Allosteric Activation of Protein Phosphatase 2C by D-*chiro*-Inositol−Galactosamine, a Putative Mediator Mimetic of Insulin Action†

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ABSTRACT: Insulin-stimulated glucose disposal in skeletal muscle proceeds predominantly through a nonoxidative pathway with glycogen synthase as a rate-limiting enzyme, yet the mechanisms for insulin activation of glycogen synthase are not understood despite years of investigation. Isolation of putative insulin second messengers from beef liver yielded a pseudo-disaccharide consisting of pinitol (3-*O*-methyl-D-*chiro*-inositol) β-1,4 linked to galactosamine chelated with Mn^{2+} (called INS2). Here we show that chemically synthesized INS2 has biological activity that significantly enhances insulin reduction of hyperglycemia in streptozotocin diabetic rats. We used computer modeling to dock INS2 onto the known three-dimensional crystal structure of protein phosphatase 2C (PP2C). Modeling and FlexX/CScore energy minimization predicted a unique favorable site on PP2C for INS2 in a surface cleft adjacent to the catalytic center. Binding of INS2 is predicted to involve formation of multiple H-bonds, including one with residue Asp163. Wild-type PP2C activity assayed with a phosphopeptide substrate was potently stimulated in a dose-dependent manner by INS2. In contrast, the D163A mutant of PP2C was not activated by INS2. The D163A mutant and wild-type PP2C in the absence of INS2 had the same Mn^{2+}-dependent phosphatase activity with *p*-nitrophenyl phosphate as a substrate, showing that this mutation did not disrupt the catalytic site. We propose that INS2 allosterically activates PP2C, fulfilling the role of a putative mediator mimetic of insulin signaling to promote protein dephosphorylation and metabolic responses.

Insulin promotes the disposal of glucose from the bloodstream into target tissues (*1*). Insulin stimulates recruitment of the GLUT4 glucose transporter to the plasma membrane surface and activates glucose uptake (*2*). Glucose metabolism via oxidative and nonoxidative routes is controlled by the rate-limiting enzymes pyruvate dehydrogenase (PDH)[1] (*3*) and glycogen synthase (GS) (*4*), respectively. Both these enzymes are activated by dephosphorylation in response to insulin (*5*, *6*). Protein phosphatase 2C (PP2C) has been known as a GS phosphatase since the 1980s (*7*), and recently, PP2C was identified as a positive regulator of PI3K by dephosphorylation and activation of the p85 subunit (*8*). Signaling pathways for activation of PDH have not been well defined (*9*), but PDH phosphatase and PP2C are members of the PPM family of enzymes with similar structures (*10*, *11*). Other proposed signaling pathways for activation of GS include inhibition of GSK3 via Ser9 phosphorylation involving a PI3K-dependent process (*12*), as well as activation of protein phosphatase-1 (PP1) by an unknown mechanism (*13*). Overall, activation of phosphatases of the PPM family could account for multiple intracellular effects of insulin.

The search for intracellular second messengers generated in response to insulin has had a checkered history. Approximately 25 years ago, different laboratories found low-molecular weight, acid- and heat-stable putative mediators produced in response to insulin by rat skeletal muscle (*14*, *15*) and isolated fat cells (*16*). Experiments by Jarrett et al. (*16*) showed clearly that adipocyte cell membranes treated

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[1] Abbreviations: PP2C, divalent metal ion-dependent protein phosphatase of the PPM type; PDH, pyruvate dehydrogenase; GS, glycogen synthase; GSK3, glycogen synthase kinase-3; PI3K, phosphatidylinositol 3-phosphate kinase; GPI, glycosylphosphoinositol; INS2, synthetic Mn^{2+} chelate of D-*chiro*-inositol−galactosamine; STZ, streptozotocin; GST, glutathione *S*-transferase.

