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

ASSOCIATION *IN VIVO* AND *IN VITRO* WITH KINESIN*

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γ-Aminobutyric acid$_A$ receptor-interacting factor (GRIF-1) is a 913-amino acid protein proposed to function as a GABA$_A$ receptor β_2 subunit-interacting, trafficking protein. GRIF-1 shares ~44% amino acid sequence identity with *O*-linked *N*-acetylglucosamine 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 α-amino-3-hydroxy-5-methylisoxazole-4-propionate glutamate excitatory neurotransmitter receptors to dendrites.

γ-Aminobutyric acid$_A$ receptor-interacting factor (GRIF-1)[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$_A$ receptor-trafficking factor (1). GRIF-1 was also recently identified as a protein that interacts with the enzyme uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase or *O*-GlcNAc transferase (OGT) (2, 3). OGT catalyzes the post-translational modification of proteins by β-*O*-linked *N*-acetylglucosamine (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, pGADT7GRIF-1-(1–913), pGAD10GRIF-1-(8–633), and pGADT7GRIF-1-(124–283), pMBL-33GABA$_A$ receptor β_2 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 SalI/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 pCRII 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 SalI were used to amplify KIF5C. The full-length OIP106

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[1] The abbreviations used are: GRIF-1, GABA$_A$ receptor-interacting 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 pGADT7OIP106-(1–953) and pMBL33KIF5C-(1–957). Full-length OIP106 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-(283–913) and OIP106-(124–283) were obtained by PCR amplification using pGADT7GRIF-1 and pGADT7OIP106 as templates, respectively, and cloned into the EcoRI/XhoI restriction sites of the pGADT7 vector (Clontech, Palo Alto, CA) to generate pGADT7GRIF-1-(283–913) and pGADT7OIP106-(124–283). GRIF-1-(283–913), GRIF-1-(124–283), and OIP106-(124–283) were obtained by PCR amplification using pGADT7GRIF-1 and pGADT7OIP106 as templates, respectively, and cloned into the EcoRI/XhoI restriction sites of a modified pCMV4a plasmid to generate pCMVGRIF-1-(283–913), pCMVGRIF-1-(124–283), and pCMVOIP106-(124–283), 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 CNSGGSLLGGLRRNQSL that corresponds to GRIF-1-(874–889) with an N-terminal cysteine was coupled to thyroglobulin via the cysteine residue, and both rabbit and sheep anti-GRIF-1-(874–889) polyclonal antibodies were generated as described (8). Anti-kinesin monoclonal antibodies were from Cytoskeleton Inc. (Denver, CO); anti-β-actin and anti-β-tubulin monoclonal antibodies were from Sigma; anti-dynein monoclonal antibodies were from Chemicon International Inc. (Temecula, CA); anti-His G antibodies were from Invitrogen; anti-c-Myc antibodies were from Upstate (Charlottesville, VA); and anti-KIF5A-(1007–1027), KIF5B-(376–396), and KIF5C-(938–957) antibodies were from Abcam Ltd. (Cambridge, UK). Affinity-purified anti-Kv1.2 potassium channel antibodies were a generous gift of Professor J. O. Dolly (9). pCMVtag1*milton* and the anti-Milton monoclonal antibody, αMilton 5A 124, were generous gifts of Dr. T. L. Schwarz (Boston, MA).

Mammalian 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 mM HEPES, pH 7.5, 145 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, benzamidine (1 μg/ml), bacitracin (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), chicken egg trypsin inhibitor (1 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) and 1% (v/v) Triton X-100 for 60 min at 4 °C. Detergent-solubilized extracts were collected by centrifugation for 30 min at 4 °C at 100,000 $\times$ *g*.

Immunochemical Analyses of Mammalian Cells Transfected with pCISGRIF-1, pCISOIP106, and pCMVtag1milton—For immunocytochemical studies, HEK 293 cells were transfected with either pCISGRIF-1, pCISOIP106, or pCMVtag1*milton* as described previously (1). Transfected cells were cultured for 20 h, cell culture media were removed, and cells were rinsed with Hanks' balanced salt solution at 37 °C. Cells were incubated with freshly prepared 100 nM MitoTracker Red CMXRos (Molecular Probes, Inc., Eugene, OR) for 15 min at 37 °C followed by a wash with cell culture media and a wash with Hanks' balanced salt solution also at 37 °C. Cells were fixed with 4% (w/v) paraformaldehyde in Hanks' balanced salt solution for 10 min at 37 °C and washed with 3$\times$ 20 mM Tris-HCl, pH 7.4, 145 mM NaCl (TBS) for 5 min each at 20 °C. They were permeabilized with 0.03% (v/v) Triton X 100 in TBS for 10 min at 20 °C and incubated with 10% (v/v) goat serum, 4% (w/v) bovine serum albumin, 0.1% (w/v) DL-lysine in TBS for 10 min at 20 °C. Primary antibodies were diluted in 5% (v/v) goat serum, 2% (w/v) bovine serum albumin, 0.1% (w/v) DL-lysine in TBS and incubated with transfected cells overnight at 4 °C. Cells were washed three times with TBS for 5 min each at 20 °C and then incubated with a 1:180 dilution of secondary antibody, either goat anti-rabbit AlexaFluor 488 or goat anti-mouse AlexaFluor 488 antibodies (Molecular Probes) for 50 min at 20 °C. Coverslips were washed with 5$\times$ TBS for 5 min at 20 °C, mounted in Citifluor (Citifluor Ltd., London, UK), and viewed with a Zeiss LSM-510 confocal microscope.

