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Tyrosine Phosphorylation of Phosphatase Inhibitor 2

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Abstract Inhibitor 2 is a heat-stable protein that complexes with the catalytic subunit of type-1 protein phosphatase. The reversible phosphorylation of Thr 72 of the inhibitor in this complex has been shown to regulate phosphatase activity. Here we show that inhibitor 2 can also be phosphorylated on tyrosine residues. Inhibitor 2 was ^{32}P-labeled by the insulin receptor kinase in vitro, in the presence of polylysine. Phosphorylation of inhibitor 2 was accompanied by decreased electrophoretic mobility. Dephosphorylation of inhibitor 2 by tyrosine phosphatase 1B, restored normal electrophoretic mobility. Phosphotyrosine in inhibitor 2 was detected by immunoblotting with antiphosphotyrosine antibodies and phosphoamino acid analysis. In addition, following tryptic digestion, one predominant phosphopeptide was recovered at the anode. The ability of inhibitor 2 to inhibit type-1 phosphatase activity was diminished with increasing phosphorylation up to a stoichiometry of 1 mole phosphate incorporated/mole of inhibitor 2, where inhibitory activity was completely lost. These data demonstrate that inhibitor 2 can be phosphorylated on tyrosine residues by the insulin receptor kinase, resulting in a molecule with decreased ability to inhibit type-1 phosphatase activity.

Key words: insulin, dephosphorylation, type 1 phosphatase, tyrosine kinase, polylysine, insulin receptor

Insulin stimulates the phosphorylation of some proteins while at the same time stimulating the dephosphorylation of other proteins [Denton, 1990]. Most investigations have focused on the effects of insulin on protein kinases, but recently a mechanism has been proposed for activation of phosphatases in response to insulin [Dent et al., 1990]. Protein phosphatases can be divided into distinct protein families, tyrosine, and serine/threonine phosphatases [see review, Shenolikar and Nairn, 1991]. Serine/threonine protein phosphatases have been classified functionally into two groups: type 1 phosphatases, which are susceptible to inhibition by two heat-stable inhibitor proteins (inhibitor 1 and inhibitor 2) and preferentially dephosphorylate the β-subunit of phosphorylase kinase; and type 2 phosphatases, which are unaffected by the inhibitor proteins and preferentially dephosphorylate the α-subunit of phosphorylase kinase [Ingebritsen and Cohen, 1983].

One of the type 1 phosphatase inhibitor molecules, inhibitor 2, is currently thought to be present in the cytosol in a 1:1 heterodimer with the catalytic subunit of type 1 phosphatase [Ballou and Fisher, 1986; DePaoli, 1989]. However, others have argued that the heterodimer is not present in cells or fresh extracts, but rather is formed during purification [Vandenheede et al., 1989; Gruppuso et al., 1987; Brautigan et al., 1991]. In the heterodimer, phosphorylation of Thr 72 in inhibitor 2 by glycogen synthase kinase 3 is rapidly followed by dephosphorylation, triggering conformational changes in the catalytic subunit, resulting in increased type 1 phosphatase activity [Li et al., 1985]. In addition, inhibitor 2 is phosphorylated by casein kinase II on serines 86, 120, and 121, making it a better substrate for glycogen synthase kinase 3, promoting a further increase in activity of the Mg · ATP-dependent phosphatase [DePaoli, 1984].

Inhibitor 2 has been reported to be phosphorylated on tyrosine residues in vitro by the insulin receptor kinase, but no supporting data were shown [Cohen et al., 1985]. We reexamined the possibility that inhibitor 2 may be tyrosine phosphorylated. We report here that inhibitor 2 was tyrosine phosphorylated by highly purified insulin receptors in an insulin- and time-dependent reaction. Phosphorylation decreased the electro-

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phoretic mobility of inhibitor 2, and inhibitor 2 activity decreased in parallel with increasing tyrosine phosphorylation.

MATERIALS AND METHODS

Protein Purification

Bovine brain calmodulin was purified by standard methods [Gopalakrishna and Anderson, 1982]. Protein phosphatase inhibitor 2 was purified from rabbit skeletal muscle [Tonks and Cohen, 1984] with a final chromatography step on Mono Q (Pharmacia). Insulin receptors were purified from human placenta (less than 1 hour post partum) [Jo et al., 1992]. Tyrosine phosphatase 1B agarose was purchased from UBI (Lake Placid, NY).

Antibody Preparations

Polyclonal antibodies were raised against inhibitor 2 in sheep and affinity-purified as previously described [Gruppuso et al., 1987]. Anti-phosphotyrosine monoclonal antibodies were purchased from UBI (Lake Placid, NY).