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with insulin generated a transferable factor that stimulated mitochondrial PDH phosphatase activity. The chemical composition and structure of such putative mediators were not elucidated at the time, though evidence suggested they contained inositol and amino hexoses (*17*, *18*) and therefore were likely to be inositol glycans derived from membrane glycolipids (GPIs) and/or GPI-linked proteins. Work on cytoplasmic insulin second messengers was eclipsed by discovery in the early 1980s of the intrinsic protein Tyr kinase activity of the insulin receptor. The subsequent pursuit of stepwise phosphorylation events, including the MAP kinase pathway, sought to account for all the cellular effects of insulin. The concept of second messengers for insulin fell into disfavor among researchers in part because the Tyr phosphorylation pathway did not afford an obvious mechanism for generation of inositol glycans.

Nonetheless, our laboratory has persisted in studies of possible messengers for insulin. We demonstrated that in response to insulin, GPI precursors in BC3H1 myocytes were cleaved to release soluble alkaline phosphatase (the first known GPI protein), and diacylglycerol and a soluble putative mediator that activated PDH phosphatase (*19*). Our purifications yielded two separate inositol glycan species from rat liver (*20*), one containing *myo*-inositol and glucosamine, which inhibits cAMP-dependent protein kinase and the catalytic subunit of adenylate cyclase (*21*, *22*), and a second species containing D-*chiro*-inositol and galactosamine, which activates phosphatase PP2C (*24*) and PDH phosphatase (*15*, *16*, *19*, *20*, *25*−*27*). Consistent with a role as potential mediators, the levels of both the *myo*-inositol and D-*chiro*-inositol species increased in rat muscle, liver, and fat tissues following in vivo insulin administration (*14*, *15*, *20*, *22*, *23*). This was the first identification of the D stereoisomer of *chiro*-inositol derived from animal tissues, which was previously found in only plants and insects (*28*). Most recently, we have published the isolation, purification, structure determination, and chemical synthesis of a novel β-1,4-linked pseudo-disaccharide called INS2 that is composed of pinitol and galactosamine chelated with Mn^{2+} (*27*). Chemically synthesized INS2 elicited dose-dependent reduction of hyperglycemia in streptozotocin (STZ) diabetic rats at low micromolar concentrations. INS2 activated PDH phosphatase in vitro and enhanced insulin-stimulated incorporation of [^{14}C]glucose into [^{14}C]glycogen in hepatoma H4 cells (*27*). INS2 activated PDH phosphatase by enhancing its sensitivity to Mg^{2+} (*27*), and insulin treatment of adipocytes enhances the Mg^{2+} sensitivity of PDH phosphatase in broken cell preparations (*5*). These effects of INS2 reproduced the biological responses to earlier preparations of the putative mediator that were partially purified from tissues following insulin treatment (*29*, *30*), consistent with INS2 being the primary bioactive component of those mediator preparations.

Here we show that in severely diabetic rats pretreatment with INS2 sensitizes insulin reduction of hyperglycemia, showing in vivo biological activity of INS2. We use molecular modeling and quantitative scoring to locate an energetically favorable site for binding of INS2 to PP2C, and show dose-dependent activation of phosphatase activity. These results support the concept that naturally produced or synthetic small molecules may allosterically activate PP2C to enhance insulin signaling.