Preparation of Detergent-solubilized Extracts of Rat Tissues—Forebrain, heart, and liver were dissected from adult rats and processed immediately. Forebrain was homogenized using a Wheaton/Dounce glass/glass homogenizer in ~40 ml (~20 volumes) of 10 mM HEPES, pH 7.5, 145 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, benzamidine (1 μg/ml), bacitracin (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), chicken egg trypsin inhibitor (1 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) and 1% (v/v) Triton X-100 for 60 min at 4 °C. Liver was minced using scissors and then homogenized as above; heart tissue was also minced and then homogenized using an Ultra-Turrax (three 10-s pulses at medium speed) prior to detergent solubilization as before. Soluble extracts were collected by centrifugation for 30 min at 4 °C at 100,000 $\times$ *g*, and immunoprecipitation assays were carried out.

Immunoblotting—Immunoblotting was performed as previously described using 25–50 μg of protein/sample precipitated using the chloroform/methanol method and SDS-PAGE under reducing conditions in 7.5 or 12% polyacrylamide slab minigels (1). Rabbit and mouse horseradish-linked secondary antibodies (Amersham Biosciences) were used at a final dilution of 1:2000, and immunoreactivities were detected using the ECL Western blotting system.

Immunoprecipitation Assays—Detergent-solubilized extracts of either transfected HEK 293 cells (diluted to 1 mg of protein/ml with a final Triton X-100 concentration of 0.5% (v/v); 20 ml) or rat forebrain, heart, and liver (diluted to 2 mg of protein/ml with a final Triton X-100 concentration of 0.5% (v/v); 10 ml) were incubated for polyclonal antibodies with either affinity-purified anti-GRIF-1-(8–633) antibodies (10 μg) or protein A-purified nonimmune IgG (10 μg of protein) for 1.5 h at 37 °C. Protein A-Sepharose (20 μl) was added, and samples were incubated for 1 h at 37 °C. For monoclonal antibody immunoprecipitations, the primary antibody used was anti-His G (5 μg), and the control was anti-dynein (5 μg) with an incubation overnight at 4 °C. Protein G-Sepharose (20 μl) was added, and the samples were treated as for immunoprecipitation with polyclonal antibodies. For both polyclonal and monoclonal immunoprecipitations, pellets were collected by centrifugation for 3 s at 2500 $\times$ *g* and washed with 3 $\times$ 1.5 ml of 10 mM HEPES, pH 7.5, 145 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, benzamidine (1 μg/ml), bacitracin (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), chicken egg trypsin inhibitor (1 μg/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, pMBL33, and the fish vector is pGADT7.[2] Briefly, the bait vector, pMBL33, was constructed as follows. The LexA/NLS sequence was amplified from plasmid, pEG202NLS, (DuplexA System, OriGene Technologies Inc., Rockville, MD) using primers ADH_prom (CACCATATCCGCAATGACAA) and ADH_term (GAGCGACCTCATGCTATACC) and the following profile: initial denaturation at 94 °C for 4 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 pGBKT7 (linearized with XhoI, NotI, and EcoRI; 1 μl) were transformed into competent AH109 cells (25 μl; BD Biosciences, Clontech). From 10 individual 5-ml cultures, DNA was prepared by lyticase digest, followed by minipreparation of plasmid DNA. Isolated yeast DNA was screened by PCR (primer ADH_plus and ADH_minus) and digested with BclI (expected 797 and 424 bp) and EcoRV (expected 742 and 479 bp). Positive DNA was transformed into competent DH5α *Escherichia coli*, prepared and sequenced by standard methods. Yeast cultures were grown on standard solid or in liquid media using either YPAD (yeast, peptone, adenine, dextrose; 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, 0.003% (w/v) adenine) or SD (synthetic defined medium; 0.67% (w/v) yeast nitrogen base (BD Biosciences), 2% (w/v) glucose, 1$\times$ dropout supplement (Clontech)) as appropriate for the selection involved. All transformations were performed using the lithium acetate/polyethylene glycol method (10). The S*accharomyces cerevisae* strain L40 (MATa *his3*Δ*200 trp1*-901 *leu2-3112 ade2 LYS2*::(4lexAop-*HIS3*) *URA3*::(8lexAop-*lacZ*) *GAL4*) was used. Resulting colonies were assessed for reporter gene activation by initial nutritional selection (growth on SD −His) and subsequent *lacZ* activity assay. *lacZ* activity was determined by filter lift analysis including appropriate positive and negative interaction controls.

Preparation of Yeast Lysates—Yeast L40 lysates were prepared according to the Yeast Protocols Handbook (Clontech). Briefly, for each transformed yeast strain to be assayed, 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

[2] M. Beck, L. Demmel, A.-L. Schlaitz, P. Hsu, J. Havlis, A. Shevchenko, E. Krause, and C. Walch-Solimena, manuscript in preparation.

