Identification and Cloning of a Novel Family of Coiled-coil Domain Proteins That Interact with O-GlcNAc Transferase*

Sai Prasad N. Iyer‡‡, Yoshihiro Akimoto¶¶, and Gerald W. Hart††

From the ‡Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185, the §Graduate Program, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294, and the ¶Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan

The abundant and dynamic post-translational modification of nuclear and cytosolic proteins by β-O-linked N-acetylglucosamine (O-GlcNAc) is catalyzed by O-GlcNAc transferase (OGT). Here we used the yeast two-hybrid approach to identify and isolate GABA_4 receptor-associated protein, GRIF-1 (Beck, M., Brickley, K., Wilkinson, H. L., Sharma, S., Smith, M., Chazot, P. L., Pollard, S., and Stephenson, F. A. (2002) J. Biol. Chem. 277, 30079–30090), and its novel homolog, OIP106 (KIAA1042), as novel OGT-interacting proteins. The proteins are highly similar to each other but are encoded by two separate genes. Both GRIF-1 and OIP106 contain coiled-coil domains and interact with the tetratricopeptide repeats of OGT. GRIF-1 and OIP106 are modified by O-GlcNAc and therefore are substrates for OGT. However, unlike another high affinity protein substrate, such as nucleoporin p62, OIP106 and GRIF-1 co-immunoprecipitate with OGT, exhibiting stable in vitro and in vivo associations. Whereas GRIF-1 has been reported to be expressed only in excitable tissue, OIP106 is expressed in all human cell lines that were examined. Confocal and electron microscopy show that OIP106 localizes to nuclear punctae in HeLa cells and co-localizes with RNA polymerase II. Co-immunoprecipitation experiments confirm the presence of an in vitro RNA polymerase II-OIP106-OGT complex, suggesting that OIP106 may target OGT to transcriptional complexes for glycosylation of transcriptional proteins, such as RNA polymerase II, and transcription factors. Similarly, GRIF-1 may serve to target OGT to GABA_A receptor complexes for mediating GABA signaling cascades.

Dynamic modification of Ser/Thr residues of nucleocytoplasmic proteins by single β-O-linked N-acetylglucosamine (O-GlcNAc) is ubiquitous in multicellular eukaryotes (1–5). The proteins modified by O-GlcNAc are myriad and diverse in form and function, ranging from transcription factors (4–6) to RNA polymerase II (7), oncoproteins (8), tumor suppressors (9), viral proteins (10), cytoskeletal proteins (11, 12), kinases (13) and phosphatases (14). Furthermore, O-GlcNAc-modified proteins are phosphoproteins as well, often belonging to large multimeric and reversible protein complexes. Indeed, in many cases, the sites of O-GlcNAc modification are either the same or adjacent to those modified by O-phosphate (15). Thus, there is mounting evidence supporting the hypothesis that O-GlcNAc is a regulatory modification analogous to phosphorylation. For example, the transcription factor Sp1 is extensively modified by O-GlcNAc, and it has been shown that the presence of the sugar in the transactivation domain inhibits its homomultimerization and transcriptional capability (4, 16). O-GlcNAc modification of p67 regulates protein synthesis by controlling the phosphorylation status of eukaryotic initiation factor-2α (17, 18). In cases where the sites of modification of O-GlcNAc and O-phosphate are the same, a reciprocal relationship between the two modifications has been suggested (19, 20).

Enzymes that cycle the O-GlcNAc modification are analogous to those that catalyze phosphorylation (i.e. kinases and phosphatases). The enzyme that attaches the saccharide to proteins is uridine diphospho-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyltransferase, or O-GlcNAc transferase (OGT), and its counterpart is an N-acetylglucosaminidase known as the O-GlcNAcase. Both of these enzymes have been purified and characterized (21–26). Recently, the cDNAs that encode for OGT (25, 26) and O-GlcNAcase (23), have been cloned from rat, Caenorhabditis elegans, and human. The OGT is a highly unique and ubiquitous glycosyltransferase, encoded by a single gene. OGT is highly conserved throughout evolution from C. elegans to humans. The gene for OGT has been mapped to the X chromosome (Xq13 in humans) (27, 28). Targeted deletion of the OGT gene results in ES cell lethality in mice (27). Thus, OGT is essential for life at the single cell level.

OGT localizes to both the nucleus and cytoplasm of cells, but it is present at higher levels in the nucleus (25). Aside from being tyrosine-phosphorylated and O-GlcNAc-modified, the rat 110-kDa OGT enzyme contains 11½ tetratricopeptide repeats (TPRs), a protein-protein interaction domain found in many proteins (25, 29, 30). TPRs have been shown to mediate protein-protein interactions in a variety of proteins (37). Recently, the crystal structure of the TPR domain of two of these proteins, protein phosphatase 5 (31) and Pex5p (32), were solved. OGT exists as a homotrimer, and the TPR domain was shown to be expressed only in excitable tissue, OIP106 is expressed in all human cell lines that were examined. Confocal and electron microscopy show that OIP106 localizes to nuclear punctae in HeLa cells and co-localizes with RNA polymerase II. Co-immunoprecipitation experiments confirm the presence of an in vitro RNA polymerase II-OIP106-OGT complex, suggesting that OIP106 may target OGT to transcriptional complexes for glycosylation of transcriptional proteins, such as RNA polymerase II, and transcription factors. Similarly, GRIF-1 may serve to target OGT to GABA_A receptor complexes for mediating GABA signaling cascades.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication, September 12, 2002, and in revised form, October 25, 2002
Published, JBC Papers in Press, November 14, 2002, DOI 10.1074/jbc.M209384200

* The abbreviations used are: O-GlcNAc, β-O-linked N-acetylglucosamine; OGT, uridine diphospho-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyltransferase; O-GlcNAcase, N-acetyl-β-N-glu- cosaminidase; TPR, tetratricopeptide repeat; HBS, Heps-buffered sa- line; PBS, phosphate-buffered saline; CTD, carboxyl-terminal domain; IP, immunoprecipitation; SD, synthetic dropout medium; AD, activa-

