Mapping the GRIF-1 Binding Domain of the Kinesin, KIF5C, Substantiates a Role for GRIF-1 as an Adaptor Protein in the Anterograde Trafficking of Cargo**

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γ-Aminobutyric acid, type A (GABA A) receptor interacting factor-1 (GRIF-1) and N-acetylglucosamine transferase interacting protein (OIP) 106 are both members of a newly identified coiled-coil family of proteins. They are kinesin-associated proteins proposed to function as adaptors in the anterograde trafficking of organelles to synapses. Here we have studied in more detail the interaction between the prototypic kinesin heavy chain, KIF5C, kinesin light chain, and GRIF-1. The GRIF-1 binding site of KIF5C was mapped using truncation constructs in yeast two-hybrid interaction assays, co-immunoprecipitations, and co-localization studies following expression in mammalian cells. Using these approaches, it was shown that GRIF-1 and the KIF5C binding domain of GRIF-1, GRIF-1-(124–283), associated with the KIF5C non-motor domain. Refined studies using yeast two-hybrid interactions and co-immunoprecipitations showed that GRIF-1 and GRIF-1-(124–283) associated with the KIF5C non-motor domain. Substantiation that the GRIF-1-KIF5C interaction was direct was shown by fluorescence resonance energy transfer analyses using fluorescently tagged GRIF-1 and KIF5C constructs. A significant fluorescence resonance energy transfer value was found between the C-terminal EYFP-tagged KIF5C and ECFP-GRIF-1, the C-terminal EYFP-tagged KIF5C non-motor domain and ECFP-GRIF-1, but not between the N-terminal EYFP-tagged KIF5C nor the EYFP-KIF5C motor domain and ECFP-GRIF-1, thus confirming direct association between the two proteins at the KIF5C C-terminal and GRIF-1 N-terminal regions. Co-immunoprecipitation and confocal imaging strategies further showed that GRIF-1 can bind to the tetrameric kinesin light-chain/kinesin heavy-chain complex. These findings support a role for GRIF-1 as a kinesin adaptor molecule requisite for the anterograde delivery of defined cargoes such as mitochondria and/or vesicles incorporating β2 subunit-containing GABA A receptors, in the brain.

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γ-Aminobutyric acid, type A (GABA A) receptor interacting factor-1 (GRIF-1) was initially identified from rat brain by a yeast two-hybrid screen searching for GABA A receptor clustering and trafficking proteins (1). It was shown to associate at least in vitro with GABA A receptor β2 subunits. GRIF-1 is highly expressed in excitable tissue, most notably in the brain and in the heart (1). It is the orthologue of the human protein, β-O-linked N-acetylgalactosamine transferase (OGT) interacting protein 98 (OIP98, also termed ALS2CR3, huMilt2, or TRAK2), and it is the homologue of the protein OIP106 (also termed huMilt1 and TRAK1 (2)). GRIF-1 is also probably the orthologue of the Drosophila protein, Milton, a kinesin-associated protein that is involved in the transport of mitochondria to the synapses in retina (3, 4). GRIF-1 and OIP106 have also been shown to aggregate mitochondria following over-expression in mammalian cells (5). Recently, the gene encoding OIP106 (TRAK1), Trak1, was identified as the mutated gene in hyt mice, an animal model of hypertonia (6). hyt mice were shown to have a deficit of GABA A receptors; wild-type OIP106 (TRAK1) was shown to co-immunoprecipitate with GABA A receptor α1 subunits in extracts of mouse brain stem and spinal cord (6). Although the mutant OIP106 was shown to still associate with GABA A receptors, it was concluded that OIP106 (TRAK1) may play a crucial role in regulating endocytic trafficking of receptors and dysfunction disrupts receptor homeostasis leading to hypertonia (6). Thus, GRIF-1, OIP106, and Milton belong to a newly identified family of coiled-coil proteins putatively involved in the trafficking of organelles and GABA A receptors to synapses.

