The Regulation of Glycogen Synthase by Protein Phosphatase 1 in 3T3-L1 Adipocytes

EVIDENCE FOR A POTENTIAL ROLE FOR DARPP-32 IN INSULIN ACTION*

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The stimulation of glycogen-targeted protein phosphatase 1 (PP1), glycogen synthase, and glycogen synthesis by insulin was examined during the differentiation of 3T3-L1 fibroblasts into adipocytes. Insulin treatment barely changed the low levels of glycogen synthesis measured in fibroblasts. Following differentiation into adipocytes, insulin increased glycogen synthesis up to 40-fold. After further culturing of the adipocytes for a week, insulin stimulated glycogen accumulation 700-fold. Differentiation of 3T3-L1 cells also resulted in the increased expression of glycogen synthase and in increases in both total glycogen synthase activity and fold-stimulation by insulin. While the levels of PP1 protein were unchanged by differentiation, PP1 specific activity decreased over 60%, although sensitivity to insulin treatment was augmented. Concurrently, levels of the PP1 inhibitor protein DARPP-32 were dramatically induced upon 3T3-L1 adipogenesis. DARPP-32 in both 3T3-L1 and primary rat adipocytes was exclusively localized to the particulate fractions, including the glycogen-enriched pellet. PP1 activity from 3T3-L1 adipocytes exhibited a kinetic lag in vitro, which was not present in fibroblast extracts. Insulin pretreatment of the adipocyte cells overcame the in vitro lag in PP1 activity, resulting in up to 5-fold stimulation of PP1 activity being measured at early assay time points. These results suggest that in 3T3-L1 adipocytes, DARPP-32 may maintain glycogen-targeted PP1 activity in a low basal state, priming the phosphatase for stimulation by insulin.

Protein phosphorylation plays a critical role in the regulation of lipid and glucose metabolism (1). As the major anabolic hormone regulating glucose utilization and storage, insulin exerts many of its effects by promoting the net dephosphorylation of enzymes such as glycogen synthase, glycogen phosphorylase, and phosphorylase kinase, resulting in the stimulation of glycogen synthesis. Several lines of evidence indicate that these insulin-stimulated dephosphorylations are catalyzed by protein phosphatase 1 (PP1). This phosphatase is found in nearly all cellular compartments and is thought to be targeted intracellularly by specific proteins (2, 3). Such targeting proteins have been identified that localize PP1 to the nucleus (4), the sarcoplasmic reticulum (5), glycogen, and myofibrils (reviewed in Ref. 3). The best characterized of these are the mammalian glycogen-targeting proteins. Three related members have been described: GM, isolated from skeletal muscle (6, 7), the hepatic GL protein (8, 9), and the recently cloned PTG protein (10, 11). Unlike the extremely restricted expression of GM and GL, PTG is highly expressed in the major insulin-responsive tissues, including fat, skeletal muscle, heart, and liver.2

PP1 activity is controlled by targeting proteins in several ways. First, by localizing PP1 to subcellular compartments, such as glycogen, targeting proteins serve to increase PP1 specific activity against co-localized substrates. The two glycogen-targeting subunits, GM and GL, have also been reported to differentially regulate PP1 activity toward the same glycogen-bound substrates (9, 12). Phosphorylation of GM has been proposed to directly regulate PP1 activity in response to both anabolic and catabolic signals (12), although this model has been questioned (13–17). To add to the uncertainty, the two putative regulatory phosphorylation sites of GM are not conserved in PTG or GL, and both proteins are poorly phosphorylated in vitro (9, 18). Since it is unlikely that PTG is regulated by phosphorylation in vivo (18), other models for the stimulation of glycogen-targeted PP1 need to be explored.

PP1 activity is also regulated by inhibitor (Inh) proteins (reviewed in Ref. 19). There are three inhibitors of PP1 activity, Inh-1, its neuronal homologue DARPP-32, and Inh-2. Phosphorylation of a conserved threonine residue on Inh-1 and DARPP-32, by cAMP-dependent protein kinase, converts these molecules into highly potent, reversible, and very specific inhibitors of PP1 activity. Dephosphorylation of these proteins results in their inactivation and disassociation from PP1. Inh-2 is also regulated by phosphorylation, but it acts more as a regulatory subunit. The direct regulation of glycogen-targeted PP1 by these inhibitors has not been extensively investigated.