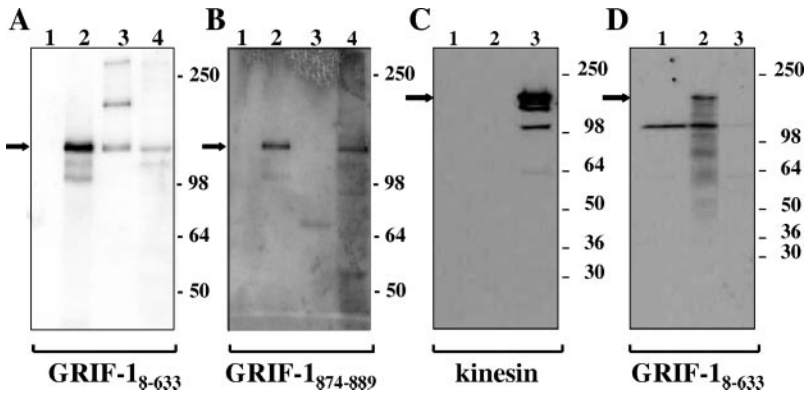

FIG. 1. **Demonstration of the specificity of anti-GRIF-1-(8–633), anti-GRIF-1-(874–889), and anti-kinesin antibodies.** In *A* and *B*, homogenates were prepared from HEK 293 cells transfected with pCISGRIF or pCISOIP106, and the P2 membrane fraction was prepared from adult rat forebrain. Both were analyzed by immunoblotting following SDS-PAGE under reducing conditions using affinity-purified anti-GRIF-1-(8–633) antibodies (1 μg/ml) (*A*) and affinity-purified anti-GRIF-1-(874–889) antibodies (4 μg/ml) (*B*), all as described under "Experimental Procedures." *Lane 1*, untransfected cells; *lane 2*, cells transfected with pCISGRIF-1; *lane 3*, cells transfected with pCISOIP106; *lane 4*, P2 membranes prepared from adult rat forebrain with the antibody specificities as shown. In *C* and *D*, yeast lysates were prepared from untransformed yeast or yeast transformed with either pGADT7GRIF-1-(1–913) or pMBL33KIF5C, and each was analyzed by immunoblotting using both anti-kinesin (*C*) and anti-GRIF-1-(8–633) (*D*) antibodies as indicated. *Lane 1*, untransformed yeast; *lane 2*, pGADT7GRIF-1-(1–913) transformed yeast; *lane 3*, pMBL33KIF5C transformed yeast. The *arrows* denote the positions of the GRIF-1 and KIF5C fusion proteins of 181 and 163 kDa, respectively. Lower molecular weight bands are probably proteolytic species, since they are not observed in untransformed yeast cells. It can clearly be seen that anti-GRIF-1-(8–633) antibodies recognize GRIF-1 only and that anti-kinesin antibodies recognize KIF5C but not GRIF-1. The positions of molecular weight standards (kDa) are shown on the *right*.

inoculate a 50-ml aliquot of YPAD, and cultures were incubated at 30 °C with shaking until the A_{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_2O 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 bromphenol blue, 8.8% (v/v) β-mercaptoethanol, 6 μg/ml pepstatin A, 13 μg/ml leupeptin, 23 μg/ml aprotinin (100 μl per 7.5 A_{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 protein, and the molecular size concurs with that reported by Iyer *et al.* (2) for OIP106 (*i.e.* 106 kDa). Because of the observed antibody cross-reactivity, a second, different specificity anti-GRIF-1 antibody was raised. This antibody was directed against the sequence GRIF-1-(874–889) that shares no amino acid sequence homology with OIP106. Anti-GRIF-1-(874–889) antibodies did not recognize OIP106 when expressed alone in HEK293 cells (Fig. 1). Further, in adult rat forebrain, anti-GRIF-1-(874–889) antibodies recognized a single band of 115 kDa. This corresponds to the molecular mass of the major band detected by anti-GRIF-1-(8–633) antibodies and demonstrates clearly that GRIF-1 is indeed expressed in brain tissue. The lower molecular weight species not recognized by anti-GRIF-1-(874–889) antibodies may be proteolytic fragments of OIP106. Alternatively, GRIF-1 is known to be expressed as three splice forms. One splice variant is truncated at the C terminus and does not contain the GRIF-1-(874–889) epitope (1). The lower molecular weight species may thus correspond to this splice variant detected by anti-GRIF-1-(8–633) but not anti-GRIF-1-(874–889) antibodies (1).

GRIF-1 and OIP106 Both Co-associate with Endogenous Kinesin in Heterologous Expression Systems and in the Brain—Affinity-purified anti-GRIF-1-(8–633) antibodies successfully immunoprecipitated both GRIF-1 and OIP106 from detergent extracts of HEK 293 cells transfected separately with each of 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 also 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 these three antibodies (results not shown).