Two additional proteins that associate with high affinity with both GRIF-1 and OIP106 have been identified. These proteins are the enzyme, OGT (2), and, consistent with the role in trafficking, the molecular motor protein, kinesin (5). Kinesin was also shown to be immuno precipitated from detergent extracts of Drosophila heads with anti-Milton antibodies, thereby dem-

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**The abbreviations used are: GABA, γ-aminobutyric acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; APP, amyloid precursor protein; cfu, colony forming units; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GRIF-1, GABA A receptor interacting factor-1; GRIP1, glutamate-receptor-interacting protein 1; HAP-1, huntingtin-associated protein; HEN, human embryonic kidney; KHC, kinesin heavy chain; IL, intracellular loop; KIF, kinesin superfamily protein; KLC, kinesin light chain; N-methyl-D-aspartate, N-methyl-D-aspartate; OGT, O-GlcNAc transferase; OIP, OGT interacting protein; JNK, c-Jun N-terminal kinase; CMV, cytomegalovirus; AD, activation domain; SD, synthetic defined media.

5 K. Pozo, K. Brickley, M. Beck, and F. A. Stephenson, unpublished observations.
Kinesins belong to the kinesin superfamily, i.e. KIFs. KIFs are microtubule-based mechanoochemical motors that are involved primarily in the anterograde transport of organelles and protein complexes (reviewed in Refs. 7 and 8). They are particularly important in transport processes in neurons where it is necessary to deliver defined cargoes from the cell body along axons and dendrites to pre- and post-synaptic sites. In brain, GRIF-1 associates predominantly with KIF5A, whereas in heart association is predominantly with KIF5B (5). Following overexpression in human embryonic kidney (HEK) 293 cells, GRIF-1 will co-associate with exogenous KIF5C (5). KIF5A, KIF5B, and KIF5C are members of the kinesin-1 family. KIF5B is ubiquitously expressed, whereas KIF5A and KIF5C are only expressed in neurons (7). They are conventional kinesin heavy chains belonging to the kinesin-1 family, and they share at least 60% amino acid identity. It is probable that each has a binding site for GRIF-1 and that GRIF-1 is promiscuous with regard to association with KIF5 subtypes.

Kinesin-1 proteins are tetramers formed by the association of two kinesin heavy chains (KHC or alternatively KIF5) and two kinesin light chains (KLC). The KHC is formed from an N-terminal motor domain that contains the microtubule and ATP binding sites and a C-terminal non-motor domain. This domain includes a neck, a coiled-coil stalk region, and a cargo binding site in its C-terminal region. The KLC interacts with the KHC via the stalk region. Cargoes bind to either the KLC or to the KHC of kinesin-1 proteins. Increasing evidence suggests that this interaction is mediated by an adaptor protein. For example, mitochondria and syntaxin-1-containing vesicles are attached to the KHC cargo binding domain by the adaptor protein, syntabulin, for their transport to synapses (9); JIP-3, a c-Jun N-terminal kinase (JNK) signaling pathway protein, binds to a six tetrapeptide motif in KLCs to transport the cargo, amyloid precursor protein (APP; 10); KIF17 forms a complex with mLin10 in the transportation of N-methyl-D-aspartate receptor NR2B subunits to the synapse (11, 12); and glutamate receptor interacting protein 1 (GRIP1) is an adaptor protein linking the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) subtype of glutamate receptor containing vesicles to KIF5 (13).

In this report, we have studied the interaction between GRIF-1 and the prototypic kinesin-1, KIF5C, and kinesin light chain. The results obtained provide supporting evidence that GRIF-1 is a kinesin adaptor protein involved in motor-dependent trafficking of organelles and/or proteins. Please note a preliminary report of some of these findings (14).

