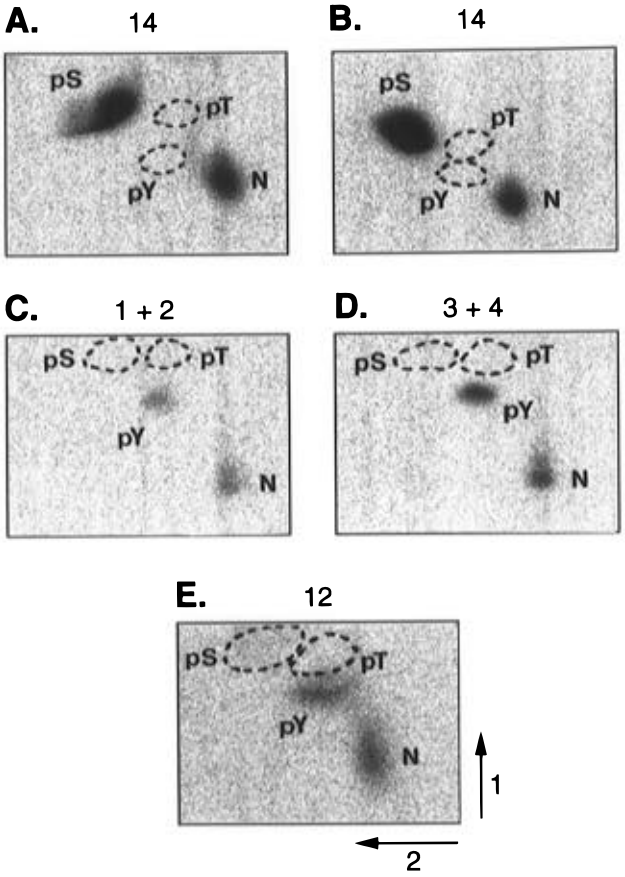

FIGURE 7: Phosphoamino acid analysis of phosphopeptides derived from calmodulin phosphorylated *in vivo*. Phosphoamino acid analysis was performed on phosphopeptides isolated from the two-dimensional tryptic phosphopeptide maps of calmodulin immunoprecipitated from CHO/IR cells (depicted in Figure 3). (A, B) Phosphopeptide 14 eluted from the maps of calmodulin immunoprecipitated from control CHO/IR cells (A) and cells treated with insulin for 30 min (B). (C−E), phosphopeptides eluted from the map of calmodulin immunoprecipitated from CHO/IR cells treated with insulin for 30 min. Phosphopeptides 1 and 2 (C), 3 and 4 (D), and 12 (E) are shown. All phosphopeptides were hydrolyzed with 6 M HCl, and phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis as described under Materials and Methods. The positions of migration of phosphoserine (pS), phosphothreonine (pT), phosphotyrosine (pY) and nonhydrolyzed peptide (N) are depicted. Arrows 1 and 2 indicate the direction of electrophoresis in the first and second dimensions, respectively.

sis by densitometry revealed that approximately 3-fold more ^{32}P was incorporated into Tyr-99 than into Tyr-138.

DISCUSSION

Insulin enhances the phosphorylation of calmodulin in rat hepatocytes on serine, threonine, and tyrosine residues (Sacks et al., 1992a), but the specific phosphorylation sites were not identified in that study. Without overexpressing the protein and/or kinase of interest, it is rarely possible to isolate adequate amounts of radiolabeled protein to identify *in vivo* phosphorylation sites on endogenous proteins by direct phosphopeptide sequencing. An approach employed by several investigators is comparison by two-dimensional phosphopeptide mapping of phosphopeptides derived from proteins phosphorylated *in vivo* with proteins phosphorylated *in vitro* (Tavare & Denton, 1988; Krieg & Hunter, 1992; Piccitto et al., 1992; Wang et al., 1993; Uchida et al., 1994). This approach was employed in our study using phosphocalmodulin immunoprecipitated from CHO/IR cells and calmodulin phosphorylated *in vitro* by the insulin receptor kinase or casein kinase II. Individual phosphopeptides were then directly isolated from two-dimensional peptide maps of the *in vitro* samples and sequenced manually. Since the amino acid sequence of calmodulin is known (Watterson et al., 1980), proteases with known site specificity, e.g., trypsin, were used, and the type of phosphoamino acid (phosphoserine, phosphothreonine, or phosphotyrosine) was determined experimentally. The observed cycle of release of radioactivity during manual Edman degradation allowed logical assignment of the specific sites of phosphorylation.