This paper is available on line at http://www.jbc.org
to mediate the trimerization (33). Studies performed on recombinant OGT overexpressed and purified in baculovirus (33) and Escherichia coli (13) have shown that the TPR domain plays a key role in intrasubunit interaction and in substrate recognition. Unlike the large numbers of genes encoding kinases, thus far, there is evidence for only a single OGT catalytic subunit despite the myriad of different O-GlcNAc-modifying proteins (1). Virtually nothing is known about the regulation of OGT’s subcellular localization or substrate specificity. The presence of multiple TPR repeats implies that proteins might specifically interact with the enzyme, targeting the catalytic domain to specific protein substrates within the nucleus and cytoplasm. To examine this hypothesis, we screened a rat brain library with OGT using the yeast two-hybrid approach, in order to begin to identify potential OGT-interacting proteins (OIPs). Initially, 250 positively interacting clones were identified. Here, we report the cloning and characterization of GRIF-1, a recently cloned GABAA receptor-associated protein, and its novel homolog KIAA1042/OIP106 as novel coiled-coil domain proteins that interact strongly with the TPR domain of OGT. Our findings suggest that these proteins possibly function to target OGT to RNA polymerase II and GABA_A receptor complexes to mediate transcriptional and signaling events.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The OGT-Gal4 BD fusion bait plasmid pIL59-OGT was generated by inserting the rat OGT cDNA into pIL59. The negative control pIL59-C-termOGT plasmid was generated by inserting the C terminus (residues 476–1037) of OGT. The plasmids encoding the full-length OGT, C-termOGT, and TPR domain for E. coli expression were generated by subcloning the respective cDNAs into pET32 (Novagen) as thioredoxin fusion proteins. Constructs encoding for an N-terminal hexa-His-tagged TPR and C-termOGT fusion proteins (pRSET-TPR and pRSET-C, respectively) were generated by subcloning the TPR and C-termOGT fragments into the pRSET vector (Invitrogen). The pACT2 plasmid encoding the partial rat OIP98 (AD-OIP98) clone (residues 105–578) fused to the activation domain of Gal4 was obtained from the initial yeast two-hybrid screen as a result of plasmid rescue. Full-length rat OIP98 was generated by performing 5’-rapid amplification of cDNA ends using the rat brain Marathon-Ready cDNA library (Clontech) and by assembling the 5’-rapid amplification of cDNA product with the preexisting insert derived from the yeast two-hybrid screen using overlapping restriction sites. OIP98 is a partial clone of GRIF-1, lacking exon 15. The missing exon was obtained by using a QuickClone rat brain cDNA library (Clontech) as template and GRIF-1 gene-specific primers flanking exon 15 in a PCR and inserted into the OIP98 cDNA using overlapping restriction sites. An E. coli expression plasmid of rat GRIF-1 (OIP98) was generated by subcloning the cDNA into pET32. Plasmids for in vitro transcription/translation reactions and mammalian overexpression were generated by subcloning the GRIF-1 cDNA into pCITE4C (Novagen) and pCITE3A His A (Invitrogen), respectively. The insert encoding KIAA1042/OIP106 cDNA was obtained from Dr. Takahiro Nagase from the Kazusa DNA Research Institute (Chiba, Japan) in pBluescript II SK+(KIAA1042). This region is specific and unique to KIAA1042. Affinity-purified antibodies were generated by purifying SAI1 antiserum over a GRIF-1 (OIP98) affinity column as previously described (36). KIAA1042/OIP106-specific polyclonal antibodies were generated and purified under native, nondenaturing conditions via nickel affinity chromatography using HiTrap chelating columns (Amersham Biosciences) according to the manufacturer’s instructions. His-tagged C-termOGT (pRSET-C) was expressed and purified in a similar manner for use as an antigen to raise anti-OGT polyclonal antibodies generated in rabbits. Soluble thioredoxin-tagged TPR (pET22/TPR) was overexpressed and purified under native, nondenaturing conditions via nickel affinity chromatography.

**Blot Overlay Assays**—Equal amounts of E. coli expressed recombinant OGT, TPR, and C-termOGT and BSA proteins were separated on SDS-PAGE and blotted on polyvinylidene difluoride membranes. Polyclonal antibodies—anti-OGT (in renaturation buffer of 100 mM NaCl, 150 mM potassium acetate, 1 mM dithiothreitol, 5 mM MgCl2, 1 mM EDTA, 0.1% (v/v) Tween 20, 0.1 mM zinc, 5% milk (w/v), and 0.1% M Met) at 4 °C. [35S]Met-labeled GRIF-1 and OIP106 probes were synthesized in vitro using the TnT coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions. Reactions were desalted and added to 15 ml of cold renaturation buffer and used to probe the blots containing the immobilized proteins at 4 °C. Following probing, the blots were washed extensively with renaturation buffer, dried, and subjected to autoradiography by exposure to Biomax MR film (Eastman Kodak Co.) at ~80 °C. Typical exposure times were 8 h.

**Preparation and Purification of Rabbit and Chicken Polyclonal Antibodies**—Rabbit and chicken antisera to GRIF-1 and OIP106 were generated by immunizing rabbits and chickens with recombinant His-tagged GRIF-1 (OIP98) purified as described above. Gel-purified protein was used as an immunogen by Covance Research Products (Denver, PA) to produce polyclonal antisera in one rabbit designated as AL28 and to a chicken designated as AL29. AL28 antiserum was affinity-purified by using Aves Laboratories (Tigard, OR) to produce chicken IgY antibodies. Affinity-purified antibodies were generated by purifying JH 3286 antisera or chicken IgY over a GRIF-1 (OIP98) affinity column as previously described (36). KIAA1042/OIP106-specific polyclonal antibodies were generated by immunizing one rabbit designated as SA1 with a peptide corresponding to the first 20 amino acids of the N terminus (N20) of KIAA1042. This region is specific and unique to KIAA1042. Affinity-purified antibodies were generated by purifying SA1 antisera over an N20 peptide affinity column as previously described (36). The anti-OGT antibody AL28 was generated in a similar manner by using His-tagged C-termOGT protein to immunize two rabbits designated AL28 and AL29. AL28 antisera was affinity-purified over a thioredoxin-C-termOGT (pET32-C) column as previously described (36).

**Antibodies and Western Blot Analysis**—AL28 was used at a final concentration of 25–50 ng/ml in 5% (w/v) milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 for 16 h at 4 °C. Similarly, JH 3286 was used at a final concentration of 100 ng/ml. Chicken anti-OIP106/ KIAA1042 was used at a final concentration of 0.1 M Met. Chicken anti-OIP106/KIAA1042 conjugate (Novagen) was used at 1:5,000 according to the manufacturer’s instructions. Anti-actin (Sigma) and anti-Rb p107 (Santa Cruz Biotechnology, Inc.) antibodies were used at 1:10,000. Mouse Omni- actin (Cedarlane Laboratories Ltd.) and anti-actin (Santa Cruz Biotechnology, Inc.) antibodies were used at 1:2000. Monoclonal antibodies to hypophosphorylated RNA polymerase II (Neoclon) and anti-tubulin (Sigma) were used at 1:1,000. Mouse anti-OGT (Promega) was used at 1:5000. Mouse anti-OGT conjugate (Novagen) was used at 1:5,000 according to the manufacturer’s instructions. Anti-OGT conjugate (Promega) was used at 1:5,000, and anti-O-GlcNAc CTD 110.6 mouse monoclonal antibody was used at 1:2500 as described before, either in the absence or presence of 50 mM GlcNAc (51). All blots were developed with the enhanced chemiluminescence (ECL) reagent (Amersham Biosciences).

**Cell Culture**—HeLa and HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal bovine serum containing 0.1 mM nonessential amino acids, penicillin, and streptomycin.

**Preparation of Tissue Extracts**—For preparation of whole tissue extracts, frozen tissue from male Sprague-Dawley rats were homogenized in ice-cold radiomimune precipitation assay lys buffer (PBS, 0.1%
Novel O-GlcNAc Transferase-interacting Proteins

5401

SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate containing proteins, inhibitors, and soluble crude extracts were generated by spinning the homogenate at 100,000 \( \times g \) for 90 min at 4 °C. Extracts were assayed for protein concentration via the Bio-Rad protein assay reagent, and equal amounts of each tissue extract were separated on SDS-PAGE for JH 3286 Western blot analysis. HeLa whole cell lysate was prepared in a similar manner. For analysis of human cell line extracts, Crust-Blot B was purchased (Santa Cruz Biotechnology) and analyzed with SAH1 and \( \alpha \)-tubulin antibodies.