**EXPERIMENTAL PROCEDURES**

**Constructs and Antibodies**—pCISGRIF-1, splice form GRIF-1a, hereafter referred to as GRIF-1, pCMVTag4aGRIF-1 (C-terminal FLAG-tagged GRIF-1), pGADT7GRIF-1 (1–913), pGADT7GRIF-1-(8–633), pGADT7GRIF-1-(124–283), and affinity-purified rabbit anti-GRIF-1 (837–889) antibodies were as previously described (1). pMBL33 GABAA receptor β2 intracellular loop (303–427; IL), pcDNAHisMaxKIF5C, and affinity-purified sheep anti-GRIF-1 (874–899) were as described in Bickley et al. (5). The kinesin heavy-chain KIF5C fragments, corresponding to the motor domain (1–335), the non-motor domain (336–957), coil 1 (336–542), coil 2 (594–804), and the cargo binding domain, coil 3 (827–957), were PCR-amplified from the construct, pBluescriptK11I/KIAA0531 and cloned in-frame into the EcoR1 and Sall restriction sites of the modified mammalian expression vector, pCMVTag4a, to generate the constructs pCMV-KIF5C-(1–335), pCMV-KIF5C-(336–957), pCMV-KIF5C-(336–542), pCMV-KIF5C-(593–804), and pCMV-KIF5C-(827–957) each with an N-terminal c-Myc tag. The same fragments were also cloned into the EcoR1 and Sall restriction sites of the DNA binding domain yeast expression vector, pMBL33, to generate the constructs, pMBL33-KIF5C-(1–335), pMBL33-KIF5C-(336–957), pMBL33-KIF5C-(336–542), pMBL33-KIF5C-(593–804), and pMBL33-KIF5C-(827–957). The kinesin light chain was amplified by PCR from a kinesin light chain cDNA in the pHA vector and subcloned in-frame into pMBL33 using EcoR1/Sall and into pCMVTag4a using EcoR1/Sall to generate an N-terminal c-Myc-tagged KFC. Full-length GRIF-1 was amplified from pCISGRIF-1 by PCR and subcloned in-frame in the EcoR1 and Sall restriction sites of pECFP-C1 (BD Biosciences, Clontech, Palo Alto, CA) to generate pECFP-GRIF-1, i.e. GRIF-1 with an N-terminal enhanced cyan fluorescent protein (ECFP) tag. KIF5C was amplified from pcDNAHisMaxKIF5C and subcloned into either the EcoR1 and Sall restriction sites of pEYFP-C1 (BD Biosciences, Clontech) to generate pEYFP-KIF5C, i.e. KIF5C with an N-terminal tag, or into the Nhel site of pEYFP to generate pKIF5C-enhanced yellow fluorescent protein (EYFP), i.e. KIF5C with a C-terminal EYFP tag. The KIF5C motor domain, KIF5C-(1–335), and KIF5C non-motor domain, KIF5C-(336–957), were amplified by PCR from pcDNAHisMaxKIF5C. KIF5C-(1–335) was subcloned in-frame in the XhoI/EcoR1 sites and KIF5C-(336–957) in the Nhel site, respectively, of pEYFP to generate pEYFP-KIF5C-(1–335) with an EYFP tag at the N terminus and pEYFP-KIF5C-(336–957) with an EYFP tag at the C-terminal end of KIF5C. All constructs were verified by DNA sequencing (MWG-Biotech AG, Ebersberg, Germany). Further, for all constructs used in yeast two-hybrid interaction assays, the expression of fusion proteins was verified by immunoblotting (data not shown). A schematic of all constructs used is shown in Fig. 1.

Anti-GFP and anti-KIF5C338957 antibodies were from Abcam Ltd. (Cambridge, UK); anti-c-Myc clone 4A6 antibodies were from Upstate (Charlottesville, VA); and anti-mouse-Ig Alexa Fluor 633 antibodies from Invitrogen. Anti-FLAG antibodies were raised in-house against the FLAG amino acid sequence, DYKDDDDK, with an N-terminal cysteine coupled to thyroglobulin and affinity-purified for use with CDYKDDDDK covalently coupled via the cysteine to thiopropyl-activated Sepharose.

**Mammalian Cell Transfection and Preparation of Detergent-solubilized Extracts of Transfected Cells**—For immunoprecipitation assays, HEK 293 cells were transfected with combinations of FLAG-tagged pCMVGRIF-1 (1–913) together with c-Myc-tagged pCMV-KIF5C-(1–335), pCMV-KIF5C-(336–957), pCMV-KIF5C-(336–542), pCMV-KIF5C-(593–804), or pCMV-KIF5C-(827–957) using the calcium phosphate method using a 1:1 ratio with a total of 10 μg of DNA per...
250-ml culture flask. In pCISGRIF-1, pEYFP-KIF5C, and pCM-VKLC triple transfections, a ratio of 1:1:3 was used with a total of 10 μg of DNA per 250-ml culture flask. For the characterization of the ECFP- and EYFP-tagged proteins, HEK 293 cells were transfected with either pCISGRIF-1 plus pEYFP-KIF5C; pCISGRIF-1 plus pKIF5C-EYFP; pCISGRIF-1 plus pEYFP-KIF5C-(336–957); pCISGRIF-1 plus pEYFP-KIF5C-(1–335); or pcDNAHismaxKIF5C plus pECFP-GRIF-1 and pECFP-GRIF-1 plus pKIF5C-EYFP all using a 1:1 ratio with a total of 10 μg of DNA per 250-ml culture flask. Cells were harvested 24–48 h post-transfection, and cell homogenates were either analyzed by immunoblotting or, alternatively, transfected cell homogenates were solubilized with 10 mM HEPES, pH 7.5, 145 mM NaCl, 1 mM EGTA, 0.1 mM MgCl₂, 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 following centrifugation for 40 min at 4 °C at 100,000 g (6). For confocal microscopy studies, HEK 293 cells or COS-7 cells were plated onto poly-D-lysine (0.1 mg/ml)-coated coverslips and transfected using the calcium phosphate method and plasmid DNA ratios as above. Transfections using pDsRed1-Mito to visualize mitochondria used a ratio of 1 pDsRed1-Mito:19 ECFP-GRIF-1 for double transfections, and 1 pDsRed1-Mito:8 ECFP-GRIF-1:8 EYFP-KIF5C for triples. Immunoblotting—Immunoblotting was performed as previously described using 25–50 μg of protein/sample precipitated.

