GRIF-1 and OIP106, Members of a Novel Gene Family of Coiled-Coil Domain Proteins

ASSOCIATION IN VIVO AND IN VITRO WITH KINESIN

Kieran Brickley, Miriam J. Smith, Mike Beck, and F. Anne Stephenson

From the Department of Pharmaceutical and Biological Chemistry, School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, United Kingdom

Received for publication, August 9, 2004, and in revised form, December 21, 2004
Published, JBC Papers in Press, January 11, 2005, DOI 10.1074/jbc.M409095200

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Printed in U.S.A.

γ-Aminobutyric acid receptor-interacting factor (GRIF-1) is a 913-amino acid protein proposed to function as a GABA_ receptor subunit-interacting, trafficking protein. GRIF-1 shares ~44% amino acid sequence identity with O-linked N-acetylgalactosamine transferase-interacting protein 106, OIP106. Both proteins contain predicted coiled-coil domains and probably constitute a novel gene family. The Drosophila orthologue of this family of proteins may be Milton. Milton shares ~44% amino acid homology with GRIF-1. Milton is proposed to function in kinesin-mediated transport of mitochondria to nerve terminals. We report here that GRIF-1 and OIP106 also associate with kinesin and mitochondria. Following expression in human embryonic kidney 293 cells, both GRIF-1 and OIP106 were shown by co-immunoprecipitation to be specifically associated with an endogenous kinesin heavy chain species of 115 kDa and exogenous KIF5C. Association of GRIF-1 with kinesin was also evident in native brain and heart tissue. In the brain, anti-GRIF-1-(8–633) antibodies specifically co-immunoprecipitated two kinesin-immunoreactive species with molecular masses of 118 and 115 kDa, and in the heart, one kinesin-immunoreactive species, 115 kDa, was immunoprecipitated. Further studies revealed that GRIF-1 was predominantly associated with KIF5A in the brain and with KIF5B in both the heart and in HEK 293 cells. Yeast two-hybrid interaction assays and immunoprecipitations showed that GRIF-1 associated directly with KIF5C with the GRIF-1/KIF5C interaction domain localized to GRIF-1-(124–283). These results further support a role for GRIF-1 and OIP106 in protein and/or organelle transport in excitable cells in a manner analogous to glutamate receptor-interacting protein 1, in the motor-dependent transport of 3-hydroxy-3-methylisoxazole-4-propionate glutamate excitatory neurotransmitter receptors to dendrites.

γ-Aminobutyric acid receptor-interacting factor (GRIF-1) is a 913-amino acid protein first identified as a protein expressed in rat brain that has been proposed to function as a GABA_ receptor-trafficking factor (1). GRIF-1 was also recently identified as a protein that interacts with the enzyme uridine diphaso-N-acetylgalactosamine:polypeptide β-N-acetylgalactosaminyltransferase or O-GlcNAc transferase (OGT) (2, 3). OGT catalyzes the post-translational modification of proteins by β-O-linked N-acetylgalactosamine (GlcNAc) in the cell cytoplasm. GRIF-1 thus has the alternative name OGT-interacting protein 98 (OIP98). GRIF-1 (OIP98) shares ~44% amino acid sequence identity over the full-length sequence with the human protein, OIP106, also known as KIAA1042 (4). OIP106 was also shown to associate with OGT, suggesting that GRIF-1 (OIP98) and OIP106 form a new gene family (1, 2).

The human orthologue of GRIF-1 (OIP98) is the protein encoded by the gene ALS2CR3 (5), a protein of unknown function. The Drosophila orthologue is probably Milton (6). Milton shares ~44% amino acid homology with GRIF-1 (OIP98). Milton, like GRIF-1, is enriched in neuronal tissue. It was identified originally by a genetic screen of Drosophila mutations, searching for effects on the cell biology of the axon and synaptic terminals. Milton is a mitochondria-associated protein (6, 7). The microtubule-based motor protein, kinesin, was found to be co-immunoprecipitated with Milton from extracts of fly heads, thus suggesting that it is required for the kinesin-mediated transport of mitochondria to nerve terminals.