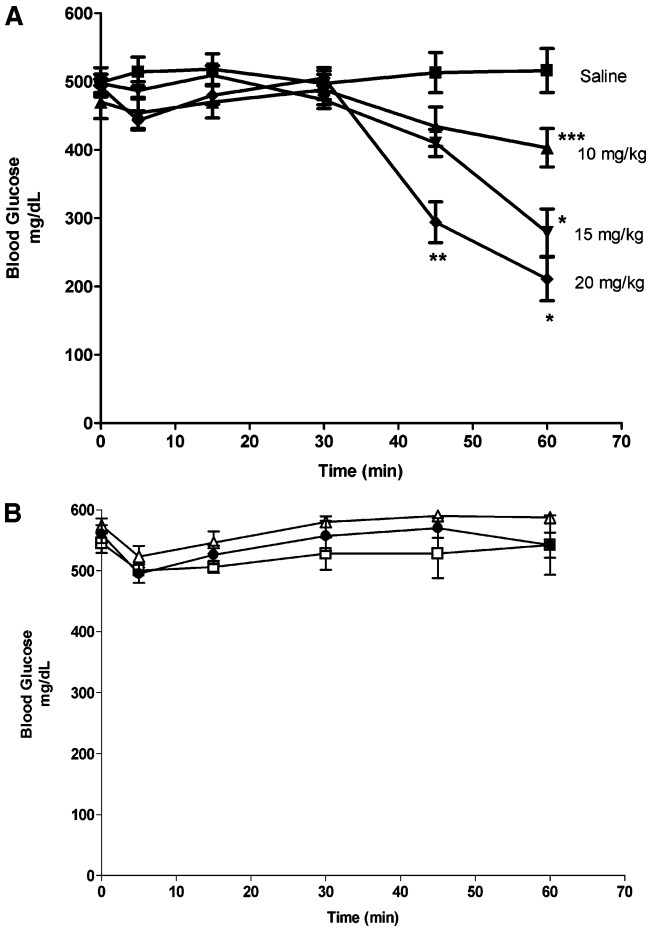

FIGURE 1: Dose-dependent reduction of the extent of hyperglycemia in STZ diabetic rats by insulin following prior INS2 administration. (A) Animals were injected with saline or different doses of INS2 followed 15 min later by insulin. The dose of 20 mg of INS2/kg was statistically different from the 10 mg/kg dose with a 57% decrease in the blood glucose level from the saline control at 60 min: (■) control of saline and insulin, (▲) 10 mg of INS2/kg and insulin, (▼) 15 mg of INS2/kg and insulin, and (◆) 20 mg of INS2/kg and insulin. Significance calculated as follows: one asterisk, $p < 0.001$ vs saline and insulin at 60 min; two asterisks, $p < 0.001$ vs saline and insulin at 45 min; and three asterisks, $p < 0.001$ vs 20 mg of INS2/kg and insulin at 60 min ANOVA, followed by Tukey post-test. (B) Animals were injected with INS2 followed 15 min later by saline (●), with insulin followed 15 min later by 20 mg of INS2/kg (□), or with insulin followed 15 min later by saline (△). As shown, all these combinations were ineffective at reducing the blood glucose level over 60 min.

MATERIALS AND METHODS

Diabetic Animal Model. Sprague-Dawley male rats weighing 110−120 g were injected in the tail vein with streptozotocin (50 mg/kg), and after 2−5 days, diabetes was confirmed by blood glucose analysis with a glucometer. Treatments with INS2 or insulin were conducted as described in the legend of Figure 1. This study was Protocol 3333, reviewed and approved by the University of Virginia Animal Care and Use Committee (ACUC).

Flexible Docking of INS2 into the PP2C Crystal Structure. INS2 and its isomeric structures were virtually constructed and adjusted to the lowest-energy conformation using the SYBYL modeling program. The crystal structure of PP2C (PDB entry 1A6Q) was downloaded from the Protein Data Bank (PDB). For the FlexX studies, the allosteric binding pocket of PP2C was defined by the following residues:

Asp146, Arg148, Phe160, Asp163, Lys165, Asp243, Gly246, and Asn247 (numbering for human PP2Cα) with a selection radius of 5 Å. INS2 and isomers were then docked into the binding pocket and scores calculated for the top 30 conformers. The top conformer was chosen from calculated scores and binding conformation.

Flexible Docking of the Phosphopeptide into the PP2C Crystal Structure. The phosphopeptide (RRRRPp-TPA) was virtually constructed and minimized in the SYBYL shell. The catalytic binding pocket was defined from the crystal structure of PP2C (PDB entry 1A6Q) using a radius selection of 8 Å centered on the inorganic phosphate in the crystal structure. The peptide was then docked into the binding pocket selecting the top 30 conformers. The highest-scoring conformer was used for the remainder of the study.