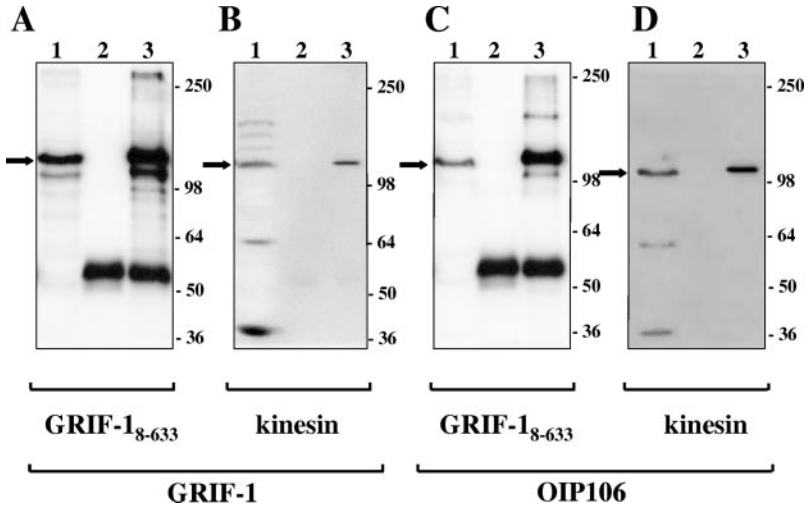

FIG. 2. **GRIF-1 and OIP106 both co-associate with endogenous kinesin following expression in HEK 293 cells.** HEK 293 cells were transfected with either pCISGRIF-1 or pCISOIP106, detergent extracts of cell homogenates were prepared 48 h post-transfection, and immunoprecipitation assays were carried out using either affinity-purified anti-GRIF-1-(8–633) antibodies or nonimmune Ig, and immune pellets were analyzed by immunoblotting using anti-GRIF-1-(8–633) (10% of immune pellet) and anti-kinesin (90% of immune pellet) antibodies all as described under "Experimental Procedures." *A* and *B*, immunoprecipitation from HEK 293 cells transfected with pCISGRIF-1; *C* and *D*, immunoprecipitation from HEK 293 cells transfected with pCISOIP106. In *A* and *C*, immunoblots were probed with anti-GRIF-1-(8–633) antibodies, and in *B* and *D*, immunoblots were probed with anti-kinesin antibodies. *Lane 1*, detergent-solubilized transfected HEK 293 cell homogenate; *lane 2*, nonimmune pellet; *lane 3*, anti-GRIF-1-(8–633) pellet. The positions of molecular weight standards (kDa) are shown on the *right*. The immunoblot is representative of at least $n = 3$ immunoprecipitations from $n = 3$ independent transfections.

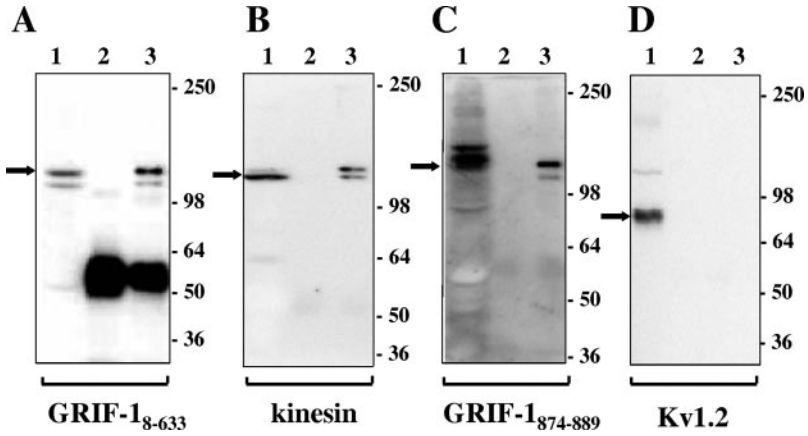

FIG. 3. **Anti-GRIF-1-(8–633) antibodies co-immunoprecipitate kinesin from detergent extracts of rat forebrain.** Triton X-100 extracts of rat forebrain were incubated with either affinity-purified anti-GRIF-1-(8–633) antibodies or nonimmune Ig, immune complexes were precipitated by the addition of protein A-Sepharose, and the immune pellets were analyzed by immunoblotting using anti-GRIF-1-(8–633) (10% of immune pellet) and anti-kinesin (90% of immune pellet) antibodies all as described under "Experimental Procedures." The gel layout is identical for *A–D* where *lane 1* is detergent-solubilized brain extract, *lane 2* is nonimmune Ig pellet, and *lane 3* is anti-GRIF-1-(8–633) pellet. Shown is immunoblotting with anti-GRIF-1-(8–633) (*A*), anti-kinesin (*B*), anti-GRIF-1-(874–889) (*C*), and anti-Kv1.2 antibodies (*D*). The positions of molecular weight standards (kDa) are shown on the *right*. The immunoblot is representative of $n = 7$ independent immunoprecipitations.

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 −W, −L, −H media and β-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-

TABLE I

GRIF-1 and the KIF protein, KIF5C, interact directly: Demonstration by yeast two-hybrid interaction assays

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.

AD vector	DNA-BD vector	Growth on −W, −L dropout medium	Growth on −W, −L, −H dropout medium	β-Galactosidase activity
pGADT7GRIF-1-(1–913)	pMBL33KIF5C-(1–957)	++	(+)	(+)
pGAD10GRIF-1-(8–633)	pMBL33 KIF5C-(1–957)	++	++	+
pGADT7GRIF-1-(283–913)	pMBL33 KIF5C-(1–957)	++	−	−
pGADT7GRIF-1-(124–283)	pMBL33 KIF5C-(1–957)	++	+++	++
pGADT7OIP106-(1–953)	pMBL33 KIF5C-(1–957)	++	−	−
pGADT7OIP106-(124–283)	pMBL33 KIF5C-(1–957)	++	−	−
pGADT7GRIF-1-(1–913)	pMBL33	++	−	−
pGAD10GRIF-1-(8–633)	pMBL33	++	−	−
pGADT7GRIF-1-(124–283)	pMBL33	++	−	−
pGADT7OIP106-(1–953)	pMBL33	++	−	−
pGADT7OIP106-(124–283)	pMBL33	++	−	−
pGADT7	pMBL33 KIF5C-(1–957)	++	−	−
pGADT7	pMBL33	++	−	−
pGAD10GRIF-1-(8–633)	pMBL33 β2-IL	++	++	+

