A hallmark of signal transduction is the dynamic and inducible post-translational modification of proteins. In addition to the well characterized phosphorylation of proteins, other modifications have been shown to be regulatory, including O-linked β-N-acetylglucosamine (O-GlcNAc). O-GlcNAc modifies serine and threonine residues on a myriad of nuclear and cytosolic proteins, and for several proteins there appears to be a reciprocal relationship between phosphorylation and O-GlcNAc modification. Here we report further evidence of this yin-yang relationship by demonstrating that O-GlcNAc transference, the enzyme that adds O-GlcNAc to proteins, exists in stable and active complexes with the serine/threonine phosphatases PP1β and PP1γ, enzymes that remove phosphate from proteins. The existence of this complex highlights the importance of understanding the dynamic relationship between O-GlcNAc and phosphate in modulating protein function in many cellular processes and disease states such as Alzheimer’s disease and type II diabetes.

Although there are only 21, counting selenocysteine, genomically encoded mammalian amino acids, post-translational modification results in proteins that contain more than 50 different amino acids (1). Thus, covalent modification of polypeptides plays a major role in the generation of functional polypeptides and adds an extra level of complexity in attempts to understand the proteome (2). While several protein modifications are static, a subset of post-translational modifications is both dynamic and inducible. It is the study of these “regulatory” post-translational modifications and the enzymes that add and subtract them to proteins that comprises a significant portion of the signal transduction field (3).

The most well studied and understood regulatory post-translational modification is phosphorylation (3). Accordingly, a significant body of literature has focused on the kinase superfam-

ily, which makes up more than 2% of the proteins encoded by the human genome (4). Interestingly, in humans there appear to be only about 15 catalytic serine/threonine protein phosphatases (PPP subfamily) compared with literally hundreds of serine/threonine kinases (4). Thus, understanding how protein dephosphorylation is regulated has been an area of intense study for the last two decades (5, 6). One emerging theme is that the catalytic phosphatases have binding partners that regulate their localization and activity (7).

Similar to phosphorylation, O-linked β-N-acetylglucosamine (O-GlcNAc) modification of nuclear and cytosolic proteins is an abundant, dynamic, and inducible post-translational modification (8–10). However, unlike kinases, there appears to be only one O-GlcNAc transference catalytic subunit (OGT, 1 the enzyme that adds O-GlcNAc to proteins) in mammals (11). Similar to phosphatases, it has been proposed that OGT is regulated by a multitude of binding partners as well as post-translational modification and alternative splicing (12, 13). It has been demonstrated recently that OGT interacts with several proteins that appear to target it to different locations inside the cell (14–16).

Serine/threonine phosphorylation and O-GlcNAc modification are mutually exclusive at a given residue. Interestingly, several site-mapping studies have established the same site on a protein as being phosphorylated and O-GlcNAc-modified (for review, see Ref. 17). This suggested that there might be a reciprocal, competitive relationship between these two dynamic modifications. Subsequent studies established that in fact this yin-yang relationship does exist both at the global level as well as at specific sites on several proteins (for review, see Ref. 18). Thus, at a given serine/threonine residue in a protein, the amino acid could be unmodified, phosphorylated, or O-GlcNAc-modified. To further increase complexity, serine/threonine sulfation has been demonstrated recently as well (19).

Here, in further support of the yin-yang model, we demonstrate that OGT and phosphatases copurify with one another. Using a two-step purification, we are able to isolate a relatively pure complex that contains both OGT and serine/threonine phosphatases. Western blot analysis identifies the predominant copurifying phosphatases as PP1β and PP1γ. Finally, we demonstrate that this complex is active in converting a phosphorylated substrate to an O-GlcNAc-modified product.

**EXPERIMENTAL PROCEDURES**

Preparation of Rat Brain Extract—Extracts were prepared by polytron lysis of frozen rat brain (7 mL/g; Pel-Freeze) in 25 mM Tris, pH 7.5, 150 mM NaCl, 10 μM the O-GlcNAcase inhibitor O-(2-acetamidino-2-deoxy-o-glucopyranosylidene)amino-N-phenylcarbamate (FUGNAC; Ref. 20), 500 μM MnCl2, 250 μM EDTA, 200 μM dithiothreitol, and protease inhibitors. After lysis, the suspension was centrifuged at 35,000 × g for 1 h, and the resulting supernatant was filtered through a 1-μm glass fiber filter and used immediately or frozen at −80 °C.