For immunoprecipitation experiments, frozen rat brains were homogenized in Heps-buffered saline (HBS) containing 20 mM Heps pH 7.4, 0.3 M NaCl, 5 mM MgCl\(_2\), 1 mM NaF, 100 mM GlcNAc, and protease inhibitors using a Polytron homogenizer. The homogenate was then centrifuged at 100,000 \( \times g \) for 90 min at 4 °C to generate soluble crude brain extract and stored at \(-80 \) °C. Preparation of nuclear and cytoplasmic extracts from HeLa cells was performed as described (38). OIP106 Confocal and Electron Microscopy—HeLa cells were fixed in 4% (v/v) formaldehyde in PBS (pH 7.3) for 1 h at 4 °C, permeabilized with 0.5% (v/v) Triton X-100 in PBS for 5 min and treated with 5% (w/v) BSA in PBS for 10 min. The specimens were then incubated with the anti-OIP106 antibody (JH 3286) (dilution, 1:200) or with pre-immune rabbit IgG for 1 h at room temperature, washed with PBS, and subsequently incubated for 1 h with Cy3-conjugated donkey anti-rabbit IgG antibody (dilution, 1:500) (Jackson Immunoresearch). Specimens were then incubated with the mouse monoclonal anti-hypophosphorylated RNA polymerase II (dilution, 1:1000) antibody 8WG16 (Neoclon) for 1 h at room temperature, washed with PBS, and incubated for 1 h with fluoromethylisothiocyanate-conjugated donkey anti-mouse IgG (Jackson Immunoresearch). After a wash with PBS, the specimens were mounted in 90% (v/v) glycerol, 0.1% (v/v) Tris buffer (pH 8.5) containing 0.5 mM p-phenylene diamine and observed under laser-scanning confocal microscopy (MRC-1024; Bio-Rad). Electron microscopy was performed as previously described (47), using a JEM-1010 electron microscope (JEOL, Tokyo, Japan), using the above dilutions for JH 3286 and 8WG16.

Immunoprecipitation—Crude rat brain extract was filtered through a 0.22-μm syringe filter and was preclarified by anti-IgG-agarose (Promega). Preclarified extract was incubated with either 2 μg of preimmune chicken IgY or anti-OIP106/GRIF-1 IgY to immunoprecipitate native OIP106 and GRIF-1 16 h at 4 °C. OIP106/GRIF-1 immune complexes were collected by incubation with anti-IgG-agarose for 1 h at 4 °C. The beads were extensively washed in cold HBS extract buffer, and bound proteins were eluted with SDS-PAGE sample buffer. Samples were boiled, separated by SDS-PAGE, and analyzed by Western blotting with either rabbit anti-OIP106/GRIF-1 antibody JH 3286 or rabbit anti-O-GT antibody AL28.

Similarly, for OGT immunoprecipitations, crude rat brain extract was incubated with AL28 in HBS (0.3 M NaCl) or radioligand immunoprecipitation assay lysis buffer for 16 h at 4 °C. Immune complexes were collected by incubation with protein A-Sepharose (Amersham Biosciences), and bound proteins were eluted with SDS-PAGE sample buffer, followed by analysis by Western blotting.

HEK 293 cell immunoprecipitations were performed by lysing transfected cells in HBS (0.3 M NaCl) buffer containing 0.5% (v/v) Triton X-100 and 0.5 mM phenethylmethylsulfonyl fluoride for 30 min at 4 °C. Whole cell extracts were generated by centrifuging the lysates at 16,000 × g at 4 °C for 10 min. Preclarified extracts were incubated with anti-Xpress Omniprobe D-8 antibody (Santa Cruz Biotechnology) and protein A-Sepharose (Amersham Biosciences) to immunoprecipitate recombinant Xpress-tagged OIP106 and GRIF-1 for 3 h at 4 °C. Immune precipitates were washed extensively in lysis buffer and eluted with SDS-PAGE sample buffer, followed by Western blotting with Omniprobe D-8, anti-O-GT AL28, and CTD 110.6 antibodies. Immune precipitations from TnT rabbit reticulocyte lysates expressing Xpress-tagged OIP106 and GRIF-1 were performed with Omniprobe D-8 and analyzed in a similar manner as described above.

For HeLa nuclear extract immunoprecipitations, 5 μg of either preimmune chicken IgY or anti-OIP106/GRIF-1 IgY bound to anti-IgG-agarose were incubated with 330 μg of preclarified HeLa nuclear extract in 20 mM Heps, pH 7.9, 300 mM NaCl, 10 mM EDTA, 10 mM MgCl\(_2\), 20 μM O-(2-acetamido-2-deoxy-D-glucopyranosylidene)aminoo-N-phenylcarbamate, 0.1% (v/v) Triton X-100 overnight at 4 °C to immunoprecipitate native OIP106. OIP106 immune complexes were washed extensively with binding buffer containing 0.2% (v/v) Triton X-100, and bound proteins were eluted by boiling in SDS-PAGE sample buffer, followed by Western blot analysis with JH 3286, 8WG16, AL28, and anti-Rb p107 antibodies.

Protein-Protein Interactions—For OIP106/GRIF-1 pull-down experiments, S-tagged OIP106 and GRIF-1 were synthesized in TnT rabbit reticulocyte lysates (Promega). Following synthesis, lysates were incubated with S-protein-agarose to purify S-tagged OIP106 and GRIF-1 for 3 h at room temperature in HBS (0.3 M NaCl) lysis buffer containing 0.5% (v/v) Triton X-100. Pull-downs were washed extensively in lysis buffer, and bound proteins were eluted with SDS-PAGE sample buffer and analyzed by silver staining.

For the p62 and OIP106-AL28 pull-down assays, S-tagged p62 and OIP106 were synthesized in TnT rabbit reticulocyte lysates. Following synthesis, proteins were incubated with preimmune or AL28 in Tris-buffered saline containing 0.5 M NaCl and 0.1% (v/v) Triton X-100 at 4 °C. Immune complexes were collected by incubating the reactions with Amersham-A-Sepharose (Amersham Biosciences) containing the immune complexes were washed extensively with binding buffer, and bound proteins were eluted by boiling in SDS-PAGE sample buffer. Samples were then analyzed with S-protein HRP and AL28 Western blots.

Expression of OIP106 and GRIF-1 cDNAs—OIP106 and GRIF-1 were expressed in vitro in rabbit reticulocyte lysates using the TnT in vitro transcription/translation system (Promega) from either pcDNA 3.1 His or pCITE vectors. For HEK 293 cell transfections, 4 μg each of OIP106 and GRIF-1 in their respective pcDNA 3.1 His vectors were transfected using LipofectAMINE 2000 and expressed as Xpress-tagged fusion proteins. HEK 293 cell transfections were carried out for 48 h, and harvested and transfected cells were subjected to anti-Xpress Omniprobe antibody immunoprecipitation as described under “Immunoprecipitation.”

RESULTS

Yeast Two-Hybrid Screen to Identify Proteins That Interact with O-GlcNAc Transferase—to identify OGT-interacting proteins, ~7 × 10^6 clones (6–7-fold redundancy) were screened from a rat brain two-hybrid cDNA library, and 250 positive yeast colonies were isolated. We chose a rat brain library because OGT protein levels and its enzymatic activity are high in brain (23). Eighty of these colonies were screened for the presence of cDNA inserts that were larger than 2 kb, in hopes of isolating full-length cDNAs. Nine of these 80 clones encoded for cDNAs ranging in size from 2.3 to 3.5 kb and were sequenced and identified. Four of the nine clones contained a cDNA that encoded for a 744-amino acid polypeptide, which was lacking its 5′-end. We performed 5′-rapid amplification of cDNA ends using a rat brain cDNA library to isolate the 5′-end and isolated a 900-bp fragment that encoded for an additional 104 amino acids. Assembly of both cDNA fragments yielded a ~3.4-kb cDNA fragment (GenBank™ accession number AF471463) that encoded for a 878-amino acid polypeptide, with a predicted molecular mass of 98 kDa, which we named OIP98 (for OGT-interacting protein of 98 kDa). To ensure that the original interaction observed in yeast was not a false positive, we repeated the yeast two-hybrid assay using the original isolated partial OIP98 target cDNA (AD-OIP98) fused to the activation domain of Gal4 and the OGT bait cDNA (BD-OGT) fused to the binding domain of Gal4. As seen in Fig. 1A, only the yeast colonies containing both the interacting OGT bait and the OIP98 target plasmids were able to grow on plates lacking the histidine-selective marker, by activation of the HIS3 reporter gene. Furthermore, this was confirmed by additional liquid phase assays where the OIP98-OGT interaction was quantified by the activation of the \( \beta \)-galactosidase reporter gene (Fig. 1B). We performed an additional control by testing the interaction of the putative catalytic domain of OGT (BD-Cterm\(^{OGT} \)) with OIP98. As can be seen in Fig. 1B, the C terminus of OGT did not interact with OIP98, indicating that the amino-terminal half of OGT containing the TPR domain may be the region of interaction with OIP98.