cides with the first coil of the predicted coiled-coil domain of GRIF-1. It was notable that the order for the strength of interaction between GRIF-1 and KIF5C was GRIF-1-(124–283) > GRIF-1-(8–633) > GRIF-1-(1–913). Co-transformation of either pGADT7OIP106-(1–953) or pGADT7OIP106-(124–283), a region that shares ~60% amino acid identity and ~80% amino acid similarity with GRIF-1-(124–283), did not result in colony growth on −3 SD −W, −L, −H media or β-galactosidase reporter gene activity despite robust expression detected by immunoblotting of the appropriate transformed yeast lysates.

Co-association of GRIF-1 and OIP106 with KIF5C in Transfected HEK 293 Cells: Mapping of the KIF5C Binding Domain of GRIF-1—To confirm the yeast two-hybrid results, native GRIF-1, c-Myc-tagged GRIF-1-(283–913) (84 kDa), c-Myc-tagged GRIF-1-(124–283) (28 kDa), c-Myc-tagged OIP106, and c-Myc-tagged OIP106-(124–283) (32 kDa) were each co-expressed in HEK 293 cells with an N-terminal His-tagged KIF5C, and the association between the recombinant proteins was assessed by immunoprecipitation. The results are shown in Fig. 4. The primary antibody used for the immunoprecipitations was anti-His G. (Note that this was necessary, since anti-GRIF-1-(8–633) antibodies were raised against a His-tagged GRIF-1-(8–633) fusion protein (1). Unfortunately, they were found to recognize His-tagged KIF5C and other His-tagged proteins (results not shown).) For all immunoprecipitations, His-tagged KIF5C was present in the immune pellets with no immunoreactivity being detected in the control precipitations. For GRIF-1- and KIF5C-transfected cells, full-length GRIF-1 and GRIF-1-(124–283) but not GRIF-1-(283–913) were found to be specifically co-associated with KIF5C following immunoprecipitation with anti-His G antibodies (Fig. 4). These observations concur with those found in the yeast two-hybrid interaction assays (Table I). For OIP106, unexpectedly and in contrast to the yeast two-hybrid findings, full-length OIP106 was associated with KIF5C. For OIP106-(124–283), however, the results were consistent with yeast interaction assays in that it was not co-immunoprecipitated with KIF5C. Thus, in two different experimental paradigms, GRIF-1 was shown to co-associate with KIF5C with the KIF5C binding domain mapping to GRIF-1-(124–283).

Further Investigation into the Specificity of Interaction between Native and Recombinant GRIF-1 and the KIF5 Proteins—Since both the yeast two-hybrid results and the co-expression of GRIF-1 and N-terminal His-tagged KIF5C in HEK 293 cells suggested a direct association between GRIF-1 and KIF5C, further experiments were carried out to determine the KIF interaction specificity of native GRIF-1. Thus, Triton X-100 extracts of homogenates of rat forebrain or HEK 293 cells transfected with pCISGRIF-1 or pCISOIP106 were prepared and immunoprecipitated with anti-GRIF-1-(8–633) antibodies, and the 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-imunoreactive 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,