Phosphorylation Assays

Phosphorylation reactions were carried out in 100 μl buffer containing 50 mM Tris HCl, pH 7.5, 1 mM EGTA, 0.4 mM $CaCl_2$, 0.1% Triton X-100, 0–1 μM polylysine (PL), and inhibitor 2, calmodulin, and insulin receptor as indicated. Reactions were initiated with the addition of 1 mM (γ^{32}P)-ATP (250–500 cpm/mmol), incubated for the times indicated at 30°C, and terminated with 1 vol ice-cold 25% (w/v) trichloroacetic acid (TCA) and processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described [Sacks and McDonald, 1989]. Following electrophoresis, gels were either stained with Coomassie Blue, or blotted onto Immobilon-P transfer membranes [Gershoni and Palade, 1983]. Blots were probed with antibodies to inhibitor 2 or phosphotyrosine and developed using the Bio-Rad alkaline phosphatase-conjugated substrate kit. To calculate the stoichiometry of inhibitor 2 phosphorylation the protein concentration was determined by amino acid composition using a Waters Pico Tag column on a Perkin-Elmer Series 4LC HPLC with a Waters 715 Ultra Wisp Autoinjector. In parallel experiments inhibitor 2 was phosphorylated with [γ^{32}P]-ATP. ^{32}P-labeled inhibitor 2 was excised from 12.5% gels and ^{32}P incorporation quantified by liquid scintillation counting.

Dephosphorylation of Inhibitor 2

In experiments requiring dephosphorylation of inhibitor 2, phosphorylation of inhibitor 2 was terminated by heating samples in a boiling water bath for 5 minutes thus destroying enzyme activity and utilizing the known heat stability of inhibitor 2. Samples were placed on ice for 5 min, followed by a 5-min preincubation at 30°C. Dephosphorylation was initiated by the addition of tyrosine phosphatase 1B agarose (85-nM final concentration) or a similar volume of agarose beads. Reactions were terminated after 20 min by boiling in SDS sample buffer and the reaction products separated on 12.5% SDS–PAGE.

Phosphoamino Acid Analysis

Inhibitor 2 was excised from gels and washed thoroughly in H_2O, then in 50 mM NH_4HCO_3. The protein was digested with 10 μg TPCK trypsin in 0.1 M N-ethylmorpholine acetate at pH 8.5 overnight. The sample was dried, then acid hydrolyzed for 30 min in 6 N HCl at 110°C. Phosphoamino acids were separated by electrophoresis on Kodak Chromagram thin layer cellulose plates at 1 kV for 60 min in water, acetic acid, and pyridine (89:10:1) pH 3.5 [Boyle et al., 1991].

Two-Dimensional Phosphopeptide Mapping

Inhibitor 2 was tryptically digested from SDS–PAGE gel slices as described for phosphoamino acid analysis. Samples were lyophilized and then resuspended in water, glacial acetic acid, and formic acid (89.7:7.8:2.5), at pH 1.9. Peptides were separated in the first dimension by high-voltage electrophoresis at pH 1.9 in the same buffer and then by ascending chromatography (*n*-butanol, pyridine, acetic acid, water; 60:40:48:12) in the second dimension, as previously described [Boyle et al., 1991].

Assay of Inhibitor 2 Activity

Inhibitor 2 activity was assayed using the purified catalytic subunit of type 1 phosphatase [Gruppuso et al., 1987]. Phosphatase activity was monitored by measuring the release of $^{32}P_i$ from labeled phosphorylase a. To assess the effects of phosphorylation, inhibitor 2 (90 nM) was incubated in phosphorylation reactions for 60 min. Control reactions were performed by omitting a single component in the phosphorylation mixture. An additional control assay was

performed in which an otherwise complete reaction mixture had the ATP added after the 60-min incubation and was immediately boiled (0 min phosphorylated). Reactions were terminated by boiling for 5 min and assayed for inhibitory activity after 1:10 dilution in the phosphatase assay. At this dilution nonphosphorylated inhibitor 2 produced 50% inhibition of type 1 phosphatase activity and represents 100% of the inhibitor activity in the assays. All assays were performed in duplicate, with values within 5% of one another.

RESULTS

Phosphatase inhibitor 2 was included in phosphorylation reactions in an attempt to determine its effect on calmodulin phosphorylation by purified placental insulin receptors. As shown in Figure 1, when calmodulin and inhibitor 2 were both present, inhibitor 2 was a better substrate for the insulin receptor kinase than was calmodulin (lanes 4 and 8). The concentration of inhibitor 2 (0.6 μM) was one-half the concentration of calmodulin (1.2 μM) and was preferentially labeled, while the phosphorylation of calmodulin markedly decreased. This occurred both in the absence (lanes 2 and 4) and in the presence of insulin (lanes 6 and 8). Insulin increased the phosphorylation of calmodulin alone approximately twofold (lanes 2 and 6), consistent with previous observations [Sacks and McDonald, 1989]. Inhibitor 2 phosphorylation was also increased by insulin (see Fig. 3). Phosphorylation of inhibitor 2 or calmodulin by the insulin receptor required the presence of polylysine. In the absence of polylysine (Fig. 1, lanes 1, 3, 5, 7), there was no detectable phosphorylation. Maximum phosphorylation of inhibitor 2 was obtained at a concentration of 25 nM polylysine (data not shown).