**TABLE 1**

| AD vector | DNA-BD vector | Growth on − W and − L dropout media | Growth on − W, − L, and − H dropout media |
|-----------|---------------|-------------------------------------|------------------------------------------|
| pGADTr1GRIF-1-(1–913) | pMBL33KIF5C-(1–335) | ++ | ++ |
| pGADTr1GRIF-1-(1–913) | pMBL33KIF5C-(336–957) | ++ | ++ |
| pGADTr1GRIF-1-(8–633) | pMBL33KIF5C-(1–335) | ++ | ++ |
| pGADTr1GRIF-1-(8–633) | pMBL33KIF5C-(336–957) | ++ | ++ |
| pGADTr1GRIF-1-(124–283) | pMBL33KIF5C-(336–957) | ++ | ++ |
| pGADTr1GRIF-1-(124–283) | pMBL33KLC-(1–569) | ++ | ++ |
| pGADTr7 | pMBL33KIF5C-(1–335) | ++ | ++ |
| pGADTr7 | pMBL33KIF5C-(336–957) | ++ | ++ |
| pGADTr7 | pMBL33KLC-(1–569) | ++ | ++ |
| pGADTr7 | pMBL33KLC-(336–957) | ++ | ++ |
| pGADTr7 | pMBL33S2-IL | ++ | ++ |
| pGADTr1GRIF-1-(1–913) | pMBL33 | ++ | ++ |
| pGADTr1GRIF-1-(8–633) | pMBL33 | ++ | ++ |
| pGADTr1GRIF-1-(124–283) | pMBL33 | ++ | ++ |
| pGADTr7 | pMBL33 | ++ | ++ |
| pGADTr7 | pMBL33S2-IL | ++ | ++ |
| pGADTr1GRIF-1-(1–913) | pMBL33 | ++ | ++ |
| pGADTr1GRIF-1-(8–633) | pMBL33 | ++ | ++ |
| pGADTr1GRIF-1-(124–283) | pMBL33 | ++ | ++ |
| pGADTr7 | pMBL33 | ++ | ++ |
| pGAD10GRIF-1-(8–633) | pMBL33 | ++ | ++ |

* For colony growth, – = 0 cfu; + = 10–100 cfu; ++ = 100–200 cfu; +++ = 200 cfu. pGAD10GRIF-1-(8–633) and pMBL33S2-IL served as the positive control. The results are representative of n = 3 independent co-transformations.

**FIGURE 1.** A schematic diagram showing the kinesin and GRIF-1 constructs used in this study. A, KIF5C and kinesin light chain constructs used for the yeast two-hybrid and immunoprecipitation studies; B, KIF5C-EYFP constructs used for the imaging and FRET studies; C, GRIF-1 constructs used for the imaging and FRET studies depicting also, the kinesin binding domain, GRIF-1-(124–283); D, a summary of the nomenclatures in the literature for the two proteins encoded by the GRIF-1 coiled-coil gene family.
using the chloroform/methanol method and SDS-PAGE under reducing conditions in either 7.5% or 12.5% polyacrylamide slab minigels (1, 5). 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 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) were incubated for polyclonal antibodies, with either affinity-puriﬁed rabbit anti-FLAG antibodies (10 µg), protein A-puriﬁed non-immune rabbit Ig (10 µg) as control or, affinity-puriﬁed sheep anti-GRIF-1874–889 antibodies (10 µg), protein G-puriﬁed non-immune sheep Ig as control for 1.5 h at 37 °C (6). Protein A (rabbit polyclonal antibodies) or protein G (sheep polyclonal antibodies) Sepharose (20 µl) was added, and samples were incubated for 1 h at 37 °C. Immune pellets were collected by centrifugation for 10 min at 10,000 g. ECL Western blotting system.