We have investigated the regulation of glycogen synthesis by insulin in the 3T3-L1 cell line. Differentiation of these cells from fibroblasts into adipocytes results in a large increase in both total metabolic activity and insulin responsiveness. To evaluate the regulation of the components directly involved in glycogen synthesis, we examined the effects of insulin treatment on glycogen synthase and glycogen-targeted PP1 activity during differentiation of 3T3-L1 cells. The results suggest that in 3T3-L1 adipocytes, the inhibitor peptide DARPP-32 may regulate glycogen-associated PP1 activity and may be a target protein targeting to glycogen; FBS, fetal bovine serum; G6P, glucose-6-phosphate; PNS, postnuclear supernatant.

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1 The abbreviations used are: PP1, type 1 protein phosphatase; GM, glycogen-binding regulatory subunit of type 1 protein phosphatase; GL, hepatic glycogen-targeting subunit of type 1 protein phosphatase; PTG, hepatic glycogen-targeting subunit of type 1 protein phosphatase; FBS, fetal bovine serum; G6P, glucose-6-phosphate; PNS, postnuclear supernatant.

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of insulin signaling to increase glycogen synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents, phosphorylase, and phosphorylase kinase were from Life Technologies, Inc. Okadaic acid was obtained from Calbiochem. γ-[32P]ATP (3000 Ci/mmole) and (U-14C)glucose (251

2-mercaptoethanol, and 2 mg/ml glycogen) plus 10

REACTIONS were terminated by the addition of 90

Phosphate release was measured as described previously (15), or the

tant was measured by liquid scintillation counting. 32P-Phosphoryl-

fuged for 2 min at 15,000

Ref. 21). Following a 10-min incubation on ice, samples were centri-

fibroblasts into lipid-containing adipocytes, glycogen synthesis

KRBH containing 2.5 mM glucose and 0.5% bovine serum albumin.

minor modifications. Briefly, six-well dishes were serum-deprived in

described (15). Glycogen synthesis was assayed as described (15) with

activities during 3T3-L1 Differentiation—Because of the dra-

matic changes in insulin-stimulated glycogen synthesis during

The responsiveness of glycogen synthesis to insulin dramati-

cally increases during differentiation of 3T3-L1 cells—3T3-L1

fibroblasts were differentiated into adipocytes by a standard

4-day protocol, and the adipocytes were then maintained in

FBS-containing medium for an additional 1 or 7 days. The cul-

tures of the adipocytes in the FBS medium for 7 more days

did not change the percentage of cells containing lipid droplets

(>90%), but it did not result in increased lipid stores per cell (data

not shown). Basal glycogen synthesis was barely detectable in

fibroblast cells and was increased only 3-fold by 100 nM insulin

(Glycogen synthesis

TABLE I

| Cell type      | Glycogen synthesis |
|----------------|--------------------|
| Basal          | Insulin (100 nM)   |
| Fibroblast     | 0.34 ± 0.01        |
| Adipo/1        | 0.40 ± 0.01        |
| Adipo/8        | 0.27 ± 0.02        |

Values presented are in nmol of glucose incorporated per 45 min per well.

stimulated 30–40-fold by insulin, with no change in the

natal rate (Table I, Adipo/1). In day 8 adipocytes, basal glyco-

gen synthesis was still comparable with that seen in 3T3-L1

fibroblasts, but insulin caused a nearly 700-fold increase in

glycogen accumulation (Table I, Adipo/8).

Regulation of Glycogen Synthase and PP1 Expression and Activities during 3T3-L1 Differentiation—Because of the dra-

matic changes in insulin-stimulated glycogen synthesis during

adipogenesis, we sought to compare the relative expression of different enzymes that might be critical to the process. Indeed, several proteins have been reported to be expressed upon