There was a decrease in electrophoretic mobility of inhibitor 2 that was dependent on the time and extent of phosphorylation (Fig. 2). This phosphorylation was performed with 0.2 μg insulin receptor in the absence of added insulin. As phosphorylation of inhibitor 2 increased over time, the nonphosphorylated 31-kd band of rabbit skeletal muscle inhibitor 2 (migration indicated in Fig. 2A) was converted to a ^{32}P-labeled band at 34 kd (Fig. 2A). The immunoblot probed with antibody to inhibitor 2 (Fig. 2B) confirmed that these bands represent different forms of inhibitor 2. The phosphoprotein at 29 kDa (Figs. 2A, 3) was present in variable amounts in different inhibitor 2 preparations and possibly represents a proteolytic fragment of inhibitor 2 or a form that is fully dephosphorylated when purified. Although the 29-kd form was phosphorylated in a time-dependent manner, it represented a relatively minor amount of the inhibitor, as determined by the weak immunostain with the inhibitor 2 antibody (Fig. 2B). Interestingly,

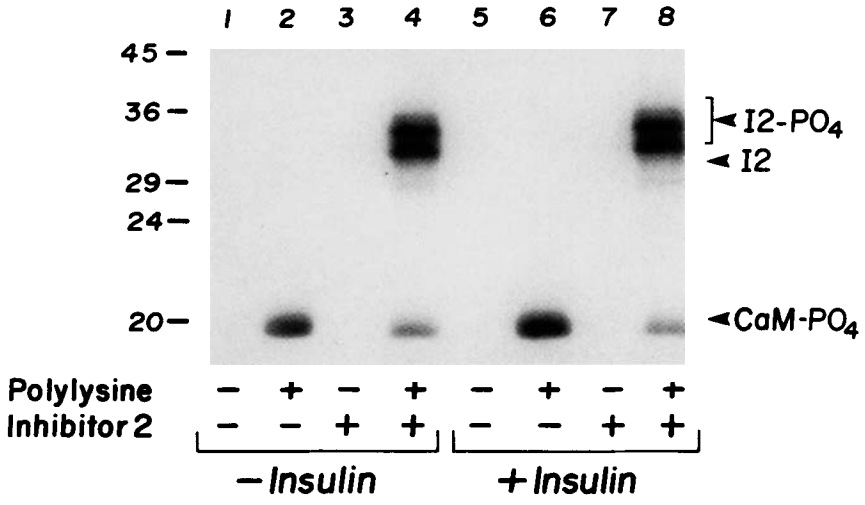

Fig. 1. Phosphorylation of inhibitor 2 and calmodulin by insulin receptor kinase. Phosphorylation assays were performed for 2 hr at 30°C. Calmodulin (1.2 μM) and insulin receptor were present in all lanes. Inhibitor 2 (0.6 μM), 60 nM polylysine (PL) and 3 nM insulin were added as indicated. An autoradiograph is shown of the reaction products after separation by polyacrylamide gel electrophoresis. Arrows indicate the migration of phosphorylated calmodulin (CaM-PO_4), nonphosphorylated inhibitor 2 (I2) and phosphorylated inhibitor 2 (I2-PO_4). The migration of molecular weight standards (kDa) are indicated on the left margin.

its mobility did not seem to be affected by phosphorylation. Inhibitor 2 phosphorylation by the insulin receptor was insulin concentration dependent (Fig. 3). Quantitation by laser densitometry of multiple independent experiments gave a $K_{0.5}$ of approximately 3 nM insulin. The apparent decreased electrophoretic mobility of inhibitor 2 is not readily apparent in Figure 3, since inhibitor 2 is phosphorylated in all lanes shown. A 34-kd form of inhibitor 2 is not readily discernible in this Figure (cf. Figs. 2, 3), but was present on the original autoradiograph. The migration of nonphosphorylated inhibitor 2 is indicated (I 2 arrow). The stimulation of inhibitor 2 phosphorylation by insulin (and the stoichiometry of labeling), was variable in different receptor preparations, ranging from 2- to 10-fold. This may be due to partial activation of the insulin receptor kinase during purification and loss of activity during storage [Fujita-Yamaguchi et al., 1989a]. The experiments illustrated in Figures 2 and 3 were conducted with the same amount of insulin receptor from different preparations.