Here we report that GRIF-1 (OIP98) and OIP106 also co-associate with kinesin and mitochondria, thus demonstrating that Milton, GRIF-1, and OIP106 are indeed species orthologues, further supporting a role for GRIF-1 in protein and/or organelle transport in excitable cells.

EXPERIMENTAL PROCEDURES

Constructs and Antibodies—pCISGRIF-1, pGAD7GRIF-1-(1–913), pGAD10GRIF-1-(8–633), and pGAD7GRIF-1-(124–283), pMBL-33GABA_ receptor β intracellular loop (residues 303–427), and affinity-purified anti-GRIF-1 (8–633) antibodies were as previously described (1). Full-length cDNA clones of KIAA1042 (1–953) (hereafter termed OIP106-(1–953)) and KIAA0531 (kinesin heavy chain, KIF5C-(1–957); hereafter termed KIF5C-(1–957)), cloned into the Sall/NotI restriction sites of the pBluescript II SK + vector, were obtained from Professor T. Nagase (Kazusa Research Institute, Chiba, Japan). Each insert was excised by restriction enzyme digestion and used as template DNA for PCR amplification and cloning into pCRRI TOPO (Invitrogen, Groningen, The Netherlands). Specific oligonucleotide primers incorporating the restriction sites for EcoRI and BamHI were used to amplify OIP106, and primers containing the restriction sites EcoRI and Sall were used to amplify KIF5C. The full-length OIP106 factor-1; GABA, γ-aminobutyric acid; GRIP1, glutamate-receptor-interacting protein 1; HAP-1, huntingtin-associated protein; HEK, human embryonic kidney; KIF, kinesin superfamily protein; OGT, O-GlcNAc transferase; OIP, OGT-interacting protein; SD, synthetic defined; cfu, colony-forming units.
and KIF5C clones were subcloned from the respective pCRII TOPO constructs in frame into the modified (m) LexA vectors pGADT7 and pMBL33 to generate the fusion constructs pGADT7FOIP106 (1–655) and pMBL33KIF5C (1–957). Full-length GPI-FOIP106 was also subcloned into the mammalian expression vector, pCIS, to generate pCISOIP106 and into pCMVTag4a engineered to include an N-terminal c-Myc tag; KIF5C was subcloned into pcDNAHisMax (Invitrogen) to generate pcDNAHisMaxKIF5C. GRIF-1 (1–283–913) and OIP106 (124–283) were obtained by PCR amplification using pGADT7GRIF-1 and pGADT7FOIP106 as templates, respectively, and cloned into the EcoRI/XhoI restriction sites of the pGADT7 vector (Clontech, Palo Alto, CA) to generate pGADT7GRIF-1 (1–283–913) and pGADT7FOIP106 (124–283). GRIF-1 (1–283–913), GRIF-1 (124–283), and OIP106 (124–283) were obtained by PCR amplification using pGADT7GRIF-1 and pGADT7FOIP106 as templates, respectively, and cloned into the EcoRI/XhoI restriction sites of a modified pCMV4a plasmid to generate pCMVGRIF-1 (1–283–913), pCMVFOIP106 (124–283), and pCMVTag1a (1–283–912), where each protein was N-terminally c-Myc-tagged. All constructs were verified by DNA sequencing using the ABI PRISM 310 Genetic Analyser. Further, for all constructs used in yeast two-hybrid interaction assays, the expression of fusion proteins was verified by immunoblotting (data not shown).

The peptide CNSSGGLLRNQSL that corresponds to GRIF-1 (574–584) was immobilized on the N-terminal cysteine for covalent coupling via the cysteine residue, and both rabbit and sheep anti-GRIF-1 (1–584–889) polyclonal antibodies were generated as described (8). Anti-kinesin monoclonal antibodies were from Cytokeleton Inc. (Denver, CO); anti-β-actin and anti-β-tubulin monoclonal antibodies were from Chemicon International Inc. (Temecula, CA); anti-His G antibodies were from Invitrogen; anti-KIF5A (1007–1027) and anti-KIF5B (376–396) were from Abcam Ltd. (Cambridge, UK); Affinity-purified anti-Kv1.2 potassium channel antibodies were a generous gift of Professor J. O. Dolly (9). pCMVTag1milton and the anti-Milton monoclonal antibody, pMilton 5A 124, were generous gifts of Dr. T. L. Schwarz (Boston, MA).