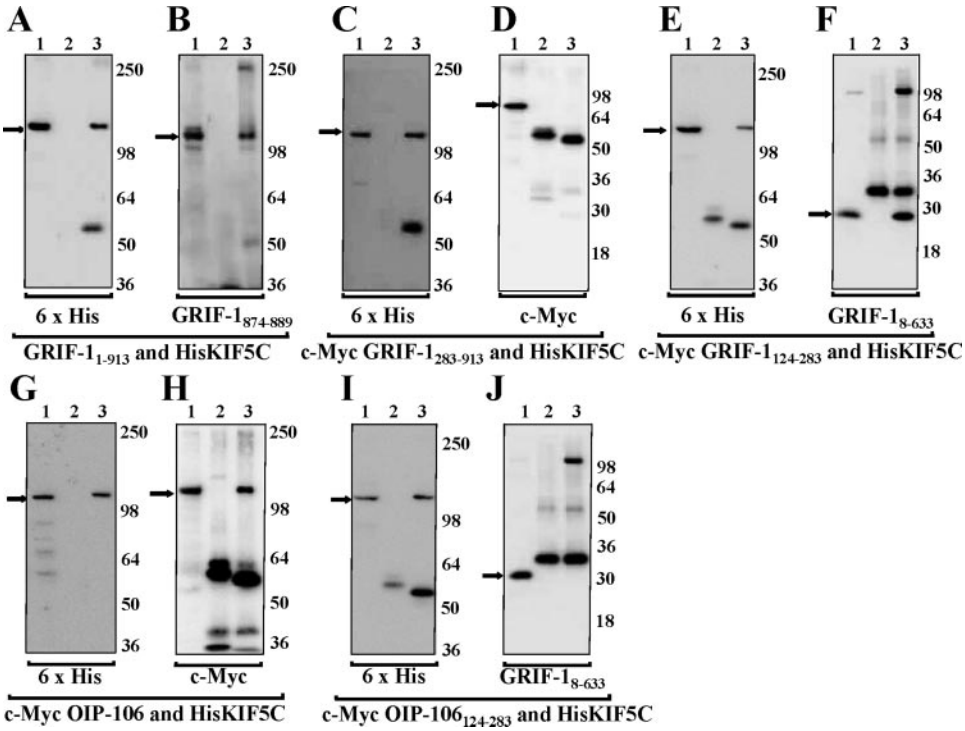

FIG. 4. **GRIF-1 and OIP106 co-associate with exogenous KIF5C following co-expression in HEK 293 cells: demonstration by immunoprecipitation.** HEK 293 cells were co-transfected with pcDNAHisMaxKIF5C and either pCISGRIF-1, pCMVTagGRIF-1-(283–913), pCMVTagGRIF-1-(124–283), pCMVTagOIP106, or pCMVTagOIP106-(124–283); detergent extracts of cell homogenates were prepared 48 h post-transfection; and immunoprecipitation assays were carried out using anti-His G or anti-dynein monoclonal antibodies and immune pellets analyzed by immunoblotting using anti-GRIF-1-(8–633), anti-GRIF-(874–889), anti-c-Myc, or anti-His G antibodies as shown, all as described under "Experimental Procedures." In *A* and *B*, cells were co-transfected with pcDNAMaxKIF5C and pCISGRIF-1; in *C* and *D*, cells were co-transfected with pcDNAMaxKIF5C and pCMVTagGRIF-1-(283–913); in *E* and *F*, cells were co-transfected with pcDNAMaxKIF5C and pCMVTagGRIF-1-(124–283); in *G* and *H*, cells were co-transfected with pcDNAMaxKIF5C and pCMVc-MycOIP106; and in *I* and *J* cells were co-transfected with pcDNAMaxKIF5C and pCMVTagOIP106-(124–283). The *gel lane* layout is identical for each. *Lane 1*, solubilized HEK 293 extract; *lane 2*, anti-dynein control immune pellet; *lane 3*, anti-His G immune pellet. Note that 10% of the immune pellet was applied to immunoblots probed with anti-His G antibodies, and 90% of the immune pellet was applied to immunoblots probed with anti-GRIF-1-(874–889), anti-GRIF-1-(8–633), or anti-c-Myc antibodies, as labeled on the *abscissae*. The immunoblots are representative of at least $n = 3$ independent transfections and immunoprecipitations. The *arrows* denote the positions of the respective immunoprecipitated proteins. The positions of molecular weight standards are shown on the *right*.

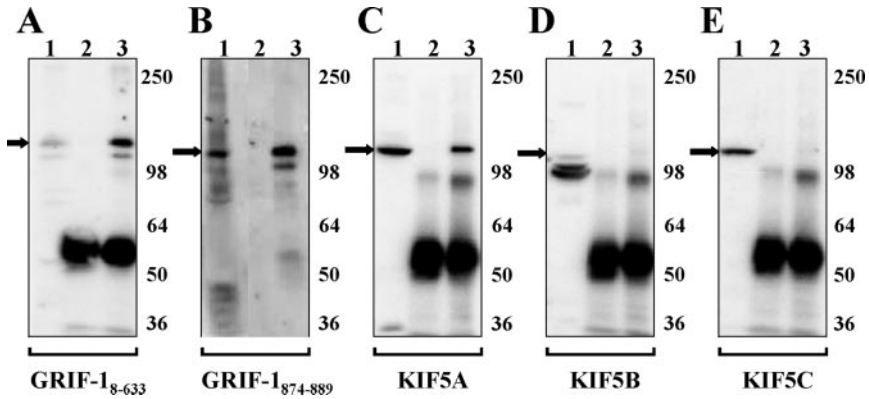

FIG. 5. **The specificity of GRIF-1/kinesin association in rat forebrain.** Triton X-100 extracts of rat forebrain were incubated with either affinity-purified anti-GRIF-1-(8–633) antibodies or nonimmune Ig, immune complexes were precipitated by the addition of protein A-Sepharose, and the immune pellets were analyzed by immunoblotting using anti-GRIF-1-(8–633) (10% of immune pellet) and anti-KIF5A, KIF5B, and KIF5C (90% of immune pellet) antibodies all as described under "Experimental Procedures." The *gel lane* layout is identical for *A–D*. *Lane 1*, detergent-solubilized brain extract; *lane 2*, nonimmune Ig pellet; *lane 3*, anti-GRIF-1-(8–633) pellet. Antibodies used for immunoblotting are indicated in the *abscissae*. The positions of molecular mass standards (kDa) are shown on the *right*. The immunoblot is representative of $n = 3$ independent immunoprecipitations.

since it was previously reported that no anti-GRIF-1 immunoreactivity 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-termi-