In a separate set of experiments, inhibitor 2 was phosphorylated in a similar manner, then incubated in the presence or absence of tyrosine phosphatase 1B (Fig. 4). Phosphorylation of inhibitor 2 resulted in the characteristic decrease in electrophoretic mobility (Fig. 4; cf. lane 4 to controls, lanes 1–3). Subsequent dephosphorylation of inhibitor 2 by tyrosine phosphatase 1B (Fig. 4, cf. lane 5 to lane 4) restored the electrophoretic mobility of inhibitor 2 to normal (M_r = 31 kDa) (Fig. 4; cf. lane 5 to lanes 1–3). The immunostained inhibitor 2 in lane 5 appears to be decreased. However, this apparent decrease was not evident in Coomassie-stained gels (data not shown).

Inhibitor 2 was phosphorylated almost exclusively on tyrosine residues by the highly purified insulin receptor kinase. That tyrosine was phosphorylated was first shown using antiphosphotyrosine antibodies (data not shown). The phosphotyrosine immunostaining of inhibitor 2 was competitively inhibited by incubating blots with the phosphotyrosine antibody plus 10 mM 0-phospho-L-tyrosine indicating its specificity (data not shown). Independently, phosphoamino acid analysis of inhibitor 2 that was phosphorylated by the purified insulin receptor kinase in the presence of polylysine as described above demonstrated that phosphotyrosine was the major product (Fig. 5A, lane b). Two-dimensional separation of tryptic peptides of ^{32}P-labeled inhibitor 2 revealed one major (immobile) and one minor highly acidic phosphopeptide both of which migrated toward the anode at pH 1.9 (Fig. 5B), while a cyanogen bromide digest of ^{32}P-labeled inhibitor 2 resulted in a single highly acidic phosphopeptide which also migrated toward the anode (data not shown).

Tyrosine phosphorylation of inhibitor 2 reduced its ability to inhibit type 1 phosphatase activity. Type 1 phosphatase activity was assayed using ^{32}P-labeled phosphorylase a as substrate [Gruppuso et al., 1985]. The amount of inhibitory activity in separate controls that omitted either insulin receptor, polylysine, or ATP were approximately the same in different experiments (column 1). Phosphorylation reactions (60 min phosphorylated) produced variable changes in inhibitory activity. In three separate experiments (Fig. 6, columns 2–4), the relative

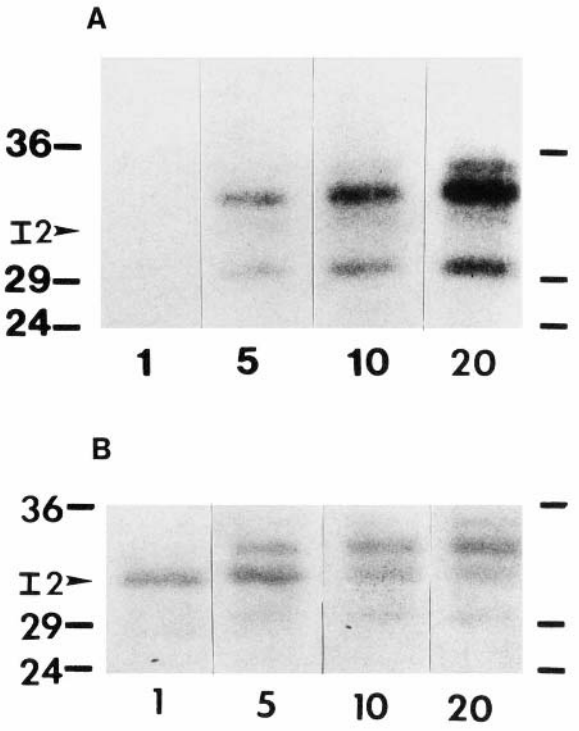

Fig. 2. Time course of phosphorylation of inhibitor 2. In the experiments shown in panels A and B, inhibitor 2 was phosphorylated in a 100-μl reaction volume in the absence of insulin with 0.2 μg of insulin receptor for 1, 5, 10, and 20 min, as described in the Materials and Methods, in the presence of 25 nM polylysine. Migration of nonphosphorylated inhibitor 2 is marked (closed arrow). The reaction products were separated by polyacrylamide gel electrophoresis, proteins transferred to Immobilon-P, and probed with the affinity-purified inhibitor 2 antibody. **A:** Autoradiograph. **B:** Alkaline phosphatase-stained immunoblot. Migration of the 24-, 36-, and 47-kd M_r standards are shown on the left margin.