At the time of its isolation, OIP98 did not bear any resemblance to any known protein; thus, we considered it a novel protein. However, during revision of this manuscript, homology searches performed in GenBank™ revealed that OIP98 dis-
played 93% identity to a rat 914-amino acid protein named GRIF-1 (GABA<sub>A</sub> receptor-associated protein) (GenBank™ accession number AJ288898) (52). GRIF-1 was found to interact with the β<sub>2</sub> subunit of the GABA<sub>A</sub> receptor and was isolated from a yeast two-hybrid screen. Rat GRIF-1 is the rat ortholog of human ALS2CR3 (40). Interestingly, sequence comparison between OIP98 and GRIF-1 revealed that the region of difference between the two proteins was from residue 620 (in GRIF-1 and OIP98) to 688 (in GRIF-1; 653 in OIP98) (positions 644–712 in Fig. 2A). This region corresponds to exon 15 in human ALS2CR3/GRIF-1. Thus, OIP98 is the GRIF-1 cDNA product but lacking exon 15; therefore, it is a partial clone of GRIF-1. We cloned the missing exon by PCR, using GRIF-1 gene-specific primers that flanked exon 15, from a rat brain cDNA library (see “Experimental Procedures”). Interestingly, sequence comparison of our assembled GRIF-1/OIP98 to the published GRIF-1 sequence (52) revealed three major differences in the predicted sequence (52). These are Leu<sup>579</sup> (our GRIF-1) to Trp<sup>579</sup> (52), Thr<sup>595</sup> (ours) to Ser<sup>595</sup> (52), and Gln<sup>596</sup> (ours) to Glu<sup>596</sup> (52) (Fig. 2B). These differences were not found when we compared our cloned GRIF-1 sequence to the human GRIF-1 (ALS2CR3) (Fig. 2B). Similar results were found when exon 15 was cloned from a Matchmaker (Clontech) rat brain cDNA library (data not shown).

Further GenBank™ searches showed considerable sequence identity (~40%) between GRIF-1 and another novel human gene of unknown function, KIAA1042 (41) (GenBank™ accession number AB028965). KIAA1042 encodes for a 953-amino acid polypeptide, with a predicted molecular mass of 106 kDa (Fig. 2A) and is mapped to 3p22.1.2 We expressed the KIAA1042 and GRIF-1 cDNAs in a rabbit reticulocyte lysate system and analyzed the [35S]Met-labeled protein products on a 7.5% SDS-PAGE, followed by autoradiography. Surprisingly, as shown in Fig. 3A (left panel), both KIAA1042 and GRIF-1 migrated similarly on SDS-PAGE, at an apparent molecular mass of about 115 kDa. Whereas the migratory pattern of GRIF-1 is consistent with the reported migration (52), our observation that KIAA1042 migrated similarly was unexpected, since KIAA1042 is 40 amino acids larger than GRIF-1. In a separate reaction using pCITE vectors, translated GRIF-1 and KIAA1042 proteins were purified via S-tag affinity chromatography. As seen in Fig. 3A (middle panel), equal amounts of S-tagged GRIF-1 and KIAA1042 were purified, as detected by S-protein HRP blot. Affinity-purified anti-GRIF-1/OIP98 polyclonal antibody (JH 3286) cross-reacted well with both purified GRIF-1 and KIAA1042 (Fig. 3A, right panel). This is not surprising, since KIAA1042 shares significant sequence identity with GRIF-1 (Fig. 2). Thus, our GRIF-1 antibody JH 3286 reacts with both KIAA1042 and GRIF-1.

We performed further JH 3286 Western blot analysis on whole rat tissue and human HeLa cell lysates. Beck et al. (52) have reported that GRIF-1 is only expressed in excitable tissue such as brain, heart, and skeletal muscle, based on Western blot analysis with a GRIF-1-specific antibody. Our JH 3286 antibody, however, reacted with a ~115-kDa band in all rat tissues that were examined, as well as HeLa cell lysates (Fig. 3B). In addition, JH 3286 reacted with a ~106-kDa band in heart, brain, lung, and smooth muscle tissue (Fig. 3B, top asterisk). An additional reactivity at ~98 kDa was observed in lung tissue (Fig. 3B, bottom asterisk). Thus, JH 3286 reacted with GRIF-1 in excitable tissue. The additional immunoreactivity and aberrant migratory patterns of GRIF-1 are consistent with the patterns reported by Beck et al. (52). Since JH 3286 reacts with both KIAA1042 and GRIF-1, the reactivity noticed in nonexcitable tissue as well as in HeLa cell lysates is probably due to the presence of KIAA1042. Interestingly, the protein species in liver and kidney migrated slightly differently from the protein reactivities in the other tissues. This could be a result of differential

---

2 Available on the World Wide Web at genome.ucsc.edu/cgi-bin/hgTracks?position=chr3:48370736-48542321&hgaid=223215.

Fig. 1. Isolation and identification of GRIF-1 (OIP98) as a novel OGT-interacting protein using the yeast interaction trap approach. A, OGT was fused to the BD of GAL4 (pJL59-OGT) and used as bait to screen a rat brain GAL4 AD cDNA fusion library for interacting proteins. Yeast strain PJ69 was co-transformed with pJL59-OGT and the Matchmaker rat brain AD fusion library (Clontech) and plated onto −Leu/−Trp plates. −Leu/−Trp transformants were plated onto −Leu/−Trp/−His/+Ade 30 mM 3-aminotriazole plates. Only colonies containing both interacting bait and target proteins survive and grow on −Leu/−Trp/−His/+Ade 30 mM 3-aminotriazole plates. B, β-galactosidase reporter assays show the interaction of BD-OGT and AD-OIP98.
post-translational modifications that might occur in these tissues. It is curious that the anti-GRIF-1 antibody generated against residues 8–633 of GRIF-1 by Beck et al. (52) did not react with KIAA1042 in nonexcitable tissue, since both KIAA1042 and GRIF-1 share a high degree of homology in that region (Fig. 2A).