**Microcinin-Sepharose Chromatography**—Purification of phosphatases and associated proteins by microcinin-Sepharose chromatography (Upstate Biotechnology) was performed as described previously start-
Absence of Okadaic Acid.

Phosphatase activity in the presence or absence of okadaic acid (OA) was examined for serine/threonine phosphatases (positive control). Resulting proteins were either immunopurified with a rabbit prebleed antibody to OKT, or a general antibody to serine/threonine phosphatases (negative control). Resulting proteins were either separated by SDS-PAGE and Western blotted with the general serine/threonine phosphatase antibody or (C) examined for phosphorylation in the presence or absence of okadaic acid.

Immunopurification, Western Blot, and Gel Analysis—The general serine/threonine phosphatase antibody (FL-18) was from Santa Cruz Biotechnology. Antibodies to OGT, PP1α, PP1β, PP1γ, PP4, and PP6 have been described previously (12, 22–27). Negative controls used normal rabbit IgG. Immunopurified proteins from rat brain extract (75 mg) were washed four times with 1% Nonidet P-40, 0.25% deoxycholate in Tris-buffered saline and were resolved on SDS-polyacrylamide gels. Gels were silver-stained as described previously (28) or transferred to membranes and Western blotted as described previously (12). For the two-step purification of the “yin-yang” complex, 250 mg of rat brain extract was immunoprecipitated, after preclearing, with the OGT antibody, and bound proteins were eluted with 4.5 ml of 100 mM glycine, pH 2.5, into 0.5 ml of 1% Tris, pH 8.0. The resulting eluted proteins were purified as described above with microcin as the general phosphatase antibody.

In-gel Digest, LC-MS/MS Analysis, and Protein Identification—In-gel digest was performed on silver-stained gels as described previously (28). The resulting peptides were separated by capillary reverse-phase HPLC, using a 45-min linear gradient of methanol in 1% acetic acid, directly into the tandem mass spectrometer (LCQ, ThermoFinnigan). Fragmentation data were collected in an automated fashion with a dynamic exclusion of 3. Data analysis was performed using TurboSequest (30), and a minimum of three peptides with Xcorr > 2.5 was required for protein identification.

Phosphatase Activity—Phosphatase activity was measured from immunoprecipitates (from 75 mg of rat brain extract) by the release of radioactive inorganic phosphate from in vitro protein kinase A-labeled myelin basic protein following the manufacturer’s protocol (New England Biolabs) in the presence or absence of (i) 50 nM okadaic acid (Calbiochem), (ii) 5 mM EDTA, or (iii) 1 mM CaCl₂. Phosphatase activity was normalized so that OGT-associated activity in the absence of inhibitor was 100%. A representative experiment is shown (Fig. 1C); although some variability was seen from experiment to experiment in total counts released, once normalized, the relative ratio between preimmune and OGT immunoprecipitates was constant (less than 10% variability).

OGT Activity—OGT activity was measured for immunoprecipitates from 75 mg of rat brain extract by the radioactive addition of GlcNAc to a synthetic unmodified or phosphorylated peptide (POGGSPVSPO₃°SPVA) for 30 min at 4 °C. The peptide was synthesized by standard FMOC (9-fluorenyl)methoxycarbonyl) chemistry, purified by reverse-phase HPLC, and analyzed by matrix-assisted laser desorption ionization time-of-flight.

In-gel Digest, LC-MS/MS Analysis, and Protein Identification—In-gel digest was performed on silver-stained gels as described previously (28). The resulting peptides were separated by capillary reverse-phase HPLC, using a 45-min linear gradient of methanol in 1% acetic acid, directly into the tandem mass spectrometer (LCQ, ThermoFinnigan). Fragmentation data were collected in an automated fashion with a dynamic exclusion of 3. Data analysis was performed using TurboSequest (30), and a minimum of three peptides with Xcorr > 2.5 was required for protein identification.

RESULTS AND DISCUSSION

Microcin-Sepharose chromatography has been used to purify multiple serine/threonine phosphatases and their binding partners (21, 32, 33). Following preclearing of rat brain extract over Sepharose 4B, OGT is retained on a microcin-Sepharose column after extensive washing (Fig. 1A). To further dem-
Either procedure gave virtually identical results, and we used both procedures to confirm the specificity of the interaction. The resulting proteins were further purified by microcystin-Sepharose chromatography, and the bound proteins were separated by SDS-PAGE with silver-stained bands or Western blotted for OGT and phosphatases (PP). Proteins were also subjected to in-gel digestion, and OGT, actin (at ~45 kDa), and IgG (heavy and light chain) were identified following capillary liquid chromatography-tandem mass spectrometry. MW, molecular weight.