3T3-L1 adipocyte differentiation, including the insulin receptor (22) and Glut-4 (23), which contribute to the regulation of glycogen synthesis by insulin. The expression of glycogen synthase and PP1 on the glycogen particle was measured during differentiation of 3T3-L1 fibroblasts into adipocytes. Across the 3T3-L1 differentiation protocol, replicate plates were stimu-

lated in the absence and presence of 100 nM insulin. Glycogen-

enriched pellets were prepared, and glycogen synthase and PP1 protein levels were examined by immunoblotting. As seen in Fig. 1A, glycogen synthesis levels in this fraction increased during the differentiation protocol (days 2–6), and continued to increase as the adipocytes were cultured in FBS medium (days 6–17). Insulin treatment had no effect on the amount of glycogen synthase present in the glycogen-containing fraction at any time. In contrast, there was no change in the amount of glycogen-associated PP1 during fibroblast differentiation or upon continued culturing of the adipocytes (Fig. 1B). At no time did insulin cause any measurable change in glycogen-targeted PP1 levels, indicating that the stimulation of PP1 activity by insulin occurs independently of translocation of the phosphatase to glycogen. It should be noted that since the total protein content of the glycogen-enriched pellet increased by at least 8-fold during differentiation, increases in the total amounts of glycogen synthase and PP1 in the adipocyte samples are significantly understated.

In parallel to the protein measurements, both glycogen synthase and PP1 activities were directly measured on the indicated days. In agreement with Fig. 1A, differentiation of the 3T3-L1 fibroblasts into adipocytes resulted in a dramatic increase in total glycogen synthase activity (Fig. 2A, +G6P). Insulin activates glycogen synthase through dephosphorylation of the enzyme, resulting in the stimulation of G6P-independent synthase activity (−G6P). Very low levels of active synthase were detected in the fibroblasts, which increased slightly in response to insulin (Fig. 2A, Fibroblast, −G6P). In day 1 adipocytes, insulin now caused a greater -fold stimulation and an absolute increase in G6P-independent synthase activity (Fig. 2A, Adipo/1, −G6P). This
trend continued as the adipocytes were cultured for another week, with active synthase being increased 7-fold in response to insulin in the day 8 adipocytes (Fig. 2A). These measurements were adjusted for protein, so the amounts of active and total glycogen synthase activities in the adipocytes are underrepresented approximately 8-fold relative to the fibroblasts.

Since glycogen synthase activation by insulin results from dephosphorylation, PP1 activity during differentiation was also examined. In contrast to observations regarding glycogen synthase, 3T3-L1 fibroblasts contained the highest PP1 basal activity, which was modestly affected by exposure to insulin (Fig. 2B). Differentiation and continued culturing of the 3T3-L1 adipocytes resulted both in a large decrease in PP1 basal activity and a corresponding increase in the stimulation of the phosphatase by insulin (Fig. 2B). In the day 8 adipocytes, insulin now caused a 2-fold increase in PP1 activity. Numerous experiments in 3T3-L1 adipocytes revealed an inverse correlation between basal PP1 activity and its stimulation by insulin (data not shown), suggesting that reducing PP1 basal activity may prime the phosphatase for activation by insulin.

Glycogen-targeted PP1 Is Regulated by DARPP-32 in 3T3-L1 Adipocytes—The significant decrease in basal PP1 specific activity without a corresponding change in protein levels during adipogenesis suggested that a PP1 inhibitor may play an important role in the regulation of phosphatase activity. Since PP1 is known to be inhibited by Inh-1 and DARPP-32, the presence of these proteins in 3T3-L1 adipocytes was examined by immunoblotting. No Inh-1 protein could be detected in 3T3-L1 lysates, although purified Inh-1 protein was recognized by the affinity-purified antibody used (data not shown). However, DARPP-32 was readily detected in 3T3-L1 adipocyte lysates using an affinity-purified mouse monoclonal antibody (Fig. 3A, lane 1). Subsequent fractionation of the PNS fraction revealed that DARPP-32 is largely absent from the cytosol and present in both the plasma membrane and glycogen-enriched fractions (Fig. 3A, lanes 2–4). Similar results were obtained when primary rat adipocytes were fractionated and subjected to anti-DARPP-32 immunoblotting (Fig. 3A, lanes 5–8), indicating that DARPP-32 expression is not a cell line artifact. In bovine parathyroid gland, DARPP-32 has recently been shown to be isoprenylated, resulting in an exclusively particulate localization (24). To examine this possibility, crude particulate fractions were prepared from 3T3-L1 adipocytes by ultracentrifugation. This fraction was resuspended in PP1 homogenization buffer containing salt or detergent and was then recentrifuged. The resulting supernatants and particulate fractions were then examined by anti-DARPP-32 immunoblotting. As seen in Fig. 3B, resuspension of the original pellet in homoge-
buffer led to the complete extraction of DARPP-32 from the 4–5 isoprenylation as described previously (24). 500 mM NaCl likely integrally associated with membranes, perhaps due to its results indicate that in 3T3-L1 adipocytes, DARPP-32 is most cubated on ice for 15 min with either buffer alone (homogenization buffer with the indicated additions. Samples were in- centrifugation and were resuspended with a 23-gauge needle in PP1 particulate fractions were prepared from 3T3-L1 adipocytes by ultra- centrifugation (100,000 g, 30 min), and subsequent pellets (lanes 1–3) and supernatants (lanes 4–6) were analyzed by DARPP-32 immunoblotting.