Preparation of the GST−PP2Cα Fusion Protein. The mouse wild-type PP2Cα fusion protein was expressed from the pGEX-2T plasmid in the BL-21 strain of *Escherichia coli* grown in 200 mL of LB medium and 2% ethanol by overnight induction with 0.1 mM isopropyl thiogalactoside at room temperature. Cells were harvested by centrifugation, lysed with 0.6 mg/mL lysozyme (Sigma Chemical Co., St. Louis, MO), and subjected to freeze−thaw cycles. The cell extract was clarified by centrifugation at 28000*g* for 45 min, and the supernatant was mixed with 3 mL of glutathione−Sepharose beads (Amersham Biosciences, Piscataway, NJ) for 30 min at 4 °C. The beads were collected in a column, washed with 50 mL of buffer, and eluted with 10 mM glutathione by following the manufacturer's instructions. The glutathione *S*-transferase (GST) fusion protein (1−2 mg per 100 mL culture) was dialyzed against 50% glycerin in 50 mM MOPS (pH 7.0), 4.5 mM 2-mercaptoethanol, 1 mM $MnCl_2$, and 0.1 mM Pefabloc (Amersham Biosciences) and stored at −20 °C. Protein concentrations were determined with serial dilutions relative to serum albumin as a standard using the Coomassie dye binding assay (Bio-Rad).

Phosphatase Assays. The phosphatase activity was assayed in 0.1 M Tris-HCl (pH 8.0), 2 mM dithiothreitol, and various concentrations of added $MnCl_2$ or INS2 at room temperature in a 96-well plate in 50 μL reaction mixtures with 1 μg of GST−PP2C fusion protein and 5 mM *p*-nitrophenyl phosphate. INS2 synthesis and preparation were carried out as previously described (*27*).

After 30 min, the reaction was stopped by addition of 200 μL of 0.5% SDS and the absorbance at 410 nm recorded with an Eldex microplate reader. Alternatively, activity of 2 μg of fusion protein was assayed in the same volume of the same buffer with a synthetic phosphopeptide substrate (R-R-R-R-P-pT-P-A) at a final concentration of 5 μM. After 15 min, the reaction was stopped by addition of 100 μL of a malachite green solution (Upstate Cell Signaling Solutions, Lake Placid, NY). After color development for 15 min, the absorbance at 650 nm was determined with a microplate reader. Values were corrected by subtracting the absorbance of blanks that were reactions without added enzyme. The assays were conducted within the linear response range with regard to the time of incubation and the amount of added enzyme. Results were replicated with different PP2C preparations.

RESULTS AND DISCUSSION

INS2 Enhances Insulin Action in Diabetic Rats. With severely diabetic rats (fasting blood glucose level of ∼500 mg/dL), INS2 itself did not promote a reduction in the level of blood glucose, but pretreatment with INS2 enhanced the response to subsequent administration of insulin. Injection with saline followed by insulin did not reduce the blood glucose level over 60 min, showing the relative insulin resistance of these animals (Figure 1). However, injection of increasing concentrations of INS2 15 min prior to the injection of insulin caused a dose-dependent reduction in blood glucose levels (Figure 1A). At the highest dose of INS2 (20 mg/kg), after 60 min the blood glucose level was reduced more than 50% relative to the control. In other control experiments, there was no reduction in the fasting blood glucose level over 60 min if insulin was injected before the INS2, or if INS2 was injected followed by saline instead of insulin (Figure 1B). These results revealed that in vivo pretreatment with INS2 sensitized diabetic animals to insulin and promoted a reduction in the blood glucose level. It was interesting that the reduction of the extent of hyperglycemia was not immediate after insulin injection but was delayed ∼30 min. Our interpretation was that ∼45 min was required for incorporation of the injected INS2 into an insulin sensitive signaling pathway. The lack of an effect of insulin alone or insulin prior to INS2 might be explained by prior observations (*31*). Examination of type 2 human diabetic muscle, hemodialysate, and urine revealed a marked decrease (ca. 50%) in putative *chiro*-inositol mediator bioactivity and inositol content compared to controls (*31*). This indicates that diabetic tissues are depleted of a basal level of mediators. We imagine that pretreatment with INS2 replenished the intracellular levels and this in turn restored insulin responsiveness.