**Materials and Methods**

**Cell Transfection and Preparation of Detergent-solubilized Extract of Transfected Cells**—Human embryonic kidney (HEK) 293 cells were transfected with either pCISGRIF-1 or pCISOIP106 alone using the calcium phosphate method with a total of 5 μg of DNA or with pCISGRIF-1/pCDNAHisMaxKIF5C, pCISOIP106/pCDNAHisMaxKIF5C, or related deletion construct combinations using 1:1 ratios with a total of 10 μg of DNA. Cells were harvested 24–48 h post-transfection, and cell homogenates were analyzed by immunoblotting. Alternatively, transfected cell homogenates were solubilized with 10 μg HEPES, pH 7.5, 145 mM NaCl, 1 mM EGTA, 0.1 mM MgCl2, benzamidine (1 mg/ml), bacitracin (1 mg/ml), soybean trypsin inhibitor (1 mg/ml), chicken egg trypsin inhibitor (1 mg/ml), and phenylmethylsulfonyl fluoride (1 mM) and 0.5% (v/v) Triton X-100 for 60 min at 4 °C. Detergent-solubilized extracts were collected by centrifugation for 3 h at 2500 x g and washed with 3 x 1.5 ml of 10 mM HEPES, pH 7.5, 145 mM NaCl, 1 mM EGTA, 0.1 mM MgCl2, benzamidine (1 mg/ml), bacitracin (1 mg/ml), soybean trypsin inhibitor (1 mg/ml), chicken egg trypsin inhibitor (1 mg/ml), phenylmethylsulfonyl fluoride (1 mM), and 0.5% (v/v) Triton X-100 and then analyzed by immunoblotting.

**Yeast Two-hybrid Interaction Assays**—Yeast two-hybrid assays were carried out using a modified LexA system that uses a modified bait vector, pMBSLM3, and the fish vector is pGADT7. briefly, the bait vector, pMBSLM3, was constructed as follows. The LexA-NLS sequence was amplified from plasmid, pEG202NLS, (DuplexA System, OriGene Technologies Inc., Rockville, MD) using primers ADH_prom (CACACATCCGCAATGCGAC) and ADH_term (GAGGGCCTCTAGGTTACTACC) and the following profile: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min 10 s; final extension at 72 °C for 5 min; and, finally, cooling of the reaction to 4 °C. The product size was 1221 bp. The PCR product (2 μl) and pGBK7T7 (linearized with XhoI, NotI, and EcoRI; 1 μl) were transformed into competent AH109 cells (25). The transformants were plated on YPD agar, incubated overnight at 30 °C, and then analyzed for β-galactosidase (β-gal) activity assay. In the next step, each interacting pair of constructs was transformed into AH109 cells (25 μl; BD Biosciences, Clontech). From 6 to 10 individual 5-ml cultures, DNA was prepared by lyticase digestion, followed by minipreparation of plasmid DNA. Isolated yeast DNA was screened by PCR (primer ADH_plus and ADH_minus) and then analyzed by immunoblotting.

**Preparation of Yeast Lysates**—Yeast lysates were prepared according to the Yeast Protocols Handbook (Clontech). Briefly, for each yeast strain, 20 ml of a 5-ml culture using a single colony dispersed in SD medium was incubated overnight at 30 °C with shaking at 220–250 rpm. Each overnight 5-ml culture was used to

inoculate a 50-ml aliquot of YPAD, and cultures were incubated at 30 °C with shaking until the A{subscript,600} reached 0.4–0.6. Each culture was centrifuged at 1000 × g for 5 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 50 ml of ice-cold H$_2$O and recentrifuged as above. Pellets were frozen immediately in liquid nitrogen. Pellets were thawed and resuspended in complete cracking buffer, 40 mM Tris-HCl, pH 6.8, 8 M urea, 5% (w/v) SDS, 0.1 mM EDTA, 9 mM benzamidine, 44 mM phenylmethylsulfonyl fluoride, 0.4 mg/ml bromophenol blue, 6 μg/ml peptatin A, 13 μg/ml leupeptin, 23 μg/ml aprotinin (100 μl per 7.5 A{subscript,600} units). Cell suspensions were incubated with 425–600-μm glass beads, heated at 70 °C for 10 min, and mixed vigorously for 1 min, and supernatants were collected by centrifugation for 5 min at 4 °C at 15,000 × g for 5 min and analyzed by immunoblotting. All fusion constructs yielded robust expression.