Currently, however, since JH 3286 recognizes both GRIF-1 and KIAA1042, we cannot distinguish between the two proteins in brain and heart tissue. In order to confirm KIAA1042 expression in HeLa cell lysates, whole cell lysates were probed with a KIAA1042 N terminus (N20; first 20 amino acids of human KIAA1042) specific antibody SAI1. As seen in Fig. 3C, SAI1 specifically reacted with a 110-kDa KIAA1042 band, consistent with the JH 3286 reactivity in Fig. 3B (right panel). This reactivity was competed away in the presence of the KIAA1042 N20 peptide antigen (Fig. 3C, right panel), thus confirming the identity of the band as KIAA1042. Further specificity of SAI1 toward KIAA1042 was examined by probing recombinant Xpress-tagged KIAA1042 and GRIF-1 with SAI1. As seen in Fig. 3D, the α-Xpress blot (right panel) shows that recombinant purified Xpress-tagged GRIF-1 or KIAA1042 was immunopurified by α-Xpress Omniprobe antibody from reticulocyte lysates expressing the cDNA for each protein. However, the SAI1 blot (Fig. 3D, left panel) clearly shows that only recombinant KIAA1042, and not GRIF-1, reacted with SAI1, indicating that SAI1 is specific for KIAA1042. Since we now had an KIAA1042-specific antibody with SAI1, we wanted to confirm its expression in human tissue, since SAI1 is specific for human KIAA1042. However, since human tissue was not easy to obtain, we decided to examine the expression of KIAA1042 on a blot containing cell lysates from a diverse range of human cell lines containing both excitable (IMR-32) and nonexcitable (HL-60, HeLa, etc.) cell lines. As seen in Fig. 3E, SAI1 signal was observed in every tissue, as seen in both short and long exposures (top and middle panels, respectively). The α-tubulin blot (Fig. 3E, bottom panel) shows the protein loading control. Thus, KIAA1042 expression was noticed in all cell types that were examined. This is consistent with its reported mRNA expression.3

Biochemical Confirmation of GRIF-1 and KIAA1042 Interaction with OGT—In order to confirm that the interactions observed in the original yeast two-hybrid screen were valid and not false positives, we performed a variety of in vitro and in vivo protein-protein interaction assays. To show the physiological existence of native GRIF-1-OGT and KIAA1042-OGT complexes from rat brain, we performed co-immunoprecipitation (IP) experiments. GRIF-1 and KIAA1042 were immunoprecipitated with anti-GRIF-1 antibody (raised in chicken) from rat

3 Available on the World Wide Web at www.kazusa.or.jp/huge/gfpage/KIAA1042/.
brain extracts. This antibody reacts with both GRIF-1 and KIAA1042, similar to JH 3286. Immunoprecipitates were separated on a 10% SDS-PAGE and analyzed by autoradiography (left panel). As seen in Fig. 4A, the JH 3286 Western blot (top panel) shows that chicken anti-GRIF-1 antibody specifically immunoprecipitated GRIF-1/KIAA1042 from rat brain extracts. Western blot analysis by anti-OGT AL28 antibody (second panel) clearly shows the presence of OGT in the anti-GRIF-1 IP, indicating that OGT co-immunoprecipitated with GRIF-1/KIAA1042. The absence of tubulin and actin (bottom two panels, respectively), which are abundant proteins in brain, in the anti-GRIF-1 IP demonstrate that the interactions between OGT and GRIF-1/KIAA1042 are specific and not a result of nonspecific binding.

To show that GRIF-1/KIAA1042 reciprocally co-immunoprecipitated with OGT, OGT was immunoprecipitated with AL28 from rat brain extract. IPs were performed in HBS (0.3 M NaCl) and high stringency radioimmune precipitation assay lysis. As seen in Fig. 4B, the JH 3286 Western blot (top panel) shows that chicken anti-GRIF-1 antibody specifically immunoprecipitated GRIF-1/KIAA1042 from rat brain extracts. Western blot analysis by anti-OGT AL28 antibody (second panel) clearly shows the presence of OGT in the anti-GRIF-1 IP, indicating that OGT co-immunoprecipitated with GRIF-1/KIAA1042. The absence of tubulin and actin (bottom two panels, respectively), which are abundant proteins in brain, in the anti-GRIF-1 IP demonstrate that the interactions between OGT and GRIF-1/KIAA1042 are specific and not a result of nonspecific binding.

To show that GRIF-1/KIAA1042 reciprocally co-immunoprecipitated with OGT, OGT was immunoprecipitated with AL28 from rat brain extract. IPs were performed in HBS (0.3 M NaCl) and high stringency radioimmune precipitation assay lysis.
Novel O-GlcNAc Transferase-interacting Proteins

Fig. 4. A, OGT co-immunoprecipitates with KIAA1042/OIP106/GRIF-1 from rat brain extracts in a reciprocal manner. KIAA1042/OIP106/GRIF-1 were immunoprecipitated with affinity-purified chicken anti-GRIF-1 polyclonal antibody, separated on 10% SDS-PAGE, and blotted with affinity-purified JH 3286, anti-OGT, anti-tubulin, and anti-actin. B, KIAA1042/OIP106/GRIF-1 co-immunoprecipitate with OGT from rat brain extracts in a reciprocal manner. OGT was immunoprecipitated with affinity-purified AL28 anti-OGT antibody and blotted with affinity-purified anti-OGT (AL28) and JH 3286. C, OGT co-immunoprecipitates with overexpressed GRIF-1 and KIAA1042/OIP106. GRIF-1 and KIAA1042/OIP106 were separately expressed in HEK 293 cells as Xpress-tagged proteins. Transfected cell lysates were subjected to anti-Xpress Omniprobe immunoprecipitation to IP Xpress-tagged proteins. Omniprobe IPs were then analyzed by Omniprobe and AL28 Western blots. As seen in Fig. 4B, AL28 immunoprecipitated OGT in both native and high stringency conditions, as analyzed by the AL28 Western blot (top panel). Western blot analysis with chicken anti-GRIF-1 (bottom left panel) clearly shows the presence of GRIF-1/KIAA1042 in OGT IPs but not in the control preimmune IP, under native and high stringency binding conditions. Since our anti-GRIF-1 antibodies do not distinguish between GRIF-1 and KIAA1042, it was not clear which protein interacts with OGT. We addressed this issue by transiently transfecting KIAA1042 and GRIF-1 cDNAs or vector alone (mock control) separately in HEK 293 cells as Xpress-tagged fusion proteins. Xpress-tagged GRIF-1 and KIAA1042 were immunoprecipitated using anti-Xpress Omniprobe antibody, and IPs were analyzed by Omniprobe and AL28 Western blots. As seen in Fig. 4C, the Omniprobe blot (left panel) clearly shows the presence of immunoprecipitated Xpress-tagged GRIF-1 and KIAA1042 in the Omniprobe IPs. The AL28 Western blot (bottom panel) shows that endogenous OGT in HEK 293 cells specifically co-immunoprecipitated with Xpress-tagged GRIF-1 and KIAA1042 but not from mock-transfected lysates (bottom panel, right lane). Therefore, OGT interacts with both KIAA1042 and GRIF-1 individually, confirming the previous rat brain native IP results.

These data clearly indicate that KIAA1042 and GRIF-1 interact with OGT quite strongly (even in the presence of 0.1% SDS) and probably exist in a complex in vivo, thus validating the interactions observed from the yeast two-hybrid experiments. Since we identified KIAA1042 as an O-GlcNAc transferase-binding protein, we named it OIP106 (for OGT-interacting protein of 106 kDa), based on its predicted molecular mass.

The TPR Domain of OGT Interacts with OIP106—Since the OGT contains a TPR domain at its amino terminus, which is a protein-protein interaction domain, we hypothesized that the TPR domain may be the region of OGT that interacted with GRIF-1 and OIP106. To examine this hypothesis, OGT and its individual domains were tested for interactions with GRIF-1 and KIAA1042 in vitro blot overlay interaction assay. Recombinant OGT, TPR, and C-term OGT (C) or BSA (−) control were blotted on polyvinylidene difluoride membranes. BSA was similarly blotted to use as a negative control. Immobilized proteins were probed by anti-GRIF-1 antibodies. As is seen in the middle panel, CTD 110.6 reacted well with both GRIF-1 and OIP106. The CTD 110.6 reactivity is competed away by probing in the presence of 50 mM GlcNAc. As is seen in the right panel, CTD 110.6 reactivity is competed away by probing in the presence of 50 mM GlcNAc antibody CTD 110.6, indicating that the reactivity observed is due to O-GlcNAc modification and not due to nonspecific binding.