**Yeast Two-hybrid Interaction Assays**—Yeast two-hybrid assays were carried out using a modiﬁed LexA system as described previously (5). The Saccharomyces cerevisiae strain, L40 (MATa his3Δ200 trpl-901 leu2–3112 ade2 lys2–1(4lexAop-HIS3) URA3::(4lexAop-lacZ) GAL4), was transformed with combinations of GRIF-1 fragments in the activation domain (AD) plasmid, pGADT7, together with each of the KIF5C fragments in the DNA binding domain plasmid, pMBL33. Negative control transformations were carried out using empty AD or DNA binding domain vectors. Positive control transformations were carried out using AD-GRIF-1-(8–633) with pMBL33β2-IL, i.e. the DNA binding domain vector containing the residues 303–427 of the intracellular loop of the GABA_A receptor β2 subunit. Resulting colonies were assessed for reporter gene activation by growth on nutritional selection agar lacking tryptophan, leucine, and histidine.

**Confocal Microscopy and FRET Efﬁciency Determinations**—Transfected cells 20–40 h post-transfection were rinsed three times with ice-cold phosphate-buffered saline followed by ﬁxation for 10 min with 4% (v/v) ice-cold paraformaldehyde. Coverslips were rinsed three times with phosphate-buffered saline and mounted onto a microscope slide using 10 µl of mounting solution containing an anti-fading agent (Citifluor, Citifluor Ltd., Leicester, UK). The coverslips were sealed and kept at 4 °C until analysis. For visualization of cells transfected with pCMVKLC, post-ﬁxation coverslips were incubated with anti-c-Myc antibodies (1:4000) overnight at 37 °C, washed as described (1), and incubated with anti-mouse-Ig Alexa Fluor 633, and again washed as previously described (1). Cells were imaged using an inverted LSM510 META Zeiss confocal microscope in the multitrack mode. EYFP was excited with the λ = 514 nm laser line, and emitted light was collected with a long pass ﬁlter LP530. ECFP was excited at λ = 458 nm, and light was collected with a band-pass ﬁlter BP 475–525. The absence of bleed-through was checked prior to each experiment by using the Meta software of the microscope. This software enables the decomposition of the mixed emission spectra (i.e. generated from cells co-transfected with both ECFP and EYFP) into the emission of the single ﬂuorescent dyes, i.e ECFP and EYFP, according to reference spectra generated from cells transfected with either ECFP or EYFP alone. Images were analyzed using the Image Browser software (Zeiss) available with the microscope.

For FRET analyses, again the Meta software mode of the LSM510 META Zeiss confocal microscope was used. FRET efﬁciency was measured by acceptor photobleaching as described previously by Liu et al. (15) and Nashmi et al. (16).

That is, co-transfected HEK 293 cells were firstly imaged with the λ = 458 nm laser to visualize ECFP-tagged proteins. A deﬁned area of co-localization within one cell was selected, and EYFP was photobleached for ~30 s using the λ = 514 nm laser at full power. Cells were imaged post-photobleaching with the λ = 458 nm laser. FRET was measured as the increase of ECFP ﬂuorescence after photobleaching where values were taken at t = 1.6 s prior to photobleaching and t = 32 s post-photobleaching and were corrected for background ﬂuorescence (usually of the order of ~10%) determined by the imaging of untransfected HEK 293 cells. The relative FRET efﬁciency was calculated by the following: 1 – [pre-bleached intensity of ECFP/
Mapping of the GRIF-1 Binding Domain of KIF5C

TABLE 2

| AD vector         | DNA-BD vector | Growth on −W and −L dropout media | Growth on −W, −L, and −H dropout media |
|-------------------|---------------|-----------------------------------|----------------------------------------|
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(336–542) | ++ +                               | -                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(593–804) | +                                  | -                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(827–957) | -                                  | -                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(827–957) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(124–283) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(336–542) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(593–804) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(827–957) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(124–283) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(336–542) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(593–804) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(827–957) | +                                  | +                                       |
| pGADT7GRIF-1-(1–913) | pMBL33KIF5C-(124–283) | +                                  | +                                       |

* For colony growth, − = 0 cfu; + = 10–100 cfu; ++ = 100–200 cfu; +++ = 200 cfu. As for Table 1, pGAD10GRIF-1-(8–633) and pMBL33B2-1L served as the positive control.