RESULTS

Anti-GRIF-1 Antibody Specificities—GRIF-1 and OIP106 have 42% amino acid identity overall but 52% identity over the sequence GRIF-1-(8–633) that was used for the production of anti-GRIF-1 antibodies. It is thus probable, as suggested by Iyer et al. (2), that anti-GRIF-1 antibodies recognize both GRIF-1 and OIP106. Indeed, when each was expressed alone in HEK 293 cells, anti-GRIF-1-(8–633) antibodies recognized three molecular mass species of 115, 106, and 98 kDa as reported previously for GRIF-1-transfected cells (1) and two molecular mass species of 170 and 115 kDa for OIP106-transfected cells (Fig. 1). OIP106 is a polypeptide consisting of 953 amino acids; therefore, the 115-kDa species is the monomeric molecular mass species of 170 and 115 kDa for OIP106-transfected cells. No anti-kinesin immunoreactivity was detected in control immunoprecipitations with the individual respective clones (Fig. 2). Since Milton was found to co-immunoprecipitate with kinesin, it was of interest to determine whether such an association occurred in mammalian systems. In Fig. 2, it can be seen that indeed, using a broad spectrum anti-kinesin heavy chain antibody, kinesin immunoreactivity, 115 kDa, was found in immune pellets resulting from precipitation with anti-GRIF-1-(8–633) antibodies for both GRIF-1- and OIP106-transfected cells. No anti-kinesin immunoreactivity was detected in control immunoprecipitations where the primary antibody was nonimmune Ig (Fig. 2). Further specificity control experiments using yeast lysates expressing either GRIF-1 or KIF5C showed that anti-kinesin antibodies do not recognize GRIF-1 and vice versa (i.e. anti-GRIF-1 antibodies do not recognize kinesin) (Fig. 1). GRIF-1 and kinesin were also found to co-associate in native brain tissue. The results are shown in Fig. 3. Anti-GRIF-1-(8–633) antibodies immunoprecipitated the three molecular weight species observed in the immunoblots. Probing with anti-GRIF-1-(874–889) confirmed that the M$_r$ 115,000 immunoreactive band was indeed GRIF-1, and probing with anti-kinesin antibodies revealed the presence of kinesin in anti-GRIF-1-(8–633) but not control immune pellets. In the brain, two kinesin-immunoreactive bands of 118 and 115 kDa were detected (Fig. 3). An additional control using an anti-Kv1.2 potassium channel antibody showed no immunoreactivity in test and control immune pellets. Further, immune pellets from both the GRIF-1 HEK 293-transfected cells and the brain were screened for reactivity with anti-β-actin, anti-β-tubulin, and anti-dynein antibodies. All antibodies yielded robust signals in detergent-solubilized extracts; however, no immunoreactivity was detected in the immune pellets for any of the three antibodies (results not shown).
The Association between GRIF-1 and Kinesin is Direct: Demonstration and Specificity Using the Yeast Two-hybrid Interaction Assay—

Association of two proteins by co-immunoprecipitation does not demonstrate definitively that two proteins interact directly, since they may co-associate via an intermediary adaptor protein molecule. Thus, to test whether GRIF-1 and OIP106 do associate directly with kinesin, yeast two-hybrid interaction assays were carried out.

Kinesin is a member of the kinesin superfamily of proteins (i.e., the KIFs). In the human genome, there are 45 KIF members; 38 of these are expressed in the brain (11). The anti-kinesin antibody employed initially in the immunoprecipitation studies was a monoclonal antibody, SUK4, which was raised against the 130-kDa kinesin heavy chain of sea urchin egg (12). This antibody was originally reported to cross-react with a 120-kDa heavy chain of bovine brain kinesin; hence its use in mammalian species (12). However, more detailed information about the KIF subtype specificity of SUK4 is unfortunately unavailable. In the absence of this knowledge, KIF5C was selected for the yeast two-hybrid studies, since it is described as neuron-specific (11). Also, the predicted size of KIF5C is similar to that of the kinesin-immunoreactive bands seen in the co-immunoprecipitation experiments (Figs. 2 and 3). The results of the yeast two-hybrid interaction assays are summarized in Table I.