The *in vitro* phosphorylation sites identified here were Thr-79, Ser-81, Ser-101, and Thr-117 for calmodulin phosphorylated by casein kinase II and Tyr-99 and Tyr-138 for calmodulin phosphorylated by the insulin receptor. These sites are identical to those previously determined by HPLC, manual sequencing (Williams et al., 1994), automated sequencing (Sacks et al., 1992a), and mass spectrometry[3] (Quadroni et al., 1994). We were initially surprised that phosphopeptides 1−4 were present in the maps obtained from calmodulin phosphorylated by casein kinase II and the insulin receptor (see Figure 2A,B). However, sequencing of these phosphopeptides indicated that all four included the sequence of calmodulin Asn-95 to Arg-106 (see Figure 5 and Table 2), which contains both Tyr-99 and Ser-101 (Watterson et al., 1980). The technique of two-dimensional phosphopeptide mapping cannot distinguish the peptides phosphorylated on Tyr-99 from those phosphorylated on Ser-101.

The results presented here clearly demonstrate that insulin promotes the phosphorylation on both Tyr-99 and Tyr-138 of calmodulin. Our data verify and extend previous observations showing that insulin stimulates tyrosine phosphorylation of calmodulin (Colca et al., 1987; Sacks et al., 1992a). In our study, insulin enhanced tyrosine phosphorylation of calmodulin by 5-fold at 10 min and phosphorylation remained at a high level at 30 min. The time course of tyrosine phosphorylation of insulin receptor substrates is variable. For example, tyrosine phosphorylation of IRS-1 (Sun et al., 1992) and shc (Sasaoka et al., 1994) is rapid and declines after a few minutes, whereas that of the fos-related protein, pp82, reaches maximum at 30 min (Kim & Kahn, 1994). Probing of anti-calmodulin immunoblots with anti-phosphotyrosine antibody demonstrated phosphotyrosine on calmodulin in insulin-treated cells (data not shown), but the signal was extremely weak and of poor quality. This result is not very surprising as it is well recognized that anti-phosphotyrosine antibodies do not react well with many proteins with molecular mass less than 30 kDa (Kamps & Sefton, 1988; Kozma et al., 1991). Phosphotyrosine comprised 18% of the total phosphate incorporated into calmodulin. The proportion of phosphotyrosine in calmodulin is similar to that seen in IRS-1 isolated from insulin-treated cells (Sun et al., 1992). Interestingly, phosphorylation on tyrosine residues *in vitro* by the insulin receptor kinase alters the ability of calmodulin to activate selected calmodulin-dependent enzymes (Williams et al., 1994; Sacks et al., 1995). Since, insulin significantly decreased the activity of the erythrocyte (Davis et al., 1985) and adipocyte (Pershadsingh & McDonald, 1979) plasma membrane Ca^{2+}-ATPase, it is possible that tyrosine phosphorylation may serve as a mechanism to regulate the activity of individual calmodulin-dependent enzymes *in vivo*.