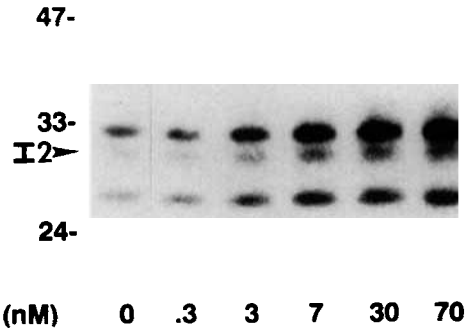

Fig. 3. Insulin dependence of inhibitor 2 phosphorylation. The insulin dependence of inhibitor 2 phosphorylation was examined by phosphorylating with 0.2-μg-purified placental insulin receptor at 30°C for 5 min in the presence of 25 nM polylysine and increasing concentrations of insulin, as indicated in a 100-μl reaction volume. Migration of nonphosphorylated inhibitor 2 is marked (closed arrow). The reaction products were separated by electrophoresis on a polyacrylamide gel and an autoradiograph was obtained. Migration of the 24-, 33-, and 47-kd molecular-weight standards are shown in the left margin.

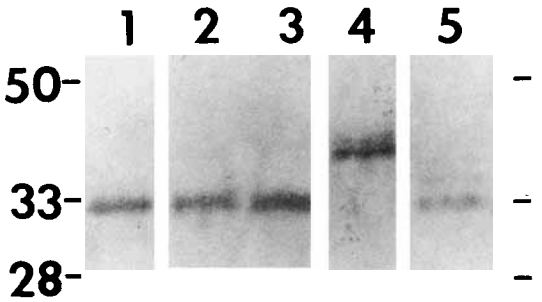

Fig. 4. Tyrosine phosphorylated inhibitor 2 is dephosphorylated by tyrosine phosphatase 1B. Inhibitor 2 (0.35 μM) was phosphorylated by purified insulin receptor kinase (0.2 μg) for 0 min (lanes 2, 3) or 60 min (lanes 4, 5). Lane 1, three separate controls in which either insulin receptor, ATP or polylysine, was deleted from the reaction. Reactions were terminated by boiling for 5 min. Following 5 min on ice, samples were incubated in the absence (lanes 1, 2, 4) or presence (lanes 3, 5) of tyrosine phosphatase 1B (85 nM) for 20 min. Reactions were terminated by boiling with SDS–PAGE sample buffer, the reaction products were separated on 12.5% polyacrylamide gels and then transferred to Immobilon and probed with polyclonal inhibitor 2 antibody.

change in inhibitor 2 activity was inversely related to the stoichiometries of phosphorylation of inhibitor 2. The activity of phosphorylated inhibitor ranged from 73% to 5% of nonphosphorylated inhibitor 2 activity, while the stoichiometry of phosphorylation of inhibitor 2 in the same experiments ranged from 0.40 to 1.06 mol phosphate/mol of inhibitor 2. The relationship between the phosphotyrosine content of inhibitor 2 and the phosphatase inhibitory activity of inhibitor 2 is shown in Figure 6. In each case