Co-transformation of yeast with pGADT7GRIF-1-(1–913) with pMBL33KIF5C-(1–957) revealed a weak but significant interaction of the resulting two fusion proteins as determined by both nutritional selection on SD/H11002W, H11002L, H11002H media and /H9252-galactosidase reporter gene activity. The GRIF-1 truncated constructs, GRIF-1-(8–633) and GRIF-1-(124–283), were also found to interact with KIF5C, whereas GRIF-1-(283–913) did not interact. This therefore identifies the kinesin binding domain of GRIF-1 to within residues 124–283. This region coin-
The results are representative of HEK 293 cells suggested a direct association between GRIF-1 expression of GRIF-1 and N-terminal His-tagged KIF5C in proteins—two different experimental paradigms, GRIF-1 was shown to that it was not co-immunoprecipitated with KIF5C. Thus, in the results were consistent with yeast interaction assays in contrast to the yeast two-hybrid findings, full-length OIP106 interaction assays (Table I). For OIP106, unexpectedly and in c-Myc-tagged OIP106-(124–283) (32 kDa) were each co-expressed and immune pellets were analyzed by immunoblotting using anti-KIF5A, KIF5B, and KIF5C antibodies. The results are shown in Figs. 5 and 6. As before, both GRIF-1-(8–633) and GRIF-1-(874–889) immunoreactivity was detected in the immune pellets. In the brain, KIF5A and KIF5C were the most abundantly expressed, but all three KIF antibodies recognized the appropriate molecular mass immunoreactive species in Triton X-100 extracts (i.e. KIF5A (118 kDa), KIF5B (115 kDa), and KIF5C (115 kDa)). A strong immunoreactive signal for KIF5A was detected in the immune pellets, demonstrating an association between GRIF-1 and KIF5A. Weak signals of similar intensity were detected for both KIF5B and KIF5C (Fig. 5).

In HEK 293 cells, only KIF5B was detected in the detergent extracts, but it was specifically immunoprecipitated from both GRIF-1- and OIP106-transfected cell homogenates (Fig. 6). A weak band was seen with ~115 kDa in the immune pellet of GRIF-1-transfected cells probed with anti-KIF5C antibodies that could be KIF5C. A lack of detection in the detergent extract may be due to sensitivity; the primary antibodies are incubated with ~30 times the amount of protein applied to the immunoblot for the detection of immunoreactivity in the detergent extracts. Other bands seen in the immunoblot are probably IgG molecules.

Association of Anti-GRIF-1-(8–633) Immunoreactivity with Endogenous Kinesin in the Heart—We previously reported that anti-GRIF-1-(8–633) immunoreactivity was found only in excitable tissues (1). Notably, as well as the 115- and 106-kDa GRIF-1-immunoreactive species, an 88-kDa immunoreactive band was expressed in the heart. It was suggested that this may be the GRIF-1b C-terminal truncated splice variant; GRIF-1a is the nomenclature for the 913-amino acid protein (1). It was thus of interest to investigate the possible association of GRIF-1 with kinesin in the heart. In Fig. 7, it is shown that anti-GRIF-1-(8–633) antibodies immunoprecipitated the three major bands of 115, 106, and 87 kDa observed in original immunoblots from detergent extracts of the heart (1). When these immune pellets were probed with anti-kinesin antibodies, a single immunoreactive band of 115 kDa was detected compared with the two immunoreactive bands, 118 and 115 kDa, observed for brain. Liver was used as a negative control.

### Table I

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<td>pGAD10GRIF-1-(8–633)</td>
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The yeast strain L40 was co-transformed with bait and fish constructs and transformants grown on –2 SD (i.e. –W, –L) and –3 SD (i.e. –W, –L, and –H). Colonies from –2 plates were restreaked onto new plates, and β-galactosidase activity was measured by filter lift assays all as described under “Experimental Procedures.” Colony growth is shown as follows. (+), <10 cfu; (+), 10–100 cfu; ++, 100–200 cfu; +++, >200 cfu. The results are representative of n = 3 independent co-transformations.
since it was previously reported that no anti-GRIF-1 immuno-reactivity was detected in soluble, P2, or nuclear crude subcellular fractions (1). Here, detergent extracts of whole cell homogenates were used for the immunoprecipitations, and weak anti-GRIF-1-(8–633) immunoreactivity was found in both homogenates and immune pellets. Kinesin immunoreactivity was not detectable in the pellets.