The results are representative of at least n = 3 independent co-transformations.

post-bleached intensity of ECFP]) × 100% (14). For each FRET experiment, pseudo-FRET was always determined by applying the photobleaching protocol to cells transfected with pECFP alone. Pseudo-FRET values (typically ~2.9%) were always subtracted from the calculated FRET efficiencies of the test samples. As a further control, an area of the cell that was not photobleached was also analyzed in parallel for FRET to ensure that the FRET efficiency values determined were not artifactual due to the photobleaching protocol. A positive control, an ECFP-EYFP tandem construct linked by two amino acids (17), and a negative control, co-transfection of the separate clones pECFP and pEYFP, were both used to validate the system.

RESULTS

**GRIF-1 Associates with the KIF5C Non-motor Domain: Demonstration by Both Yeast Two-hybrid Interaction Assays and Immunoprecipitation Strategies**

We have previously shown that GRIF-1 associates with endogenous kinesin in the brain and in the heart by co-immunoprecipitation of GRIF-1 and kinesin using anti-GRIF-1(874–889) antibodies. Association was also demonstrated, again by co-immunoprecipitation, between exogenous GRIF-1 and exogenous KIF5C both co-expressed in HEK 293 cells. Further, yeast two-hybrid interaction assays using GRIF-1 and KIF5C as fish and bait, respectively, suggested that association between the two proteins is probably direct and involves the first coiled-coil domain of GRIF-1, GRIF-1-(124–283) (5). To delineate the GRIF-1 binding domain of KIF5C initially, the KIF5C motor domain, i.e. KIF5C-(1–335), and KIF5C non-motor domain, i.e. KIF5C-(336–957), were subcloned in-frame into either the yeast two-hybrid DNA binding domain vector, pMBL33, or the modified mammalian expression vector, pCMVTag4a, to yield N-terminal c-Myc-tagged fusion proteins. The association between GRIF-1 and KIF5C was then studied by both yeast two-hybrid interaction assays and immunoprecipitation strategies following the co-expression of GRIF-1 and KIF5C truncation constructs in HEK 293 cells.

Co-transformation of yeast with pGADT7GRIF-1-(1–913) and pCMV-KIF5C-(336–957) revealed a significant interaction between the two proteins as determined by nutritional selection on SD media −W, −L, and −H media (Table 1). An
Mapping of the GRIF-1 Binding Domain of KIF5C

Characterization of ECFP-GRIF-1 and EYFP-KIF5C Constructs—The following in-frame constructs were generated by molecular cloning: full-length N- and C-terminally tagged KIF5C-EYFP, an N-terminal EYFP-tagged KIF5C-(1–335) motor domain, a C-terminal EYFP-tagged KIF5C-(336–957) non-motor domain, and an N-terminal ECFP-GRIF-1 (Fig. 1). Each was characterized with respect to molecular size by immunoblotting following expression in HEK 293 cells using anti-GFP antibodies and anti-GRIF-1 or anti-KIF5C antibodies. This was to ensure that the respective EYFP- or ECFP-tagged proteins did not undergo proteolytic digestion, thus the fluorescent moieties remained attached to the target proteins. The results for the full-length KIF5C constructs and GRIF-1 are shown in Fig. 4 and for the KIF5C-(1–335) motor domain and KIF5C-(336–957) non-motor domains in Fig. 5. In all cases, an increase in molecular weight for the tagged proteins was observed consistent with the addition of ECFP or EYFP. Further, a single band was found for all constructs in immunoblots using anti-GFP antibodies. The molecular masses of the tagged proteins were: GRIF-1, 115 kDa; ECFP-GRIF-1, 143 kDa; KIF5C, 115 kDa; EYFP-KIF5C, 143 kDa; c-Myc-tagged KIF5C-(1–335), 39 kDa; EYFP-KIF5C-(1–335), 66 kDa; c-Myc-tagged KIF5C-(336–957), 98 kDa; and EYFP-KIF5C-(336–957), 139 kDa.