FIG. 3. Characterization of DARPP-32 from adipocytes. A, DARPP-32 subcellular localization in 3T3-L1 and primary rat adipocytes. Fully differentiated 3T3-L1 adipocytes or rat primary adipocytes were fractionated into PNS, plasma membrane, cytosolic, and glycogen pellet fractions as described under “Experimental Procedures.” Equal cell equivalents were subjected to SDS-polyacrylamide gel electrophore- sis and transferred to nitrocellulose. DARPP-32 was then visualized immunohistochemically using ECL. Lanes 1–4 are from 3T3-L1 adipocytes, while lanes 5–8 are from primary rat adipocytes. Lanes 1 and 5, PNS; lanes 2 and 6, plasma membrane; lanes 3 and 7, cytosol; lanes 4 and 8, glycogen pellet. B, extraction of particulate bound DARPP-32. Crude particulate fractions were prepared from 3T3-L1 adipocytes by ultra- centrifugation and were resuspended with a 23-gauge needle in PP1 homogenization buffer with the indicated additions. Samples were incu- babed on ice for 15 min with either buffer alone (lanes 1 and 4), buffer plus 0.5 mM NaCl (lanes 2 and 5), or buffer plus 0.1% Triton X-100 (lanes 3 and 6). Samples were then subjected to ultra-centrifugation (100,000 × g, 30 min), and subsequent pellets (lanes 1–3) and supernatants (lanes 4–6) were analyzed by DARPP-32 immunoblotting.

Levels of DARPP-32 protein in the glycogen-enriched pellet were next measured during the differentiation of 3T3-L1 cells. As seen in Fig. 4, no DARPP-32 was detected in the 3T3-L1 fibroblasts. However, the inhibitor was expressed upon adipocyte differentiation and continued to increase during culturing of the adipocytes in FBS medium. At no time did insulin treat- ment result in any detectable change in the amount of DARPP-32 present in the glycogen-enriched fraction (Fig. 4). The expression of DARPP-32 correlated with the reduction in PP1 basal specific activity seen during 3T3-L1 differentiation and suggests a possible role for DARPP-32 in the regulation of glycogen-targeted PP1 activity.

The Stimulation of PP1 Activity by Insulin Results in In- creased Initial Enzymatic Rates Measured in Vitro—Elucidation of the mechanism by which PP1 is activated by insulin has been hampered by the failure to detect in vitro marked elevation of the phosphatase by the addition of hormone to cells in culture. One possible explanation for the small observed effect might lie in an artificially elevated basal activity, which may arise from the disassociation of inhibitor peptides upon cell lysis. The kinetics of PP1 activity were evaluated in full differ- entiated 3T3-L1 adipocyte lysates. Extracts from basal adipocytes were rapidly prepared, and PP1 activity was measured in the PNS fraction for indicated times at 37 °C (Fig. 5A, basal). During the first 4 min of the assay, PP1 activity was low. After

FIG. 4. DARPP-32 is expressed in the glycogen pellet during 3T3-L1 adipocyte differentiation. A, DARPP-32 immunoblot is shown. During differentiation of 3T3-L1 fibroblasts into adipocytes, two replicate plates were serum-starved for 3 h and treated in the absence (–) or presence (+) of 100 nm insulin for 15 min. Glycogen-enriched pellets were prepared as described under “Experimental Procedures.” 25 μg of protein were resolved by SDS-polyacrylamide gel electrophore- sis, transferred to nitrocellulose, and probed with an anti-DARPP-32 monoclonal affinity-purified antibody; immunoreactivity was detected by ECL. Day 0, confluent fibroblasts. Differentiation (D) was initiated on day 2 and completed on day 6 (A). Adipocytes were maintained in PBS-containing medium for up to 11 more days (days 6–17). Two confluency plates of fibroblast cells were maintained in parallel for 17 days in calf serum-containing medium (CS). The immunoblot is repre- sentative of four independent experiments.