The kinesin specificity was investigated by probing the anti-GRIF-1-(8–633) immune pellets with the anti-KIF5A, -5B, and -5C antibodies. The results are shown in Fig. 7, C–G. First, anti-GRIF-1-(874–889) recognized two bands of 118 and 115 kDa in the immune pellets, showing that GRIF-1 was present. The 88-kDa species was not detected by the GRIF-1 C-terminal antibodies, but this was expected, since the GRIF-1b C-term-
nal truncated splice form comprises amino acids 1–672 and therefore does not contain the GRIF-1-(874–889) peptide epitope. KIF5B and KIF5C were both expressed in heart tissue, but only KIF5B (115 kDa) immunoreactivity was detected in the immune pellets (Fig. 7). Thus, the band seen with the pan kinesin antibody is probably KIF5B.

Do GRIF-1 and OIP106 Associate with Mitochondria following Expression in HEK 293 Cells?—The Drosophila protein, Milton, was proposed to be a mitochondria-associated protein required for kinesin-mediated transport of mitochondria to nerve terminals (6). We have shown here that GRIF-1 and its homologue, OIP106, both associate with kinesin, thus establishing that Milton is indeed a species ortholog of GRIF-1 and OIP106. It was thus of interest to determine whether GRIF-1 and OIP106 also associated with mitochondria. HEK 293 cells were transfected with either GRIF-1, OIP106, or Milton clones. The distribution of mitochondria and the expressed proteins was determined post-transfection using MitoTracker Red CMXRos and the appropriate respective antibodies. The results are shown in Fig. 8. For Milton, similar results to those reported by Stowers et al. (6) were found (i.e. Milton-transfected cells showed a tight aggregation of mitochondria that co-local-
FIG. 8. Localization of GRIF-1, OIP106, Milton, and mitochondria in HEK 293 cells transfected with pCISGRIF-1, pCISOIP106, and pCMVtag1_milton. HEK 293 cells adhered to poly-l-lysine-coated coverslips were transfected with the appropriate clones by the calcium phosphate method, incubated for 20 h, and stained with MitoTracker Red CMXRos and anti-GRIF-1-(8–633) or anti-α-milton 5A 124 antibodies, all as described under “Experimental Procedures.” A–L, a single confocal section; A–D, pCMVtag1_milton-transfected cells; E–H, pCISGRIF-1-transfected cells; I–L, pCISOIP106-transfected cells. A, E, and I, staining with MitoTracker Red CMXRos; B, staining with anti-α-milton 5A 124 antibodies; F and J, staining with anti-GRIF-1-(8–633) antibodies; C, A and B merged; G, E and F merged; K, I, and J merged; D, H, and L, enlargements of the boxes depicted in C, G, and K, respectively, where yellow shows areas of co-localization. In the field shown in I, note the clear difference in distribution of mitochondria found for both transfected (green cells) and nontransfected cells. The results shown are representative of multiple observations found for n = 2 independent transfections. Scale bars, 5 μm.