Molecular Modeling of INS2 Binding to PP2C. We tested the hypothesis that INS2 had a specific site for interaction with PP2C, choosing this target because of the key role of PP2C in dephosphorylation of intracellular enzymes that are responsive to insulin action. We conducted computer modeling (*32*) experiments with INS2 and the reported X-ray crystal structure of PP2C, employing the FlexX module within the SYBYL 6.8 software package (*33*). INS2 was flexibly docked into the crystal structure of PP2C. FlexX begins with the compound (ligand) structure at an infinite point and calculates the energetics of the interactions as the ligand is brought into the binding pocket defined in the protein crystal structure. This maneuver is performed while also allowing free rotations of all bonds. The program alters the conformation of INS2 and ranks each of the INS2 conformers based on a set of scoring functions which take into account hydrogen bonding, the conformational energy of the ligand, and hydrophobic interactions.

FlexX produces interactive energies based on four scoring functions: *G*-score, PMF-score, *D*-score, and *C*-score (see Table 1). The *G*-score is a scoring function whose primary contributors are strong charge−charge interactions and hydrogen bonding. This scoring function also incorporates the internal energy of the ligand conformation. The PMF-score is a complement to the *G*-score as it is determined by hydrophobic and volume displacement interactions. The *D*-score is a strictly empirical score based on the compilation

Table 1: FlexX Scores for PP2C−Ligand Interaction

ligand	*G*-score	PMF-score	*D*-score	*C*-score
INS2	−202.95	−63.3	−131.14	5
INS2α	−137.52	−61.99	−80.49	3
INS2-glucosamine	−231−0.8	−70.72	−110.36	4
INS2-*myo*	−224.2	−52.52	−114.83	4

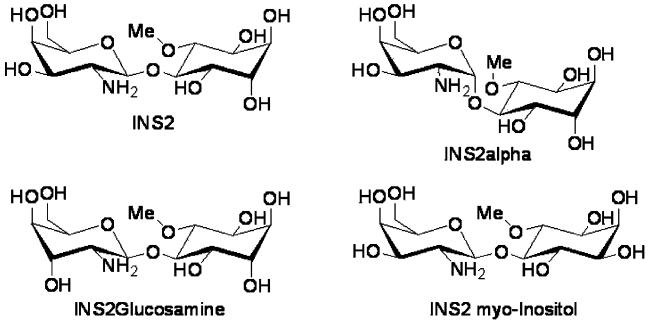

FIGURE 2: Pseudo-disaccharide structures. These four different structural variations of INS2 were generated and docked into the allosteric binding site of PP2C using computer modeling to evaluate possible preferential interactions. Analysis scores from FlexX quantitative modeling are given in Table 1.

of numerous naturally occurring protein−ligand interactions in defining positive ligand−protein interactions. The *C*-score, or cumulative scoring function, is important because it compiles the scores from the other three functions to yield a ranking from 0 to 5, 5 being the highest.

We evaluated our model for the specificity of INS2 docking into the PP2C allosteric site by designing and docking a set of alternative disaccharide structures (Figure 2). When the docking of the ligand to the protein is compared, the best binding conformer of the ligand is selected from possible conformers on the basis of its scoring functions. The scoring functions are then compared using the *C*-score value as the primary discrimination tool between various conformers. Using the *C*-score limits individual biases of the other scoring functions. To create a set of virtual alternate ligands, we introduced *myo*-inositol for D-*chiro*-inositol, glucosamine for galactosamine, and an α for a β glycosidic linkage (Figure 2). In Table 1, we analyze each of these compounds for the highest-scoring conformers (highest *C*-score) and ranked them, paying particular attention to the *C*-score function. Changes in the glycosidic bond from β to α resulted in the most significant drop in docking scores, showing that the β linkage of INS2 is a critical feature. While INS2-*myo* and INS2-glucosamine had varying results compared to INS2 in the individual scoring, the cumulative *C*-score function indicates that INS2 has the best overall binding to the allosteric site.