The TPR Domain of OGT Interacts with OIP106. Since we identified KIAA1042 as an O-GlcNAc transferase-binding protein, we named it OIP106 (for OGT-interacting protein of 106 kDa), based on its predicted molecular mass.

The TPR Domain of OGT Interacts with OIP106—Since the OGT contains a TPR domain at its amino terminus, which is a protein-protein interaction domain, we hypothesized that the TPR domain may be the region of OGT that interacted with GRIF-1 and OIP106. In addition, the carboxyl terminus of OGT failed to interact with GRIF-1 (OIP98) in the yeast two-hybrid system (Fig. 1B). To examine this hypothesis, OGT and its individual domains were tested for interactions with GRIF-1 and OIP106 using an in vitro blot overlay interaction assay. Recombinant OGT, TPR, and C-term OGT (C) proteins synthesized in E. coli were separated on SDS-PAGE and blotted onto polyvinylidene difluoride membranes. BSA was similarly blotted to use as a negative control. Immobilized proteins were probed by anti-GRIF-1 antibodies. As is seen in the middle panel, CTD 110.6 reacted well with both GRIF-1 and OIP106. The CTD 110.6 reactivity is competed away by probing in the presence of 50 mM GlcNAc antibody CTD 110.6, indicating that the reactivity observed is due to O-GlcNAc modification and not due to nonspecific binding.

The TPR Domain of OGT Interacts with OIP106. Since we identified KIAA1042 as an O-GlcNAc transferase-binding protein, we named it OIP106 (for OGT-interacting protein of 106 kDa), based on its predicted molecular mass.

The TPR Domain of OGT Interacts with OIP106—Since the OGT contains a TPR domain at its amino terminus, which is a protein-protein interaction domain, we hypothesized that the TPR domain may be the region of OGT that interacted with GRIF-1 and OIP106. Therefore, OGT interacts with both KIAA1042 and GRIF-1 individually, confirming the previous rat brain native IP results.
then renatured en blot and probed with radioactively labeled GRIF-1 and OIP106. Blots were washed and then subjected to autoradiography. As can be seen in Fig. 5, A and B, the TPR domain strongly bound radiolabeled GRIF-1 and OIP106 (middle panels). Similar binding was exhibited by the full-length OGT. In contrast, no signal was observed in the C protein lanes or the BSA control lanes, indicating that the binding was specific. Therefore, this indicates that GRIF-1 and OIP106 interact with OGT via the TPR domain.

We wanted to examine whether GRIF-1 and OIP106 bound OGT stoichiometrically. S-tagged GRIF-1 and OIP106 were synthesized in rabbit reticulocyte lysates and pulled down by S-protein-agarose. Beads were washed extensively in high salt buffer and analyzed by silver staining. As is seen in Fig. 5C, S-tagged GRIF-1 and OIP106 were purified by S-protein-agarose (middle and right lanes, respectively). The endogenous OGT band, which migrates at ~110 kDa, is clearly seen in both GRIF-1 and OIP106 pull-downs. AL28 Western blot had identified this co-purifying band as OGT in preliminary experiments (data not shown). This band is not present in the control S-protein-agarose pull-down lane (left lane). Therefore, whereas it appears that the binding of GRIF-1 and OIP106 to OGT appears to be stoichiometric, quantification of relative amounts of the protein species here is not applicable, since the response of silver staining varies from one protein to another. The above experiments, in addition to the co-IP experiments, confirmed the initial yeast two-hybrid studies and clearly show that OIP106 and GRIF-1 are a novel family of highly homologous proteins that interact strongly with OGT via its TPR repeats.

**GRIF-1 and OIP106 Are Modified by O-GlcnAc—**PROSITE analysis of GRIF-1 and OIP106 sequences revealed that the carboxyl halves of both proteins contained many potential sites of O-GlcnAc modification. Since both proteins interact strongly with OGT, we examined the potential existence of O-GlcnAc on immunoprecipitated Xpress-tagged GRIF-1 and OIP106 by probing with CTD 110.6 (51), which is an anti-O-GlcnAc-specific mouse monoclonal antibody. As is seen in Fig. 4C (middle panel), when probed in the absence of 50 mM GlcnAc, both GRIF-1 and OIP106 reacted strongly with CTD 110.6. This reactivity was competed away when probed in the presence of 50 mM GlcnAc (Fig. 4C, right panel), indicating that the reactivity observed was due to the presence of the O-GlcnAc modification and not due to nonspecific binding of the antibody. This indicates that both GRIF-1 and OIP106 are modified by O-GlcnAc and are substrates for OGT.

We wanted to further examine whether other substrates of OGT also interacted with OGT in a similar manner. Nucleoporin p62 is a well studied, high affinity substrate for OGT (13). We performed in vitro binding experiments with p62 and looked for its ability to stably interact with OGT. An identical experiment was performed with OIP106 to serve as a positive control for OGT binding. OIP106 and p62 were synthesized as S-tagged proteins in reticulocyte lysates. Following synthesis, lysates were incubated with anti-OGT AL28 antibody to immunoprecipitate endogenous OGT, and IPs were assayed for the presence of either OIP106 or p62 by S-protein HRP blot. As seen in the left bottom panel of Fig. 6 (long exposure), the AL28 blot on AL28 IPs shows that the antibody immunoprecipitated OGT from lysates, as expected. No OGT was immunoprecipitated by the preimmune IgG (lane 4, bottom panel). The S-protein HRP blot in Fig. 6 (long exposure; left top panel) shows that OIP106 clearly co-immunoprecipitated with OGT (lane 5) but not with the preimmune IgG (lane 4). In contrast, no p62 was detected in the AL28 IP (lane 2). The short exposure panels on the right show that the concentration of OIP106 used in the input for the IPs was less than that of the amount of p62 used (right top panel; compare lane 1 with lane 3), indicating that the binding observed are specific and not effects of mass action. This indicates that p62 did not stably interact with OGT in this system, but OIP106 clearly did, as consistent with previous data.

We performed a similar experiment on rat brain extracts, which would reflect an in vivo native system, and obtained the same result (data not shown). These results indicate that p62 does not interact with OGT in a stable complex, but OIP106 and GRIF-1 do, although all three proteins are substrates for OGT.

**OIP106 Localizes to the Nucleus in HeLa Cells—**Since the characterization of GRIF-1 and its subcellular localization have already been reported by Beck et al. (52), we decided to focus on OIP106. In order to more closely examine subcellular distribution of OIP106, we performed biochemical fractionation on HeLa cells. HeLa cells were biochemically fractionated into nuclear and cytosolic extracts, and equal amounts of each extract were analyzed by JH 3286. As seen in Fig. 7A, OIP106 was only detected in the nuclear (N) fraction (Fig. 7A, top panel). OGT, which is a nucleocytoplasmic enzyme, is present in higher levels in the nucleus and is shown as a control in the bottom panel of Fig. 7A by AL28 Western blotting (25). Similar results were obtained using the OIP106-specific S11 antibody (data not shown). Thus, in HeLa cells, OIP106 is a nuclear protein.