DISCUSSION

GRIF-1 and OIP106 are thought to be members of a novel coiled-coil domain family by virtue of the similarity in their primary structures (1, 2). Their function is unknown. It was speculated that GRIF-1 is a neuronal trafficking molecule because of its enrichment in excitable tissues, its association with β2 subunit-containing GABA<sub>A</sub> receptors, and its homology with other proteins such as huntingtin-associated protein (HAP-1). HAP-1 binds to the p150<sub>flied</sub> subunit of dynactin, glutamate neurotransmitter receptor-GluR2-interacting protein (GRIP1), the inositol 1,4,5-trisphosphate receptor type 1, and kinesin 5C (see Refs. 13–17, respectively). It was reported to play a role in the regulation of vesicular trafficking from early endosomes to late endocytic compartments (18). More recently, HAP1 was shown to be involved with huntingtin in the enhancement of vesicular transport of brain-derived neurotrophic factor along microtubules (17). Both GRIF-1 and OIP106 also associate with the enzyme OGT (2). Many classes of proteins are post-translationally, dynamically modified by O-glycosylation catalyzed by OGT. O-GlcNAc-glycosylated proteins are particularly abundant in the central nervous system (e.g. see Ref. 19). These proteins include transcription factors. Indeed, Iyer et al. (2) showed that OIP106 formed an in vivo RNA polymerase II-OIP106-OGT complex, leading to the speculation that OIP106 may target OGT to transcriptional complexes. Interestingly, HAP-1 and huntingtin have also been shown to associate with the transcription factor, NeuroD (20), thus revealing further similarities between HAP-1 and the GRIF-1/OIP106 coiled-coil domain family of proteins.

In this paper, we have identified a second family of proteins that associate with both GRIF-1 and OIP106. We have shown by co-immunoprecipitation strategies that GRIF-1 and its homologue, OIP106, both interact in vitro and in vivo (GRIF-1) with kinesin. Furthermore, OIP106 and possibly GRIF-1 aggregate mitochondria following their expression in mammalian cells. These results provide further support of a role for both GRIF-1 and OIP106 in membrane trafficking of vesicles and/or organelles utilizing molecular motor proteins. Additionally, they provide evidence that the Drosophila protein, Milton, is indeed a species orthologue of GRIF-1 and OIP106.

The impetus for the experiments described herein came from the work of Stowers et al. (6), who showed that kinesin heavy chain was associated with Milton following immunoprecipitation from extracts of Drosophila heads by two different specificity anti-Milton antibodies. Kinesins belong to the kinesin superfamily (i.e. KIFs). KIFs are microtubule-based mechanochanical motors that are involved primarily in the anterograde transport of organelles and protein complexes. The kinesin molecule is a heterotetramer consisting of two kinesin heavy chains and two light chains; each KIF has its own cargo. The kinesin heavy chain has three functional domains, the motor domain, an α-helical stalk domain, and a globular tail region. The motor domain has two conserved sequences proximal to a Walker A ATP binding motif and a microtubule binding domain (11). Several KIFs attach to specific cargoes through interactions with adaptor proteins in nonmotor regions. Stowers et al. (6) suggested that Milton functions as an adaptor protein in the transport of mitochondria to synapses. However, they were...
unable to demonstrate a direct association between Milton and kinesin, which led them to suggest that an additional linker protein may be required to form an adaptor protein complex (6). It was stated that a precedent for such a complex was the finding that KIF17 forms a complex with mLIn10 in the transportation of N-methyl-D-aspartate receptor NR2B subunits to the synapse (21, 22). In contrast to Ref. 6, we were able to demonstrate a direct association between GRIF-1 and KIF5C in a yeast two-hybrid interaction assay (Table I). However, association between full-length GRIF-1 and KIF5C was weak compared with strong reporter gene activation observed for the GRIF-1 fragment, GRIF-1-(124–283), that contains a coiled-coil domain. Interestingly, despite robust expression, the corresponding OIP106 coiled-coil domain, OIP-(124–283), that shares 60% amino acid identity with GRIF-1-(124–283) did not result in reporter gene activity following co-transformation with KIF5C. It was found, however, that OIP106, like GRIF-1, did immunoprecipitate with exogenous KIF5C following over-expression of both in HEK 293 cells. It is difficult to rationalize these two observations. In a yeast two-hybrid interaction assay, bait and fish proteins may be concluded to associate, but this may be due to an intermediary protein supplied by the yeast cells. Thus, either the interaction between GRIF-1 or OIP106 and kinesin is indirect and, in yeast, GRIF-1 but not OIP106 binds directly to KIF5C. In this case, OIP106 must bind to an intermediary endogenous protein in HEK 293 cells. Given the high degree of amino acid identity between the KIF5C binding domain of GRIF-1 and the homologous region of OIP106 (80% similarity), one would predict it to be unlikely that the proteins would behave differently. It should be noted, however, that OIP106 does not associate with the intracellular loop of the GABA<sub>A</sub> receptor β<sub>2</sub> subunit in yeast two-hybrid assays (23). Thus, whereas GRIF-1 and OIP106 both associate with OGT and kinesin, there may be subtle differences with regard to interactions with other proteins that may reflect dissimilarities in their respective functions.