4 min, phosphatase activity significantly increased, reaching constant activity kinetics. This activity state persisted until substrate consumption exceeded 30–40% (data not shown).
Treatment of the cells with insulin largely overcame the lag in PP1 activity seen in vitro (Fig. 5A, Ins). Thus, the highest stimulation of PP1 activity by insulin was observed during the initial phase of the assay (Fig. 5A). During the linear phase of PP1 activity, however, only 20–40% stimulation was measured in extracts from insulin-treated cells, consistent with previous reports (25, 26). Performing the assays at 30 °C resulted in an extension of the basal PP1 activity lag, which was not detected in extracts prepared from insulin-treated cells (Fig. 5B). In contrast, PP1 activity from 3T3-L1 fibroblast lysates exhibited linear kinetics at all time points (data not shown). These results suggest that insulin can increase the initial rate of PP1 activity measured in vitro, by inducing the dissociation of inhibitor peptide, allowing for the full expression of catalytic activity.

**DISCUSSION**

Insulin action is characterized by the paradoxical regulation of serine/threonine phosphorylation (1). The stimulation of protein kinase cascades play a crucial role in the mitogenic effects of this hormone. However, regulation of glucose- and lipid-metabolizing enzymes by insulin occurs largely through dephosphorylation of these proteins. Although inactivation of upstream kinases may be involved (27), the key enzymes that regulate glycogen synthesis, namely glycogen synthase, glycogen phosphorylase, and phosphorylase kinase, are all excellent substrates for PP1. Further, PP1 has been shown to be specifically activated by insulin, suggesting an important role for this phosphatase in insulin action. Although PP1 is present in nearly all cellular compartments, only a specific, limited number of phosphoproteins are dephosphorylated in response to insulin (1). This paradox raises two important questions: how is PP1 targeted to the relevant substrates, and how are specific pools of PP1 preferentially activated by insulin?

We have utilized the 3T3-L1 cell line to investigate the regulation of glycogen metabolism by insulin. 3T3-L1 fibroblasts are poorly responsive to insulin; however, differentiation of these cells into lipid-containing adipocytes results in a dramatic increase in insulin-mediated glucose utilization and storage. The levels of many proteins are increased during adipogenesis, including key mediators of insulin signaling, such as the insulin receptor (22) and Glut-4 (23). In this study, we examined the hormonal regulation of glycogen synthesis during differentiation of 3T3-L1 cells. In fibroblasts, insulin only caused a 3-fold increase in glucose accumulation as glycogen; in the fully differentiated day 8 adipocytes, insulin treatment resulted in a 700-fold increase in glycogen synthesis. Correspondingly, the protein and activity levels of glycogen synthase were dramatically increased upon adipogenesis, as was the stimulation of synthase activity in response to insulin. In contrast, the specific activity of PP1 decreased by over 60% during differentiation, with no corresponding change in protein levels. However, this decrease in PP1 specific activity correlated with a significant increase in the -fold activation of PP1 by insulin. These findings suggest that mechanisms may exist that maintain PP1 in a low activity state, allowing the phosphatase to be maximally stimulated by insulin.