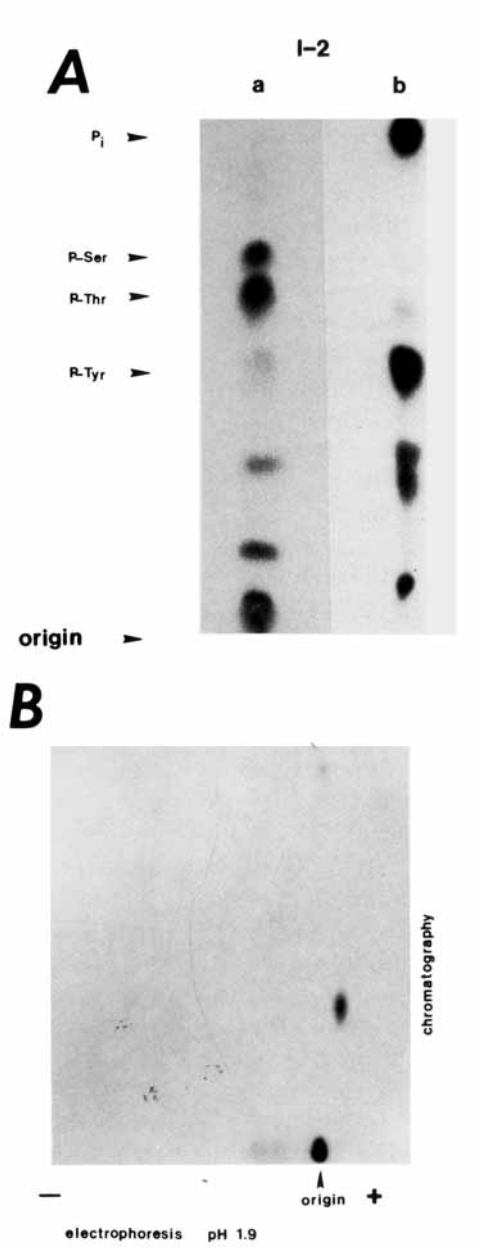

Fig. 5. Phosphotyrosine in inhibitor 2. Inhibitor 2 was phosphorylated by the purified insulin receptor (0.2 μg) in the presence of insulin and polylysine in a 100-μl reaction volume. Phosphorylated inhibitor 2 was then digested from polyacrylamide gels with TPCK trypsin and either acid hydrolysed and mixed with 1.5 μg each of phosphoamino acid standards (**A**) or separated by two-dimensional phosphopeptide mapping (**B**). Lane a (A) illustrates the ninhydrin-stained cellulose plate of inhibitor 2 after high-voltage electrophoresis, while lane b represents the corresponding autoradiogram. B: Autoradiogram obtained from phosphopeptides separated by electrophoresis in the first dimension at pH 1.9 and by ascending chromatography (*n*-butanol, pyridine, acetic acid, water; 60:40:48:12) in the second dimension. The anode (+) and cathode (−) are shown at the bottom.

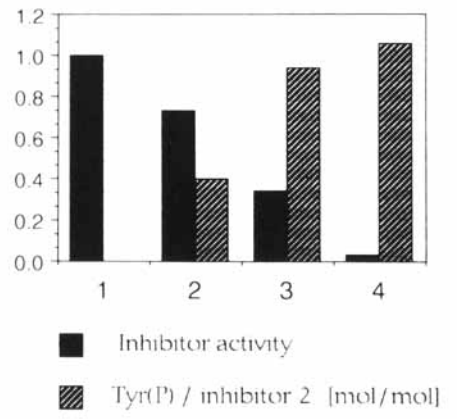

Fig. 6. Effect of tyrosine phosphorylation on phosphatase inhibitor 2 activity. The bar graph shows the relative change in units of phosphatase inhibitor 2 activity (solid bar) as a function of the stoichiometry of inhibitor 2 phosphorylation (stippled bar). Inhibitor activity is presented as the percentage of inhibitor activity remaining (1.0 being 100% of inhibitor 2 activity or 1.0 mol phosphate/mol inhibitor 2). Column 1 represents the average of three separate nonphosphorylated controls (100% of inhibitor activity), while columns 2, 3, and 4 represent the results of three separate phosphorylation experiments conducted with three separate preparations of insulin receptor.

tyrosine phosphorylation diminished the phosphatase inhibitory activity of inhibitor 2.

DISCUSSION

Tyrosine phosphorylation of inhibitor 2 is consistent with our previous studies using highly purified insulin receptor preparations, in which phosphorylation of calmodulin occurs almost exclusively on tyrosine residues [Sacks et al., 1989a; Sacks and McDonald, 1989]. Phosphorylation of inhibitor 2 by the insulin receptor kinase requires the basic polypeptide, polylysine. This requirement has been reported previously [Fujita-Yamaguchi et al., 1989a] for the phosphorylation of calmodulin by the insulin receptor kinase, but its significance is unknown. Polylysine and other polybasic proteins (i.e., polyarginine, protamine sulfate) activate a number of protein kinases (including the insulin receptor kinase) directly and, in the case of calmodulin, also interact with the substrate. It has been proposed that endogenous basic polypeptides may play an analogous regulatory role in the intact cell. One such peptide, the basic peptide tail of the c-Ki-ras oncogene product, is capable of substituting for polylysine in the in vitro system for the phosphorylation of calmodulin by the insulin receptor kinase [Fujita-Yamaguchi et al., 1989a,b; Sacks et al., 1989b].

Phosphorylation of purified skeletal muscle inhibitor 2 by the insulin receptor kinase decreased the electrophoretic mobility of inhibitor 2 and resulted in multiple phosphorylated bands of inhibitor 2. Each band presumably represented inhibitor 2 that was differentially phosphorylated. The phosphorylation of individual bands varied somewhat between preparations of inhibitor 2 and insulin receptor. However, the electrophoretic mobility of inhibitor 2 was altered substantially in each experiment. Phosphoamino acid analysis clearly demonstrated that >95% of the ^{32}P incorporated was in tyrosine. Phosphopeptide mapping was performed to determine if multiple tyrosines were phosphorylated. Tryptic phosphopeptide maps demonstrated a single, mobile phosphopeptide migrating at the anode. Migration of the phosphopeptide at the anode suggested that the peptide is multiply phosphorylated leading to the hypothesis that the phosphorylated tyrosine residue(s) probably resided in the only tryptic peptide, residues 67–101, which contains 2 tyrosine residues (75 and 85). Subsequent cleavage with cyanogen bromide, which cleaves between methionine 77 and isoleucine 78 [Holmes et al., 1986], also resulted in a single phosphopeptide migrating at the anode. We interpret this as evidence that one tyrosine residue was ^{32}P phosphorylated in this reaction. We speculate that Tyr 85 in the sequence G_{79}DDDDAYS$_{86}$ is phosphorylated because acidic sequences are preferred by tyrosine kinases. However, the peptide may also contain endogenously phosphorylated residues because of the multiple species of phosphorylated inhibitor 2 seen on SDS–PAGE. The endogenous phosphorylation state of inhibitor 2 purified from rabbit skeletal muscle utilized in these in vitro experiments was not determined. Inhibitor 2 has been reported to be phosphorylated on serine residues based on fast atom bombardment mass spectrometry [Holmes et al., 1987], while others have reported that inhibitor 2 was phosphorylated on both serine and threonine residues [DePaoli-Roach, 1984; Holmes et al., 1987; Ballou et al., 1983; Resink et al., 1983; Lawrence et al., 1988]. It seems reasonable that the endogenous phosphorylation state of purified inhibitor 2 would be variable in view of these reports. Thus, we believe that our data are consistent with ^{32}P labeling of a single tyrosine residue (probably Tyr 85) and that variations in the electrophoretic mobility of inhibitor 2 are most likely due to variations in