Insulin also enhanced the phosphorylation of at least two unidentified phosphoserine-containing peptides. The limited time course analysis revealed that phosphorylation of phosphopeptide 14 was maximal at 10 min and returned to basal levels within 30 min. Attempts to identify the site of phosphorylation of phosphopeptide 14 by manual Edman degradation were not successful due to inadequate ^{32}P labeling. Since phosphopeptide 14 did not comigrate with any of the tryptic phosphopeptides from the map of calmodulin phosphorylated *in vitro* by casein kinase II, it is extremely unlikely that it contains phosphoserine-81 or phosphoserine-101, which are constitutively phosphorylated in rat liver by casein kinase II (Quadroni et al., 1994). Calmodulin contains two additional serine residues, namely, Ser-17 and Ser-38 (Watterson et al., 1980). The migration of tryptic phosphopeptides on two-dimensional maps can be assessed by determining the mass, charge, and relative hydrophobicity of the individual peptides (Boyle et al., 1991). By these criteria, a tryptic fragment containing phosphoserine-38 but not a fragment containing phosphoserine-17 would be expected to migrate to the same position as phosphopeptide 14, possibly implicating Ser-38 as the site phosphorylated on phosphopeptide 14. Of the serine/threonine kinases that phosphorylate calmodulin, only those sites phosphorylated by casein kinase II have been identified (Sacks et al., 1992a).

By mass spectrometry Thr-79, Ser-81, and Ser-101 were identified as constitutive phosphorylation sites on calmodulin isolated from rat liver (Quadroni et al., 1994). We have determined by two-dimensional phosphopeptide mapping that these sites are also phosphorylated on calmodulin in isolated rat hepatocytes.[4] Since all of these are *in vitro* casein kinase II-catalyzed phosphorylation sites (Sacks et al., 1992a), two-dimensional phosphopeptide maps from calmodulin phosphorylated by casein kinase II *in vitro* were compared with maps of calmodulin immunoprecipitated from intact cells. None of these serine/threonine phosphorylation sites was identified on calmodulin from CHO/IR cells. The tissue-specific phosphorylation of particular sites on calmodulin may be due to variations in the relative amounts of serine/threonine kinases and/or phosphatases in the two cell types. For instance, the levels of five protein kinase C isoforms in hepatocytes was significantly different from the levels in FAO and HepG2 cells (Ducher et al., 1995). In addition, differences in the specific sites of phosphorylation in individual proteins isolated from different cell types have been described previously. For example, at least two major phosphopeptides identified on a two-dimensional map of the insulin receptor isolated from COS cells (Carter et al., 1995) were not present in maps of the insulin receptor from HepG2 cells (Tavare et al., 1991). Similarly, the phosphorylation site in eIF-4E isolated from CHO cells (Flynn & Proud, 1995) was different from the site phosphorylated in reticulocytes (Rychlik et al., 1987). Since the modulation of calmodulin activity is dependent on the site of phosphorylation (Sacks et al., 1995), it is possible that cell type specific phosphorylation may be a mechanism to differentially regulate calmodulin in various cells.

We identified phosphotyrosine on calmodulin in CHO/IR cells and previously demonstrated that phosphotyrosylcalmodulin is present in isolated hepatocytes (Sacks et al., 1992a). Carafoli's group did not detect phosphotyrosine on calmodulin in rat liver (Quadroni et al., 1994). Several factors may account for the differneces between the two studies. The results obtained here using a lysis buffer that contained a variety of phosphatase inhibitors were virtually identical to previous observations with rat hepatocytes processed by boiling SDS (Sacks et al., 1992a). High levels of phosphotyrosine phosphatases are found in the liver (Grupposo et al., 1991), and it has been suggested that boiling SDS is necessary to detect tyrosine phosphorylation of some proteins (Rothenberg et al., 1991). In addition, the effect of insulin was not examined by Carafoli's group. The amount of tyrosine-phosphorylated calmodulin in unstimulated cells may be below their level of detection.

In summary, we have documented that insulin stimulates the phosphorylation of calmodulin on Tyr-99, Tyr-138, and unidentified serine residues in CHO/IR cells. While the physiological significance is not known, we have observed that the phosphorylation at Tyr-99 mediates the binding of calmodulin to phosphatidylinositol 3-kinase.[4] Identification of the insulin-stimulated phosphorylation sites on calmodulin should facilitate efforts to elucidate the physiological role of the phosphorylation of calmodulin.

[4] J. L. Joyal and D. B. Sacks, unpublished data.

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