GRIF-1 and/or OIP106 are promiscuous with regard to association with KIF5 subtypes. In whole brain tissue, GRIF-1 is associated predominantly with KIF5A, although KIF5C is abundantly expressed; in the heart, association is with KIF5B, although both KIF5B and KIF5C are highly expressed; in HEK 293 cells, association of both proteins is with endogenous KIF5B, although both were found to associate with exogenous KIF5C. Kanai et al. (24) suggested that there is functional redundancy between KIF5A, KIF5B, and KIF5C. The three proteins share at least 63 and 73% amino acid sequence identity and similarity, respectively; thus, it is probable that each may contain a GRIF-1 binding domain within a conserved region. In general, kinesin heavy chains are thought to bind cargo in their C-terminal nonmotor regions. A ClustalW alignment of the primary structures of KIF5A, -5B, and -5C shows that within the nonmotor region, they do indeed share at least 59 and 71% amino acid identity and similarity, respectively. Preferential association with KIF5A in the brain may be due to the co-localization within defined neuronal cell types of GRIF-1 and KIF5A. Since the studies described herein use detergent extracts of whole brain homogenates for immunoprecipitations, they would not distinguish between different neuronal cell populations. In the heart, preferential association with KIF5B may be explained by a higher affinity of KIF5B for GRIF-1.

The protein GRIP1 is an α-amino-3-hydroxy-5-methylisoxazole-4-propionate glutamate neurotransmitter receptor-GluR2-interacting protein. GRIP1 was shown to interact directly with kinesin heavy chains, and thus this complex provided the molecular motor and steer to traffic α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors to dendrites in neurons (25). Based on the findings described in this paper and by Beck et al. (1) together with those of the groups of Schwarz (6) and Hart (2, 3) we propose that Milton, GRIF-1, and OIP106 may all share a similar function to GRIP-1 and

FIG. 9. A schematic diagram depicting GRIF-1/kinesin-mediated anterograde transport of organelles in excitable tissue. A, GRIF-1 is shown as forming a link between microtubules, a kinesin molecule, and a vesicle containing an assembled GABA<sub>A</sub> receptor being transported to the synapse. B, GRIF-1 is shown as forming a link between microtubules, a kinesin molecule, and a mitochondrion expressing the homotrimeric enzyme, OGT. There are two known forms of OGT, a nuclear and cytoplasmic OGT and a mitochondrial OGT (27). Since Milton has been postulated to play a role in the axonal transport of mitochondria to synapses in Drosophila, GRIF-1 is depicted as interacting with mOGT. In A and B, GRIF-1 is depicted as a dimer (G. Ojla, M. Beck, K. Brickley, and F. A. Stephenson, manuscript in preparation).
possibly HAP-1 (i.e. the anterograde trafficking of organelles such as mitochondria or vesicles containing receptor molecules to synaptic terminals). Interestingly, recently Li et al. (26) demonstrated that there is a high level of dynamism in dendritic mitochondrial distribution associated with synaptic activity and synaptic morphogenesis. It may be speculated that GRIF-1 and OIP016 play a pivotal role in the trafficking of mitochondria in concert with receptor-containing vesicles, ensuring fidelity of synaptic function during development and synaptic activity. A schematic model for the action of GRIF-1 and OIP106 is shown in Fig. 9.

Acknowledgments—We thank Dr. T. L. Schwarz and his laboratory (Division of Neuroscience, Children’s Hospital and Department of Neurobiology, Harvard Medical School, Boston, MA) for the generous gifts of pCMVtag1milton and anti-Milton antibodies; Dr. T. Nagase and colleagues (Kazusa DNA Research Institute, Chiba, Japan) for the gifts of the clones KIAA1042 and KIAA0531; Gurmail Ojla (School of Pharmacy, University of London) for expert help with Fig. 8; and Professor J. O. Dolly for the kind gift of anti-Kv1.2 potassium channel antibodies.

REFERENCES