The precise events leading to the activation of glycogen-localized PP1 activity remain unclear. Three mammalian proteins have been identified that target PP1 to glycogen: G_{M}, G_{L}, and PTG. Dent et al. (12) reported that the phosphorylation of site 1 on G_{M} by pp90^{ab} in vitro increased the activity of the bound PP1, while phosphorylation of sites 1 and 2 on G_{M} by cAMP-dependent protein kinase resulted in the dissociation of PP1 from G_{M}. This attractive model would explain how insulin, through the stimulation of the MAP kinase/pp90^{ab} cascade, could increase PP1 activity, while also explaining how catalytic agents, through activation of adenylate cyclase and cAMP-dependent protein kinase, could reduce PP1 activity at the glycogen particle. However, several groups have since convincingly shown that the MAP kinase/pp90^{ab} pathway is neither sufficient nor necessary for the increases in glucose storage or PP1 activity caused by insulin (13–17). Further, since neither site 1 or 2 of G_{M} is conserved in either PTG or G_{L} it is impossible to apply the G_{M} phosphorylation model to these proteins. Not surprisingly, PTG and G_{L} are not phosphorylated in vitro by cAMP-dependent protein kinase (9, 18). Additionally, PTG, is not phosphorylated in vivo in response to anabolic agents such as insulin or catalytic agents such as isoproterenol (18). Moreover, neither treatment had any effect on the levels of glycogen-associated PP1 in 3T3-L1 or primary rat adipocytes (Fig. 2B; data not shown; Ref. 18). Therefore, the activation of PP1 targeted to glycogen by PTG occurs independently of PP1 translocation and PTG phosphorylation.

Several lines of evidence have been presented here implicating DARPP-32 as a key regulator of glycogen-targeted PP1 activity in adipocytes. DARPP-32 was not detectable in 3T3-L1 adipocytes, but it was dramatically up-regulated during adipocyte differentiation. This expression of DARPP-32 correlated well with both the decrease in PP1 specific activity measured during differentiation and the increased stimulation of PP1 by insulin. DARPP-32 in 3T3-L1 and primary rat adipocytes is exclusively localized to particulate fractions, including the glycogen pellet. Finally, an assay of PP1 activity in 3T3-L1 adipocyte lysates revealed a lag in PP1 activity that was largely overcome by insulin treatment of the cells. The largest stimulation of PP1 activity was thus detected at the earliest assay time points. PP1 activity from fibroblast cells did not exhibit this lag, and nor was phosphatase activity significantly affected by insulin. One explanation of these results is that PP1 in fully differentiated adipocytes is regulated by DARPP-32 binding, and stimulation of PP1 by insulin occurs through inhibition of the phosphatase. Although DARPP-32 is considered to be the neuronal homologue of Inh-1, both pig brown fat and bovine adipose tissue have previously been reported to contain DARPP-32 (28, 29). DARPP-32 immunoreactivity was also detected in primary rat adipocytes (Fig. 3A), so DARPP-32 is likely to be the 32-kDa inhibitor of phosphorylase phosphatase activity in rat adipose tissue reported by Nemenoff et al. (30). These results suggest that in adipocytes DARPP-32 may play an important role in insulin signaling.

The mechanisms of regulation of glycogen-targeted PP1 by both DARPP-32 and insulin are unclear. The organic PP1 inhibitor microcystin has been used as an affinity matrix to isolate PP1 and a large number of associated proteins from tissues (8, 31). Many PP1-binding proteins, therefore, do not appear to obstruct the catalytic site, allowing targeted PP1 to interact not only with substrates but also with physiological inhibitors such as DARPP-32. Thiophosphorylated DARPP-32 could inhibit PP1 bound to recombinant glutathione S-transferase-PTG by 70% without any detectable disassociation of the phosphatase from PTG (18), indicating that a trimeric PTG-PP1-DARPP-32 complex may be formed. The possible disruption of the PP1/DARPP-32 interaction by insulin could occur in several ways. First, insulin may stimulate the dephosphorylation or prevent the phosphorylation of DARPP-32, most likely through reduction of cAMP levels and/or cAMP-dependent protein kinase activity. Alternatively, insulin stimulation of adipocytes may result in the generation of a soluble second messenger, which could disrupt DARPP-32 binding to PP1 independently of inhibitor dephosphorylation. We have previously shown that PP1 bound to PTG is less sensitive to inhibition by DARPP-32 (18). Conversely, the PP1-PTG complex may
be more easily disinhibited by insulin treatment, possibly explain-
ing the ability of insulin to specifically activate glycogen-
targeted PP1, leading to the dephosphorylation of a limited
number of substrates. Because of the high off rate and the need
to solubilize with detergent, it has thus far not been possible to
capture a complex between PP1 and DARPP-32 in vivo. Efforts
are under way to explore the effect of insulin on PP1/DARPP-32
interaction.

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