**OIP106 Co-localizes with RNA Polymerase II in Vivo—**We decided to take advantage of the observation that endogenous OIP106 was adequately detected in HeLa cells by JH 3286 (Fig. 7A) and performed native OIP106 immunofluorescence with JH 3286 via laser-scanning confocal microscopy. As seen in Fig. 7A, panel a, OIP106 localized to distinct punctate regions in the nucleus. Thus, the nuclear staining of OIP106 correlated well with the biochemical fractionation in Fig. 7A. In order to further examine OIP106’s nuclear punctate localization, we performed laser-scanning confocal microscopy co-staining for proteins that are known to be present in these punctate regions. Recently, von Mikecz et al. (44) showed that the IIA form of
was observed using a JEM 1010 electron microscope. The markers for the observed punctate staining and wanted to examine whether OIP106 possibly co-localized with RNA polymerase IIA in vivo. We performed co-staining of OIP106 and RNA polymerase IIA using JH 3286 (from rabbit) and 8WG16 (from mouse) on HeLa cells to determine their co-localization. As is seen in Fig. 7B, panels b and c, subsets of OIP106 co-localized with RNA polymerase IIA in the distinct dotlike regions, as evident in the merged image (panel c). Fig. 7B, panel d, is an enlarged image of the inset in panel c, clearly showing the co-localized subsets of OIP106 and RNA polymerase IIA in yellow. To further confirm this observation, we performed immunogold electron microscopy. This is shown in Fig. 7C, panels a and b. The large 18-nm colloidal gold particles represent OIP106 molecules, and the smaller 12-nm particles represent RNA polymerase IIA. As seen in the inset in Fig. 7C, panel b, subsets of OIP106 co-localize with RNA polymerase IIA, further supporting the confocal microscopy data.

OIP106 Exists in a Complex with RNA Polymerase II and OGT—Since OIP106 and RNA polymerase II co-localize (Fig. 7, B and C), we wanted to biochemically confirm whether the two proteins are present in a complex in vivo. We performed co-IP experiments with the anti-GRIF-1 IgY (which reacts well with OIP106) and immunoprecipitated OIP106 from HeLa nuclear extracts. IPs were washed extensively and analyzed by JH 3286, AL28, 8WG16, and anti-Rb p107 Western blotting. As is seen in Fig. 7D, a subset of RNA polymerase II (second panel, lane 3) co-immunoprecipitated with OIP106 (top panel, lane 3) but not by the preimmune antibody (lane 2). The presence of OGT in the OIP106 IP, but not in the preimmune, serves as a positive control for the IP (Fig. 7D, third panel, lanes 3 and 2, respectively). Furthermore, Rb p107, which is a nonrelated abundant nuclear protein, did not co-IP with OIP106 (bottom panel), demonstrating that the polymerase II-OIP106 interactions are specific. Similar results were obtained with the OIP106-specific SAI1 antibody (data not shown). These data support the hypothesis that OIP106 exists in a complex with a subset of RNA polymerase II and OGT, providing evidence for the possible targeting of OGT by OIP106 to transcriptional complexes.

**DISCUSSION**

OGT Interacts with Many Proteins—A myriad of different nucleocytoplasmic proteins are modified by O-GlcNac, yet there appears to be only a single OGT catalytic subunit (25). The OGT cDNA was cloned from rat (25) and human (26) 4 years ago. Subsequent knockout of the OGT gene in mouse resulted in embryonic lethality, at the single cell level (27). Mapping of the OGT gene in humans and mice revealed it to lie on the X chromosome as a single copy gene (27, 28). This clearly demonstrated that the OGT enzyme was absolutely essential for life. However, virtually nothing is known about the mechanisms regulating OGT protein specificity. The N-terminal half of the OGT contains a TPR domain, which in rat OGT, is composed of 11.5 TPR repeats. Recently, contrary to earlier reports (26), it has been shown that the human OGT has the same number of TPR repeats as the rat enzyme (28). TPR domains have been shown to be responsible for intra- and intermolecular protein-protein interactions in a variety of proteins, spanning a variety of cellular functions (29, 30). Thus, in order to identify putative binding proteins that might potentially regulate the OGT’s specificity or its subcellular localization, we performed an unbiased yeast two-hybrid screen of a rat brain library using the entire OGT protein as a bait. Our final data support the hypothesis that OIP106 exists in a complex with a subset of RNA polymerase II and OGT, providing evidence for the possible targeting of OGT by OIP106 to transcriptional complexes.
round of screening resulted in about 250 clones, which, with a
−6−7-fold redundancy, we estimated to represent at least 30
unique clones. Whereas we selected for clones that were larger
than 2 kb in hopes that we would find complete cDNAs, our
PCR screening revealed that a large number of the clones were
less than 2 kb, indicating that these either encoded for smaller
proteins or were partial clones. Thus, it is reasonable to es-
imate, even conservatively, that the number of potential OGT-
interacting proteins is quite substantial. Thus, for such a sin-
gle, unique enzyme to glycosylate a myriad of proteins, we
hypothesize that it is regulated by a large number of TPR-
interacting proteins.

**GRIF-1, OIP106, and OGT: Implications for Targeting of OGT**—GRIF-1 and OIP106 interact with OGT quite strongly and stoichiometrically. The interactions are resistant to high
salt, nonionic, and ionic detergents such as SDS. As is seen
with the blot overlay assays, these interactions occur in the
TPR domain. Interestingly, in HEK 293 cell transfection exper-
iments, binding to OGT by a FLAG-tagged OIP106 construct
was not competed away when co-transfected with increasing
amounts of Xpress-tagged GRIF-1 (data not shown), indicating
that possibly GRIF-1 and OIP106 may bind to different TPR
repeats of OGT. Thus, mutually exclusive GRIF-1-OGT and
OIP106-OGT complexes could exist in the cell. This could help
explain how each protein differently might affect OGT’s lo-
calization. GRIF-1 was recently isolated as a novel GABA receptor-interacting protein that is expressed only in excitiable
tissue (52). The function of GRIF-1 is unknown. However, by
virtue of its association with OGT, we propose that perhaps it
functions to target OGT to GABA<sub>α</sub> receptor complexes. This
would implicate O-GlcNAc, OGT, and GRIF-1 as being involved
in GABA signaling. GRIF-1 would function as an adaptor/
scaffolding protein, bridging OGT to GABA<sub>α</sub> receptor in this
model. Signaling through such scaffolding/anchoring protein
networks has been well documented, especially for protein ki-
nases such as protein kinase A and its various A kinase-
anchoring proteins and in N-methyl-d-aspartate receptor signal-
ing via PDZ domain proteins (53). Interestingly, GRIF-1
contains several PXXP motifs in its carboxyl terminus (Fig. 2).
PXXP motifs are known to bind to Src homology 3 domains (53).
The function of Src homology 3 domains in proteins that con-
tain it is to form functional oligomeric complexes at defined
subcellular sites, usually in concert with other modular do-
 mains. A canonical example of such proteins is PSD-95, which
contains both an Src homology 3 and a PDZ domain and has
been shown to mediate N-methyl-d-aspartate receptor signal-
ing via these domains (53). Thus, GRIF-1 may interact with Src
homology 3 domain proteins via its PXXP motifs, recruiting
OGT to these complexes, in the context of GABA<sub>α</sub> receptor
signaling. Furthermore, regulation of GABA<sub>α</sub> receptor via
phosphorylation has been well documented, and it is the large
intracellular cytoplasmic loops of the various receptor subunits
(α, β, γ) that have been shown to be substrates of various
kinases (protein kinase A, protein kinase C, Ca<sup>2+</sup>/calmodulin-
dependent protein kinase II) (42). GRIF-1 interacts with the
intracellular loop of the β<sub>2</sub> receptor subunit, which is phospho-
rlylated at Ser<sup>142</sup> (42). Thus, OGT could be recruited to this
domain for its potential O-GlcNAc modification and for poten-
tial O-GlcNAc modification of associated scaffolding proteins.
O-GlcNAc and O-phosphate modifications often occur on the
same or adjacent Ser/Thr residues (15), so potential O-GlcNAc
modification of GABA<sub>α</sub> receptor (complexes) may be a way of
regulating its function. GRIF-1 would mediate this regulation by
targeting OGT to these complexes.