To demonstrate the specificity of this interaction, a general serine/threonine phosphatase antibody was used to immunoprecipitate proteins from precleared rat brain lysates. This antibody, although generated against PP1, is known to react with most family members of the PP1, PP2A, and PP2B phosphatases as well as PP4. Lysates were also immunoprecipitated with an antibody to OGT as a positive control. Following SDS-PAGE separation of the proteins, OGT was detected as co-immunopurifying with the phosphatases by Western blot (Fig. 1A). Thus, using two independent methods, we were able to demonstrate that OGT copurifies with serine/threonine phosphatases.

We next wanted to determine whether phosphatases would co-immunoprecipitate with OGT. Rat brain lysates were immunoprecipitated with a polyclonal antibody to OGT, with a prebleed as a negative control, or with the general phosphatase antibody as a positive control. A serine/threonine phosphatase was detected as co-immunopurifying with OGT by Western blot analysis (Fig. 1B). To further confirm the presence of a copurifying phosphatase, OGT immunoprecipitates were used to dephosphorylate protein kinase A-phosphorylated myelin basic protein. The phosphatase activity in the OGT immunoprecipitates was found to be sensitive to okadaic acid, a potent inhibitor of PP1 and PP2A phosphatases at 50 nM (Fig. 1C). However, phosphatase activity was not abolished at 5 nM okadaic acid (data not shown), suggesting that the more sensitive PP2A was not responsible for the observed phosphatase activity (34).

To test for the presence of PP2B activity, phosphatase activity assays were conducted in the presence of divalent cations or EDTA, and no effect was seen on activity (data not shown). This suggests that the phosphatase co-immunopurifying with OGT is not PP2B, which has a divalent cation requirement for activity (35). Furthermore, the activity was inhibited by free microcystin (data not shown), and OGT purified with phosphatases upon microcystin chromatography (Fig. 1A) suggesting that the activity was not due to the microcystin-insensitive phosphatase PP2C (data not shown) (36).

We next wanted to establish the identity of the phosphatase that was in complex with OGT. As a first approach, we took rat brain extract and immunoprecipitated the complex with an antibody to OGT. The resulting proteins were further purified by either microcystin-Sepharose chromatography (Fig. 2) or immunoprecipitation with the general phosphatase antibody (data not shown). Either procedure gave virtually identical protein banding patterns upon SDS-PAGE separation and silver staining. The presence of OGT and the presence of the phosphatase were confirmed by Western blot analysis (Fig. 2). Silver-stained bands were subjected to in-gel digest with trypsin, and the resulting peptides were analyzed by capillary LC-MS/MS. Positive identifications (greater than three sequenced tryptic peptides) were made for OGT, both the heavy and light chain of the antibody, and for α-actin migrating around 45 kDa (data not shown). Unfortunately the band that represented the serine/threonine phosphatase, as identified by Western blot, was refractory to identification. Whether actin is truly in the complex or is contamination because of its overwhelming abundance in the extract is unclear. What is clear from this experiment is that OGT and a serine/threonine phosphatase, along with actin, are the predominant proteins in this complex.

As an alternative approach to identifying the phosphatase, we conducted a host of Western blots on OGT and control immunoprecipitates from rat brain extracts. Phosphatases tested for copurifying with OGT were PP1α, PP1β, PP1γ, PP4, and PP5. Only PP1β and PP1γ copurified specifically with OGT (Fig. 3A). A binding motif, Arg/Lys-Val/Il/le-Xaa-Phe (where Xaa is any amino acid), has been described previously for proteins that interact with the catalytic subunits of PP1 (37). OGT contains such a motif beginning at amino acid 889 in the rat sequence Arg-Ile-Ile-Phe. This suggests that OGT may be directly binding PP1β and PP1γ in the yin-yang complex. However, it should be noted that a synthetic peptide of OGT spanning the binding domain at a high concentration (0.5 mM) only had a minor effect on preventing OGT from copurifying with phosphatases by microcystin-Sepharose chromatography (data not shown). Thus, either the complex is difficult to disrupt once formed, or OGT is not using the consensus motif for binding in the yin-yang complexes.