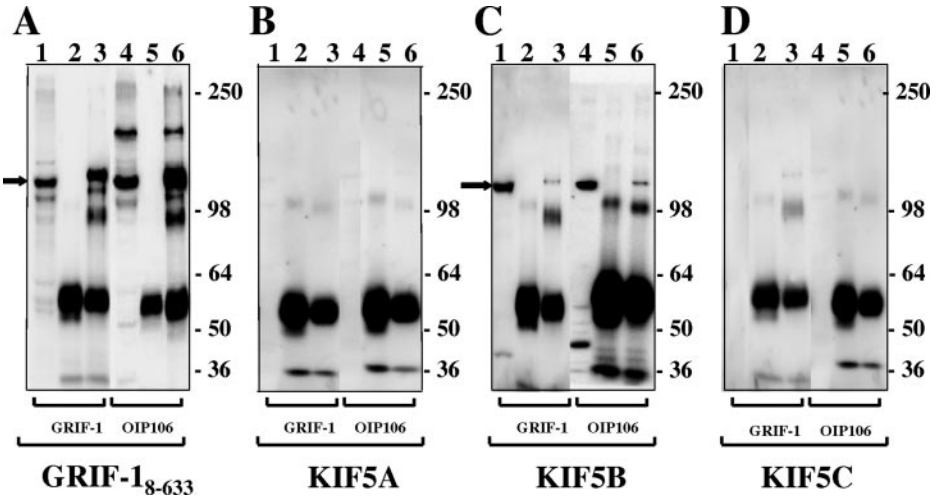

FIG. 6. **The specificity of endogenous kinesin/GRIF-1 and endogenous kinesin/OIP106 association in HEK 293 cells.** Triton X-100 extracts of HEK 293 cells transfected with either pCISGRIF-1 or pCISOIP106 were incubated with either affinity-purified anti-GRIF-1-(8–633) antibodies or nonimmune Ig, immune complexes were precipitated by the addition of protein A-Sepharose, and the immune pellets were analyzed by immunoblotting using anti-GRIF-1-(8–633) (10% of immune pellet) and anti-KIF5A, KIF5B, and KIF5C (90% of immune pellet) antibodies all as described under "Experimental Procedures." The *gel lane* layout is identical for *A–D*, where *lanes 1–3* are GRIF-1 transfected HEK 293 cells, *lanes 4–6* are OIP106-transfected HEK 293 cells, *lanes 1* and *4* are detergent-solubilized transfected cell extract; *lanes 2* and *5* are nonimmune Ig pellet; and *lanes 3* and *6* are anti-GRIF-1-(8–633) pellet. Antibodies used for immunoblotting are indicated in the *abscissae*. The positions of molecular mass standards (kDa) are shown on the *right*. The immunoblot is representative of $n = 3$ independent immunoprecipitations.

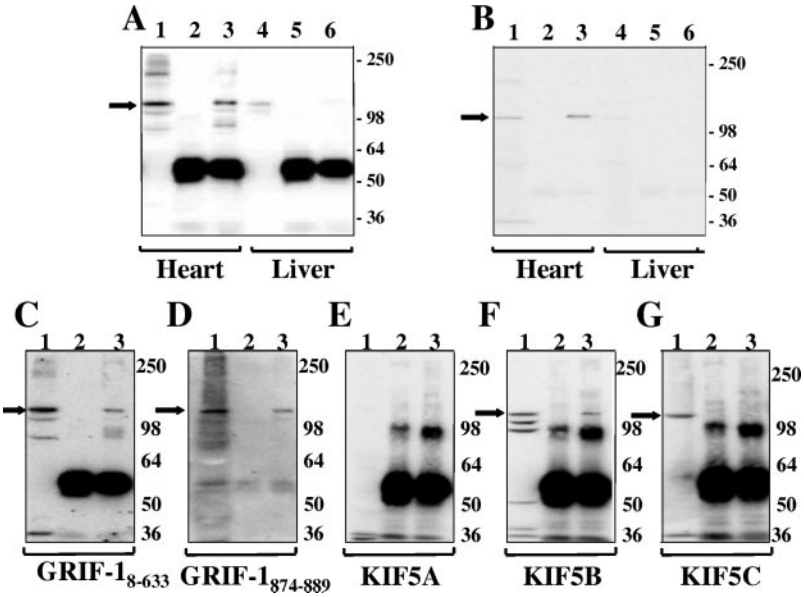

FIG. 7. **GRIF-1 associates with endogenous kinesin in the heart: specificity of GRIF-1/kinesin association.** Triton X-100 extracts of rat heart or rat liver homogenates were incubated with either affinity-purified anti-GRIF-1-(8–633) antibodies or nonimmune Ig; immune complexes were precipitated by the addition of protein A-Sepharose; and the immune pellets were analyzed by immunoblotting using anti-GRIF-1-(8–633) (10% of immune pellet), anti-kinesin, anti-GRIF-1-(874–889), anti-KIF5A, KIF5B, and KIF5C (90% of immune pellets) antibodies all as described under "Experimental Procedures." The gel layout is identical for *A* and *B*. *Lanes 1* and *4*, detergent-solubilized heart or liver, respectively; *lanes 2* and *5*, nonimmune Ig pellet from heart and liver, respectively; *lanes 3* and *6*, anti-GRIF-1-(8–633) pellet from heart and liver, respectively. *A*, probed with anti-GRIF-1-(8–633); *B*, probed with anti-kinesin antibodies. The gel layout is identical in *C–G*. *Lane 1*, detergent-solubilized heart extract; *lane 2*, nonimmune Ig pellet; *lane 3*, anti-GRIF-1-(8–633) pellet. Antibodies used for immunoblotting are indicated in the *abscissae*. The positions of molecular mass standards (kDa) are shown on the *right*. The immunoblot is representative of $n = 3$ independent immunoprecipitations.