the endogenous phosphorylation state of the purified inhibitor 2. We cannot rule out the possibility that serine and/or threonine phosphorylation is required for the tyrosine phosphorylation observed in these studies.

The tyrosine phosphorylation dependent decrease in inhibitor 2 activity is novel. The data presented in Figure 6 was obtained with 3 separate preparations of insulin receptor. The differences in stoichiometry of phosphorylation of inhibitor 2 were intended to illustrate the relationship between extent of tyrosine phosphorylation and phosphatase inhibitory activity of inhibitor 2. These data become more compelling with experiments demonstrating that the decreased electrophoretic mobility in response to phosphorylation is reversed upon incubation with the tyrosine-specific phosphatase, PTP 1B. While in vitro tyrosine phosphorylation of inhibitor 2 has been reported in the literature, no data was presented [Cohen et al., 1985] and convincing evidence of tyrosine phosphorylation of inhibitor 2 in vivo has not been reported in the literature, although it has been reported in an abstract [Williams et al., 1992]. The data presented here does not prove that the insulin receptor is the "physiological tyrosine kinase" but simply indicate that tyrosine phosphorylation of inhibitor 2 can regulate the phosphatase inhibitory activity of this protein and suggest that this modification may prove to be important in the signal transduction process of cells.

Considerable post-homogenization dephosphorylation of phosphotyrosine residues has been reported even in the presence of standard phosphatase inhibitors [Kamps and Sefton, 1988; Levenson and Blackshear, 1989]; therefore, it should not be assumed that tyrosine phosphorylation does not occur. However, conditions by which the tyrosine phosphorylation state of endogenous proteins may be more efficiently preserved in whole cells remain to be elucidated.

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REFERENCES

Ballou LM, Fischer EH (1986): Protein phosphatases. In Boyer PD, Krebs EG (eds): "The Enzymes." Vol. 17. San Diego: Academic Press, pp 312–361.

Ballou LM, Brautigan DL, Fischer EH (1983): Subunit structure and activation of inactive phosphorylase phosphatase. Biochemistry 22:3393–3399.

Boyle WJ, Van Der Geer P, Hunter T (1991): Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol 201:110–149.

Brautigan DL, Fernandez A, Lamb NJC (1991): Oscillation of protein phosphatase-1 and inhibitor 2 during mammalian cell cycle. In Merlevede W, Di Salvo J (eds): "Advances in Protein Phosphatases." Vol. 6. Leuven, Belgium: Leuven University Press, pp 375–390.

Cohen P, Parker PJ, Woodgett JR (1985): The molecular mechanism by which insulin activates glycogen synthase in mammalian skeletal muscle. In Czech M (ed): "Molecular Basis of Insulin Action." New York: Plenum Press, pp 213–233.

Dent P, Lavoinne A, Nakielney S, Caudwell FB, Watt P, Cohen P (1990): Molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature 348:302–308.

Denton RM (1990): Search for the missing links. Nature 348:286–287.

DePaoli-Roach AA (1984): Synergistic phosphorylation and activation of ATP-Mg-dependent phosphoprotein phosphatase by F_a/GSK-3 and casein kinase 11 (PC 0.7). J Biol Chem 259:12144–12152.

DePaoli-Roach AA (1989): Regulatory components of type 1 protein phosphatases. In Merlevede W, Di Salvo J (eds): "Advances in Protein Phosphatases." Vol. 5. Leuven, Belgium: Leuven University Press, pp 479–500.

Fujita-Yamaguchi Y, Kathuria S, Xu QY, McDonald JM, Nakano H, Kamata T (1989a): In vitro tyrosine phosphorylation studies on ras proteins or domains may be involved in interactions between insulin receptor and its substrate and calmodulin suggest that the polylysine-like basic proteins. Proc Natl Acad Sci USA 86:7306–7310.