Finally, we demonstrated that OGT immunoprecipitates are capable of dephosphorylating and O-GlcNAc-modifying a synthetic peptide that has been phosphorylated at the known position for O-GlcNAc modification (Fig. 3B) (31). The phosphopeptide used in this study is identical to the unmodified peptide except that it is phosphorylated at the known acceptor site for O-GlcNAc modification (31). Furthermore, we demonstrated that O-GlcNAc modification of the phosphorylated peptide requires okadaic acid-sensitive phosphatase activity (Fig. 3B). In the presence of okadaic acid, OGT is incapable of modifying the phosphopeptide but modifies the naked peptide equally well in the presence or absence of okadaic acid (Fig. 3B). This demonstrates that the OGT immunoprecipitates contain an okadaic acid-sensitive phosphatase activity that must first dephosphorylate the substrate before OGT can catalyze the addition of O-GlcNAc. Thus, the yin-yang complex is functional in the dephosphorylation and subsequent O-GlcNAc modification of a phosphorylated substrate.

Even though rat PP1β, PP1γ, and PP1α are greater than 85% identical at the amino acid level, OGT fails to copurify with PP1α from rat brain extract (Fig. 3A). Interestingly, PP1β and PP1γ have been shown to be enriched in cytoskeletal fractions of cells (38). While PP1γ appears to be the isoform mainly associated with the actin cytoskeleton, the PP1β isoform is found predominantly associated with microtubules (39). The association between PP1γ and OGT or the fact that PP1β is known to associate with the actin motor myosin (40) may explain why actin was seen to copurify in the isolation of the yin-yang complex (Fig. 2).

Several microtubule-associated proteins, including the Alzheimer’s related protein tau (41), are O-GlcNAc-modified (42). A reciprocal relationship has been established between phos-
phorylation and O-GlcNAc modification on the tau protein (43). Furthermore, hyperphosphorylated, hypoglycosylated tau is associated with the neurofibrillary tangles observed in Alzheimer’s disease and other tauopathies (for review, see Ref. 44). This suggests a working model in which the yin-yang complex serves to dephosphorylate and O-GlcNAc-modify tau in neurons. Several studies have shown a decline in brain glucose metabolism of Alzheimer’s patients compared with age-matched controls (45, 46). It has also been demonstrated that glucose utilization declines with age in neurons (47) and that the level of the donor substrate for O-GlcNAc addition, UDP-GlcNAc, is proportional to the levels of glucose in the cell. In addition, PP1β expression levels have been shown to be decreased in the neurons of Alzheimer’s disease patients (48). Taken together these data further expand the model to predict that O-GlcNAc modification prevents hyperphosphorylation and aggregation of tau and that the development of tauopathies may result from lowered levels of O-GlcNAc on tau. These lowered levels of O-GlcNAc on tau may result from reduced levels of activity of the yin-yang complex caused by transcriptional repression of PP1β and lowered donor sugar nucleotide levels for OGT. We are currently in the process of testing this attractive model.

PP1β is also the major PP1 isoform observed in insulin-responsive peripheral tissues and is targeted to glycogen (49, 50). We and others have recently established that increased O-GlcNAc levels induce insulin resistance, the hallmark of type II diabetes (51, 52). Furthermore, McClain and co-workers (53) have demonstrated that glycogen synthase is O-GlcNAc-modified. Interestingly, N-acetylglucosamine analogues have been shown to inhibit glycogen-bound PP1 (54). In adipocytes, a combination of glucose flux into the production of UDP-GlcNAc and chronic insulin treatment leads to maximal increases in the O-GlcNAc post-translational modification (51). Likewise, the insulin-stimulated dephosphorylation of metabolic enzymes that regulates glycogen and lipid metabolism is performed by PP1 isoforms (49). Thus, both O-GlcNAc modification and PP1 activity appear to be insulin-responsive and in turn to modulate insulin signal transduction. The yin-yang complex ties these two activities together and may offer insight into the mechanism of hyperglycemia- and hyperinsulinemia-induced insulin resistance in type II diabetes.

The identification here of the yin-yang complex provides further evidence for the reciprocal relationship observed between phosphorylation and O-GlcNAc modification (55). Identification of the complex suggests one possible mechanism for this dynamic relationship on certain proteins. We are currently pursuing the possible existence of a yang-yin complex that would contain a serine/threonine kinase associated with O-GlcNAcase, the enzyme that removes O-GlcNAc from protein (56, 57). Perhaps most importantly, the identification of the OGT-PP1β and OGT-PP1γ complexes suggests testable working models for the combined role of phosphorylation and O-GlcNAc modification in the development of neurofibrillary plaques in Alzheimer’s disease and insulin resistance in type II diabetes.

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