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-

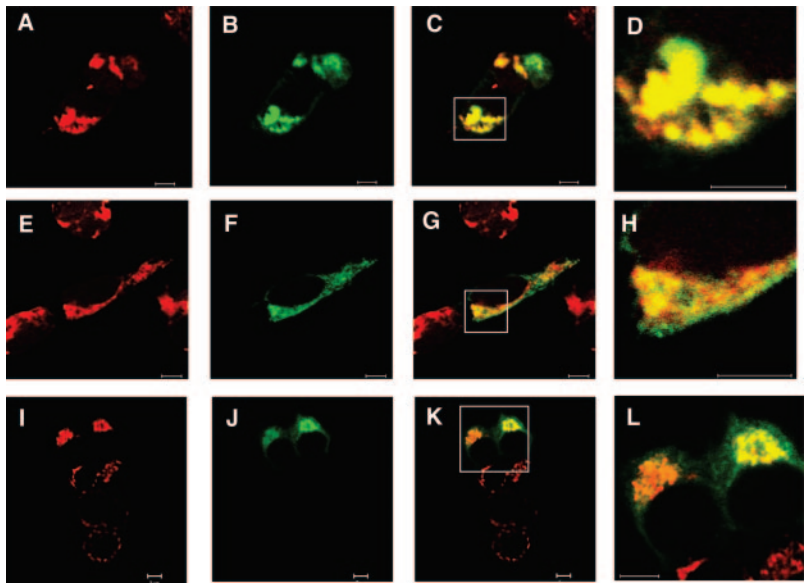

FIG. 8. **Localization of GRIF-1, OIP106, Milton, and mitochondria in HEK 293 cells transfected with pCISGRIF-1, pCISOIP106, and pCMVtag1*milton*.** HEK 293 cells adhered to poly-L-lysine-coated coverslips were transfected with the appropriate clones by the calcium phosphate method, incubated for 20 h, and stained with MitoTracker Red CMXRos and anti-GRIF-1-(8–633) or anti-αMilton 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-αMilton 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.

ized with anti-Milton immunoreactivity) (Fig. 8, *A–C*). OIP106-transfected cells had the same profile as those found for Milton in that the mitochondria were tightly clustered when compared with non-OIP106-expressing cells. Further, co-localization of OIP106 and mitochondria was clearly evident in all OIP106-transfected cells (Fig. 9, *G–I*). For GRIF-1, co-localization of GRIF-1 and mitochondria in transfected cells was not as evident. The expression of GRIF-1 compared with OIP106 was diffuse and extended throughout the cytoplasm, whereas OIP106 was always concentrated in areas of aggregated mitochondria. In some cells, mitochondria were aggregated and partially co-localized with GRIF-1; in other cells, minimal co-localization was observed. Representative results are shown in Fig. 9, *D–F*.

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 β_2 subunit-containing $GABA_A$ receptors, and its homology with other proteins such as huntingtin-associated protein (HAP-1). HAP-1 binds to the $p150^{Glued}$ 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 mechanochemical 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

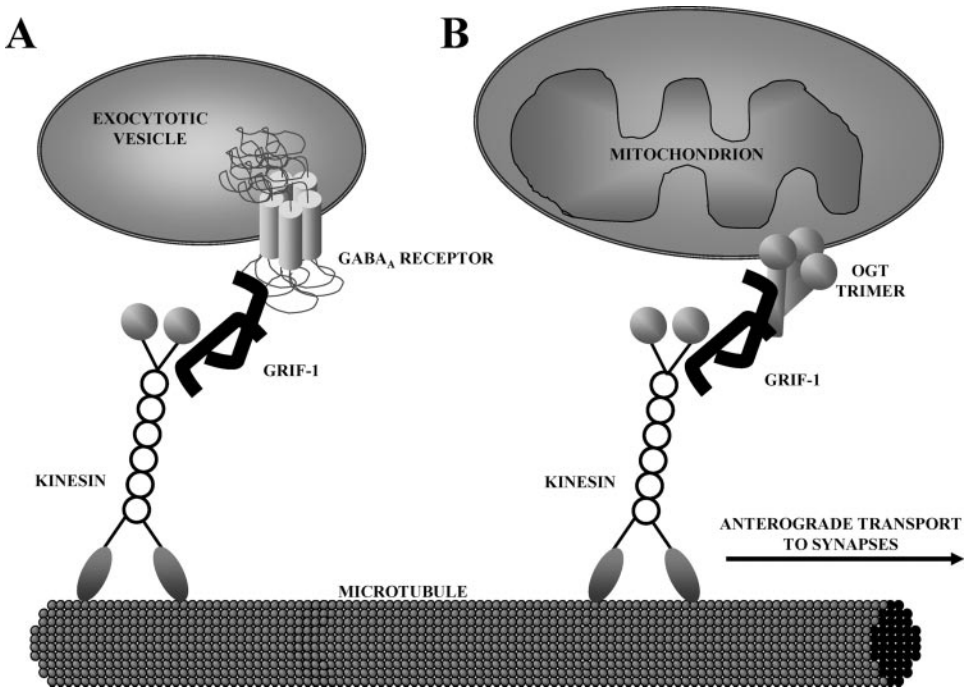

FIG. 9. **A schematic diagram depicting GRIF-1/kinesin-mediated anterograde transport of organelles in excitable tissue.** *A*, GRIF-1 is shown as forming a link between microtubules, a kinesin molecule, and a vesicle containing an assembled β_2 subunit-containing GABA$_A$ receptor being transported 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).

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 overexpression 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 to such a yeast intermediary protein, or, alternatively, 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$_A$ receptor β_2 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

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.

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