It was also requisite to demonstrate that the tagged proteins interaction was also found between both GRIF-1-(8–633) and GRIF-1-(124–283) and KIF5C-(336–957). This was in contrast to yeast co-transformed with KIF5C-(1–335) and either full-length or truncated GRIF-1 constructs where no evidence for protein-protein association was found (Table 1). The same results were obtained in immunoprecipitation studies. FLAG-tagged GRIF-1 was co-expressed in HEK 293 cells with either c-Myc-tagged KIF5C-(1–335) (43 kDa) or KIF5C-(336–957) (98 kDa), detergent extracts prepared and immunoprecipitation carried out using anti-FLAG antibodies. In each case, anti-FLAG immunoreactivity with ~114 kDa was detected in the immune pellets (Fig. 2). Anti-c-Myc antibody immunoreactivity was, however, only present for GRIF-1/KIF5C-(336–957) transfectants further demonstrating an association between GRIF-1 and the C-terminal KIF5C non-motor domain.

The above experiments were also repeated for KLC (70 kDa). No association between GRIF-1 and KLC was detected by either yeast two-hybrid or co-immunoprecipitation assays (Table 1 and Fig. 2).

GRIF-1 Associates with the KIF5C C3 Cargo Binding Domain: Demonstration by Both Yeast Two-hybrid Interaction Assays and Immunoprecipitation Strategies

The GRIF-1 binding domain was further refined by generating three truncation constructs, KIF5C-(336–542), KIF5C-(336–957), and KIF5C-(593–804), and KIF5C-(827–957), of the KIF5C non-motor domain in both yeast DNA binding domain and mammalian expression vectors. These corresponded to three predicted coiled-coil domains in the non-motor domain where KIF5C-(336–542) and KIF5C-(593–804) encompassed the KIF5C stalk region and KIF5C-(827–957) the cargo binding domain. All three constructs were used, as above, in both yeast two-hybrid interaction assays and immunoprecipitation experiments following the co-expression of GRIF-1 and KIF5C-(336–542) (37 kDa), KIF5C-(593–804) (31 kDa), or KIF5C-(827–957) (20 kDa) truncated constructs in HEK 293 cells. The results are summarized in Table 2 and Fig. 3. Both experimental paradigms demonstrate that it is only the KIF5C-(827–957) cargo binding domain that associates with GRIF-1. This domain associates with full-length GRIF-1 and the GRIF-1-(8–633) and GRIF-1-(124–283) fragments.

Confocal Microscopy Studies of the Association between GRIF-1 and KIF5C

The GRIF-1-(124–283) and KIF5C-(336–957) constructs further demonstrated an association between GRIF-1 and the C-terminal KIF5C non-motor domain.

The above experiments were also repeated for KLC (70 kDa). No association between GRIF-1 and KLC was detected by either yeast two-hybrid or co-immunoprecipitation assays (Table 1 and Fig. 2).

GRIF-1 Associates with the KIF5C C3 Cargo Binding Domain: Demonstration by Both Yeast Two-hybrid Interaction Assays and Immunoprecipitation Strategies

The GRIF-1 binding domain was further refined by generating three truncation constructs, KIF5C-(336–542), KIF5C-(336–957), and KIF5C-(593–804), and KIF5C-(827–957), of the KIF5C non-motor domain in both yeast DNA binding domain and mammalian expression vectors. These corresponded to three predicted coiled-coil domains in the non-motor domain where KIF5C-(336–542) and KIF5C-(593–804) encompassed the KIF5C stalk region and KIF5C-(827–957) the cargo binding domain. All three constructs were used, as above, in both yeast two-hybrid interaction assays and immunoprecipitation experiments following the co-expression of GRIF-1 and KIF5C-(336–542) (37 kDa), KIF5C-(593–804) (31 kDa), or KIF5C-(827–957) (20 kDa) truncated constructs in HEK 293 cells. The results are summarized in Table 2 and Fig. 3. Both experimental paradigms demonstrate that it is only the KIF5C-(827–957) cargo binding domain that associates with GRIF-1. This domain associates with full-length GRIF-1 and the GRIF-1-(8–633) and GRIF-1-(124–283) fragments.