In a similar manner, we also modeled the binding of a substrate phosphopeptide (RRRRPpTPA) to PP2C. This peptide contains a phosphorylation site known to be a substrate for PP2C, within a sequence from protein phosphatase inhibitor-1 (INH-1) (*34*). The phosphopeptide was docked into the PP2C catalytic pocket defined by a β-sandwich having two β-sheets with a 1, 2, 11, 10, 7, 8 strand order and a 3, 4, 5, 6, 9 strand order in sheets 1 and 2, respectively (Figure 3). In the same surface cleft of the protein, the catalytic site is adjacent to what we term the allosteric site, bounded by the β8 sheet and the β8−α3 loop (containing Asp163 and Lys165) distal to the catalytic site

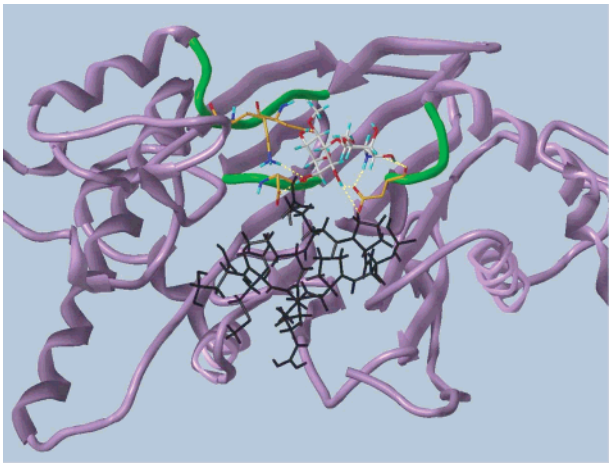

FIGURE 3: INS2 and phosphopeptide docked into the β-sandwich of PP2C. INS2 (colored ball-and-stick structure) and the substrate phosphopeptide (black ball-and-stick structure) were docked into a ribbon and tube model of PP2C showing the overall structure of the PP2C. The residues proposed to be interacting with INS2 are in three surface loops (green) that connect β8 and α3 (top left), β6 and β7 (center), and β10 and α5 (right). This face-on view of the active site shows the adjacent allosteric and catalytic sites for INS2 and phosphopeptide.

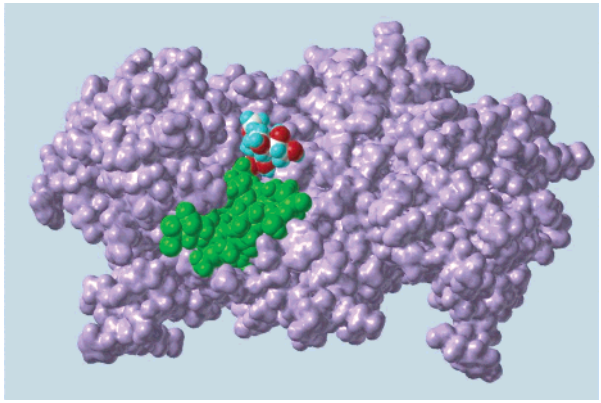

FIGURE 4: INS2 and phosphopeptide docked onto the surface of PP2C. INS2 (CPK model) and phosphopeptide (green) were docked onto a space-filling surface model of PP2C (purple) showing the potential binding to the β-sheet groove defined by the globular helical region (on the left) and the C-terminal domain of the protein (to the right).

and the β10−α5 loop (containing Asp243) proximal to the catalytic site. The "bottom" of the allosteric pocket is defined by the β7 sheet and the β6−β7 loop (containing Asp146). The residues critical for INS2 interaction are found on loops on the ends of the β-sandwich that forms the core of the protein. These loops are colored green in Figure 3. The loops of the main β-sandwich also contain six essential residues whose mutation causes the near-total loss of protein function (*35*). Our hypothesis is that allosteric activation arises in part from a conformational shift in the β10−α5 loop due to an interaction of INS2 with Asp243. This shift allows the phosphate of the peptide substrate more facile access to the catalytic core of the protein (Figure 3).