Fujita-Yamaguchi Y, Kathuria S, Sacks DB, McDonald JM, Sahal D (1989b): Effect of basic polycations and proteins on purified insulin receptor. Insulin-independent activation of the receptor tyrosine specific protein kinase by poly (L-lysine). Biochem J 263:813–822.

Gershoni JM, Palade GE (1983): Protein blotting: Principles and applications. Anal Biochem 131:11–15.

Gopalakrishna R, Anderson WB (1982): Ca^{2+}-induced hydrophobic site on calmodulin: Application for purification of calmodulin by phenyl-Sepharose affinity chromatography. Biochem Biophys Res Commun 104:830–836.

Gruppuso PA, Johnson GL, Constantinides M, Brautigan DL (1985): Phosphorylase phosphatase regulatory subunit. Western blotting with immunoglobulins against inhibitor 2 reveals a protein of M_r = 60,000. J Biol Chem 260:4288–4294.

Gruppuso PA, Shriner CL, Brautigan DL (1987): Latent forms of type-1 protein phosphatase in rabbit skeletal muscle. Biochem Biophys Res Commun 148:1174–1181.

Holmes CFB, Campbell DG, Caudwell FB, Aitken A, Cohen P (1986): The protein phosphatases involved in cellular regulation. Primary structure of inhibitor 2 from rabbit skeletal muscle. Eur J Biochem 155:173–182.

Holmes CFB, Tonks MK, Major H, Cohen P (1987): Analysis of the in vivo phosphorylation state of protein phosphatase inhibitor 2 from rabbit skeletal muscle by fast-atom bondardmant mass spectrometry. Biochim Biophys Acta 929:208–219.

Ingebritsen TS, Cohen P (1983): Protein phosphatase involved in cellular regulation. 1. Classification and substrate specificity. Science 221:331–338.

Jo H, Cha BY, Davis HW, McDonald JM (1992): Identification, partial purification, and characterization of two guanosine triphosphate-binding proteins associated with insulin receptors. Endocrinology 131:2855–2862.

Kamps MP, Sefton BM (1988): Identification of multiple novel polypeptide substrates of the v-*src*, v-*fps*, v-*ros*, and v-*erb*-β oncogenic tyrosine kinases utilizing antisera against phosphotyrosine. Oncogene 2:305–315.

Lawrence JC, Hiken J, Burnette B, DePaoli-Roach AA (1988): Phosphorylation of phosphoprotein phosphatase inhibitor 2 (I-2) in rat fat cells. Biochem Biophys Res Commun 150:197–203.

Levenson RM, Blackshear PJ (1989): Insulin-stimulated protein tyrosine phosphorylation in intact cells evaluated by giant two-dimensional gel electrophoresis. J Biol Chem *264:19984–19993.*

Li HC, Price DJ, Tabarini D (1985): On the mechanism of regulation of type 1 phosphoprotein phosphatase from bovine heart. Regulation by a novel intracyclic activation-deactivation mechanism via transient phosphorylation of the regulatory subunit by phosphatase-1 kinase (F_a). J Biol Chem 260:6416–6426.

Resink TJ, Hemmings BA, Tung HY, Cohen P (1983): Characterization of a reconstituted Mg-ATP dependent protein phosphatase. Eur J Biochem 133:455–461.

Sacks DB, McDonald JM (1989): Calmodulin as subunit for the insulin receptor kinase: Phosphorylation by receptors from rat skeletal muscle. Diabetes 38:84–90.

Sacks DB, Fujita-Yamaguchi Y, Gale RD, McDonald JM (1989a): Tyrosine-specific phosphorylation of calmodulin by the insulin receptor kinase purified from human placenta. Biochem J 263:803–812.

Sacks DB, Glenn KC, McDonald JM (1989b): The carboxyl terminal segment of the C-K_i-ras 2 gene product mediates insulin stimulated phosphorylation of calmodulin and stimulates insulin-independent autophosphorylation of the insulin receptor. Biochem Biophys Res Commun 161:399–405.

Shenolikar S, Nairn AC (1991): Protein phosphatases: Recent progress. In Greengard P, Robison GA (eds): "Advances in Second Messenger and Phosphoprotein Research." Vol. 23. New York: Raven Press, pp 1–121.

Tonks NK, Cohen P (1984): The protein phosphatases involved in cellular regulation. Identification of the inhibitor 2 phosphatases in rabbit skeletal muscle. Eur J Biochem 145:65–75.

Vandenheede JR, Agostinis P, Staquet S, Van Lint J (1989): The inactive ATP, Mg-dependent protein phosphatase. Origin, role and regulation. In Merlevede W, Di Salvo J (eds): "Advances in Protein Phosphatases." Vol. 5. Leuven, Belgium: Leuven University Press, pp 19–36.

Williams JP, Brautigan DL, McDonald JM (1992): Insulin decreases tyrosine phosphorylation of phosphatase inhibitor 2. Diabetes 41(suppl 1):165A.