OIP106-OGT complexes occur in punctate regions within the
nucleus. These nuclear punctae have been shown to contain

---

**Acknowledgments**—We thank Katie Sackstedter, Brian Geisbrecht,
and Dr. Steve Gould for the pJL59 vector and helpful advice with
the yeast two-hybrid screen. We thank Dr. Peter Agre for the FJ68-4A yeast
strain and Dr. Natasha Zachara for careful reading of the manuscript.

---

**REFERENCES**

1. Hart, G. W. (1997) *Annu. Rev. Biochem.* **66**, 315–335
2. Wells, L., Vosseller, K., and Hart, G. W. (2001) *Science* **291**, 2376–2378
3. Hanover, J. A. (2001) *FASEB J.* **15**, 1865–1876
4. Jackson, S. P., and Tjian, R. (1988) *Cell* **55**, 125–133
5. Jackson, S. P., and Tjian, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 1781–1785
6. Reason, A. J., Morris, H. R., Panicó, M., Marais, R., Treisman, R. H., Haltiwanger, R. S., Hart, G. W., Kelly, W. G., and Dell, A. (1992) *J. Biol. Chem.* **267**, 16911–16912
7. Kelly, W. G., Dahmus, M. E., and Hart, G. W. (1993) *J. Biol. Chem.* **268**, 10416–10424
8. Chou, T-Y., Dang, C. V., and Hart, G. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4417–4421
9. Shaw, P., Freeman, J., Bovey, R., and Igo, R. (1996) *Oncogene* **12**, 921–930
10. Greis, K., Gibson, W., and Hart, G. W. (1994) *J. Virol.* **68**, 9329–9349
11. Haltiwanger, R. S., Hart, G. W., Kelly, W. G., and Dell, A. (1993) *J. Biol. Chem.* **268**, 16679–16687
12. Luhan, W. A., and Hanover, J. A. (2000) *J. Biol. Chem.* **275**, 3103–3107
13. Misler, W., Smith, D. M., Kreppel, L. K., Cole, R. N., et al. (1995) *Adv. Exp. Med. Biol.* **370**, 115–123
16. Yang, X., Su, K., Roos, M. D., Chang, Q., Paterson, A. J., and Kudlow, J. E. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6611–6616
17. Chakraborty, A., Saha, D., Bose, A., Chatterjee, M., and Gupta, N. K. (1994) Biochemistry 33, 6700–6706
18. Datta, B., Ray, M. K., Chakrabarti, D., Wylie, D. E., and Gupta, N. K. (1989) J. Biol. Chem. 264, 20620–20624
19. Cheng, X., and Hart, G. W. (2001) J. Biol. Chem. 276, 10570–10575
20. Datta, B., Ray, M. K., Chakrabarti, D., Wylie, D. E., and Gupta, N. K. (1989) J. Biol. Chem. 264, 20620–20624
21. Haltiwanger, R. S., Blomberg, M. A., and Hart, G. W. (1992) J. Biol. Chem. 267, 9005–9013
22. Dong, L.-Y., and Hart, G. W. (1994) J. Biol. Chem. 269, 19321–19330
23. Gao, Y., Wells, L., Comer, F. I., Parker, G. J., and Hart, G. W. (2001) J. Biol. Chem. 276, 9838–9845
24. Wells, L., Gao Y., Mahoney, J. A., Vosseller, K., Chen, C., Rosen, A., and Hart, G. W. (2002) J. Biol. Chem. 277, 1755–1761
25. Kreppel, L. K., Blomberg, M. A., and Hart, G. W. (1997) J. Biol. Chem. 272, 1755–1761
26. Lubas, W. A., Frank, D. W., Krause, M., and Hanover, J. A. (1997) J. Biol. Chem. 272, 9316–9324
27. Shahi, R., Iyer, S. P., Elles, L. G., O’Donnell, N., Marek, K. W., Chui, D., Hart, G. W., and Marth, J. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5735–5739
28. Nolte, D., and Muller, U. (2002) Mammm. Genomics 13, 62–64
29. Goebl, M., and Yanagida, M. (1991) Trends Biochem. Sci. 16, 173–177
30. Lamb, J. R., Tungtreich, S., and Hieter, P. (1995) Trends Biochem. Sci. 20, 257–259
31. Doo, A. K., Cohen, P. W., and Barford, D. (1998) EMBO J. 17, 1192–1199
32. Gatto, G. J Jr., Geisbrecht, B. V., Gould, S. J., and Berg, J. M. (2000) Nat. Struct. Biol. 7, 1091–1095
33. Kreppel, L. K., and Hart, G. W. (1999) J. Biol. Chem. 274, 32015–32023
34. Clontech Laboratories, Inc. (1999) Clontech: Yeast Protocols Handbook, Clontech Laboratories, Inc., Palo Alto, CA
35. Comer, F. I., and Hart, G. W. (2001) Biochemistry 40, 7845–7852
36. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Tamamis, D., and Struhl, K. (1995) Genes Dev. 9, 821–831
38. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
39. Deleted in proof
40. Hadano, S., Yanaigisawa, Y., Skaug, J., Fichter, K., Nasir, J., Martindale, D., Koop, B. F., Scherer, S. W., Nicholson, D. W., Rouleau, G. A., Ikeda, J., and Hayden, M. R. (2001) Genomics 71, 200–213
41. Kikuno, R., Nagase, T., Ishikawa, K., Hirasawa, M., Miyajima, N., Tanaka, A., Kotani, H., Nemura, N., and Ohara, O. (1999) DNA Res. 6, 197–205
42. Brandon, N. J., Jovanovic, J. N., and Moss, S. J. (2002) Pharmacol. Ther. 94, 113–122
43. Deleted in proof
44. von Mikeez, A., Zhang, S., Montminy, M., Tan, E. M., and Hemmerich, P. (2000) J. Cell Biol. 150, 265–273
45. Thompson, N. E., Steinberg, T. H., Aronson, D. B., and Burgess, R. R. (1989) J. Biol. Chem. 264, 11511–11520
46. Comer, F. I., and Hart, G. W. (1999) Biochim. Biophys. Acta 1473, 161–171
47. Akimoto, Y., Kreppel, L. K., Hirano, H., and Hart, G. W. (1999) Diabetes 48, 2407–2413
48. Comer, F. I., and Hart, G. W. (1999) Biochim. Biophys. Acta 1473, 161–171
49. Deleted in proof
50. Comer, F. I. (2000) Role of O-GlcNAc on the RNA Polymerase II Carboxy-Terminal Domain, Ph.D. thesis, University of Alabama at Birmingham
51. Comer, F. I., Vosseller, K., Wells, L., Accavitti, M. A., and Hart, G. W. (2001) Anal. Biochem. 293, 169–177
52. Beck, M., Brickleley, K., Wilkinson, H. L., Sharma, S., Smith, M., Chazot, P. L., Pollard, S., and Stephenson, F. A. (2002) J. Biol. Chem. 277, 30579–30590
53. Pawson, T., and Scott, J. D. (1997) Science 278, 2075–2080