The active site and allosteric site both lie in a groove on the protein surface defined by Das et al. (*33*), seen in the space-filling model in Figure 4. The α-helical region between the β8 and β9 sheets forms a globular structure (left side of Figure 4), which helps to define the catalytic and allosteric sites within the surface groove. The docking of INS2 predicts

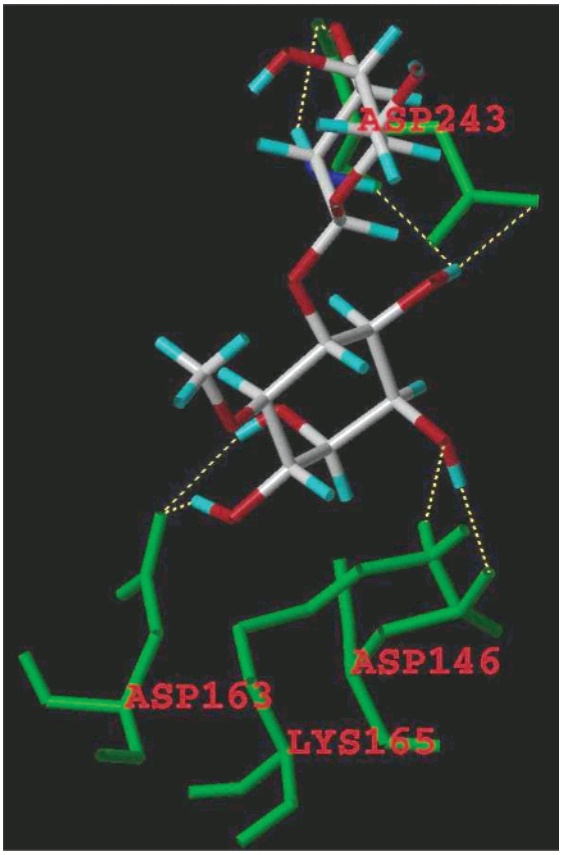

FIGURE 5: INS2 hydrogen bonding to Asp146, Asp163, Asp243, and Lys165 in PP2C. INS2 docked into the allosteric site of PP2C with only Asp146, Asp163, Asp243, and Lys165 of the protein visible, showing the potential hydrogen bonding network between INS2 and the allosteric site.

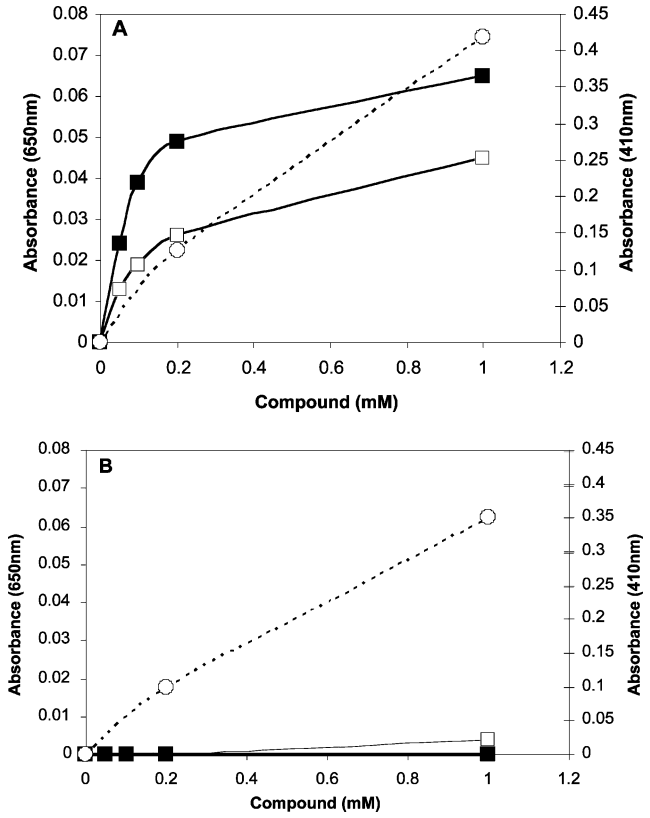

FIGURE 6: Dose-dependent activation of protein phosphatase 2C by INS2. The recombinant GST−PP2C fusion protein was assayed as described in Materials and Methods using either *p*-nitrophenyl phosphate (○ with dashed lines) or phosphopeptide (solid lines) as the substrate. Samples were assayed in duplicate and the average values plotted as a function of the concentration of added activator. (A) The wild-type GST−PP2C fusion protein was assayed at various concentrations of added $MnCl_2$ (□ and ○) or INS2 (■). (B) The D163A mutant of the GST−PP2C fusion protein was assayed at various concentrations of added $MnCl_2$ (□ and ○) or INS2 (■).

that the pinitol portion of INS2 interacts with the PP2C protein through a set of hydrogen bonds from residues Asp163, Lys165, and Asp146 and that the pinitol and galactosamine portions of INS2 hydrogen bond to Asp243 (Figures 4 and 5). Asp163 was of particular interest because the model suggested that this residue could potentially form hydrogen bonds with two *cis* vicinal hydroxyls on the pinitol (Figure 5). Asp163 was in the allosteric site but farthest from the catalytic center. Therefore, we suspected that mutation of Asp163 might not compromise catalytic function. By comparison, mutation of Asp243 in PP2Cβ, a metal ion ligand, previously was shown to markedly reduce the catalytic activity of PP2C (*35*). Our modeling studies showed that in the absence of INS2, Asp243 could form hydrogen bonds with the phosphate moiety of the phosphopeptide substrate and therefore potentially hinder its dephosphorylation. From the modeling, we developed the hypothesis that occupancy of the allosteric site by INS2 would engage Asp243 with an H-bond and thereby prevent this residue from interfering with the positioning of the phosphopeptide substrate at the catalytic center. Thus, both a conformational change and better substrate alignment are predicted to contribute to higher PP2C activity in the presence of INS2.

Phosphatase Activity of PP2C and Activation by INS2. We expressed recombinant wild-type and D163A PP2C in bacteria as GST fusion proteins and assayed the bacteria for phosphatase activity using a chromogenic substrate and a peptide substrate. As seen in the assays in Figure 6A, Mn^{2+} alone (□) as well as INS2 (■) produced dose-dependent activation of the wild-type GST−PP2Cα fusion protein. Note that INS2 was more effective than Mn^{2+} alone, in terms of the level of maximal activity. The half-maximal concentration (EC_{50}) for activation was ca. 50 μM. In contrast to the wild-type GST−PP2C fusion protein, the D163A mutant was not activated by either Mn^{2+} alone or INS2, up to 1 mM, using a phosphopeptide substrate (Figure 6B). Despite these stark differences in activity seen using the phosphopeptide substrate, both wild-type and D163A mutant forms of the GST−PP2C fusion protein had exactly the same Mn^{2+} dose dependence and the same specific activity with *p*-nitrophenyl phosphate as the substrate (○, dashed lines, Figure 6A,B). Thus, both PP2C proteins were functional, and the catalytic center was not compromised by mutation of D163; however, the effects of INS2 in promoting phosphopeptide dephosphorylation were ablated by this mutation.

These new in vivo and in vitro results reassert the possibility that there are intracellular mediators (second messengers) of insulin action and more specifically suggest a target site and mechanism for the action of INS2. We summarize the previously published supporting evidence. (1) Putative mediator activity was generated in muscles and livers of animals injected with insulin (*20*, *23*). (2) The putative mediator partially purified from muscle or liver increased PDH phosphatase activity (*15*, *16*, *19*, *20*, *25*−

27). (3) The kinetic effects of putative mediator preparations on PDH phosphatase activation (*25*) were the same as those produced by addition of insulin to adipocytes (*5*). (4) The partially purified mediator from liver and synthetic INS2 both decreased the extent of hyperglycemia in STZ diabetic rats (*27*, *29*, *30*). (5) INS2 (or a closely related inositol glycan) is present at decreased levels in human type 2 diabetic tissues compared to controls (*31*). (6) Putative mediator levels are increased in blood in normal humans, but not in type 2 diabetics administered a glucose tolerance test (*36*). (7) Putative mediators in blood were shown to contain *chiro*-inositol (*37*). Our studies show our synthetic INS2 preparation of defined composition and structure has in vivo activity in potentiating insulin lowering of the blood glucose level. One target of INS2 is the mitochondrial PDH phosphatase that activates oxidative glucose disposal. We report here molecular modeling and experimental biochemical data for PP2C, another member of the PPM enzyme family, as an intracellular target of INS2 that may be both physiologically and therapeutically important.

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