Confocal Microscopy Studies of the Association between GRIF-1 and KIF5C

Characterization of ECFP-GRIF-1 and EYFP-KIF5C Constructs—The following in-frame constructs were generated by molecular cloning: full-length N- and C-terminally tagged KIF5C-EYFP, an N-terminal EYFP-tagged KIF5C-(1–335) motor domain, a C-terminal EYFP-tagged KIF5C-(336–957) non-motor domain, and an N-terminal ECFP-GRIF-1 (Fig. 1). Each was characterized with respect to molecular size by immunoblotting following expression in HEK 293 cells using anti-GFP antibodies and anti-GRIF-1 or anti-KIF5C antibodies. This was to ensure that the respective EYFP- or ECFP-tagged proteins did not undergo proteolytic digestion, thus the fluorescent moieties remained attached to the target proteins. The results for the full-length KIF5C constructs and GRIF-1 are shown in Fig. 4 and for the KIF5C-(1–335) motor domain and KIF5C-(336–957) non-motor domains in Fig. 5. In all cases, an increase in molecular weight for the tagged proteins was observed consistent with the addition of ECFP or EYFP. Further, a single band was found for all constructs in immunoblots using anti-GFP antibodies. The molecular masses of the tagged proteins were: GRIF-1, 115 kDa; ECFP-GRIF-1, 143 kDa; KIF5C, 115 kDa; EYFP-KIF5C, 143 kDa; c-Myc-tagged KIF5C-(1–335), 39 kDa; EYFP-KIF5C-(1–335), 66 kDa; c-Myc-tagged KIF5C-(336–957), 98 kDa; and EYFP-KIF5C-(336–957), 139 kDa.

It was also requisite to demonstrate that the tagged proteins
Mapping of the GRIF-1 Binding Domain of KIF5C

FIGURE 5. Characterization of EYFP-KIF5C-(1–335) and EYFP-KIF5C-(336–957): molecular size determination and co-association properties. In A and B, HEK 293 cells were transfected with either pCMV-KIF5C-(1–335), pEYFP-KIF5C-(1–335), pCMV-KIF5C-(336–957), or pEYFP-KIF5C-(336–957), cell homogenates were prepared 24 h post-transfection, and samples were analyzed by immunoblotting with antibody specificities as shown in the abscissae. Lanes are: 1 and 4, untransfected cells; 2 and 5, cells transfected with pCMV-KIF5C-(1–335); 3 and 6, cells transfected with pEYFP-KIF5C-(336–957). Lanes are: 1 and 4, untransfected cells; 2 and 5, cells transfected with pCMV-KIF5C-(336–957); and 3 and 6, cells transfected with pEYFP-KIF5C-(336–957). Immunoblotting and co-association properties.

 Behaved as wild type. This was demonstrated here by the ability of the tagged protein to co-associate with the non-tagged or tagged binding partner. Thus the following pairwise combinations were co-expressed in HEK 293 cells, immunoprecipitations carried out with anti-GRIF-1874–889 antibodies, and tagged non-immune Ig and immune pellets analyzed by immunoprecipitations of N-terminal-tagged KIF5C and c-Myc-tagged KIF5C-(1–335), 39 kDa; EYFP-KIF5C-(1–335), 66 kDa; KIF5C-EYFP, are not shown, i.e. KIF5C-(1–335) was associated with filamentous structures thought to be microtubules, consistent with the existence of a microtubule binding site within the KIF5C-(1–335) (supplemental Fig. S2). This distribution pattern was not changed by co-expression of EYFP-KIF5C-(1–335) with ECFP-GRIF-1 (Fig. 7, E–G). EYFP-KIF5C-(336–957) expressed alone was localized as filamentous structures in the cell cytoplasm (Fig. 7C). This localization pattern is similar to that reported for the expression of GRIF-1 in HEK 293 cells (1) where the enriched areas were shown to co-localize with aggregated mitochondria (5). EYFP-KIF5C and KIF5C-EYFP expressed alone in COS-7 cells showed the same distribution profile with fluorescence visible throughout the cell cytoplasm, and, when they are present, EYFP-KIF5C was concentrated at the ends of cellular extensions (Fig. 6E). When ECFP-GRIF-1 plus EYFP-KIF5C or KIF5C-EYFP was co-expressed, the distribution of ECFP-GRIF-1 was changed. In all cells imaged, the majority of ECFP-GRIF-1 was recruited to KIF5C-enriched areas, which were particularly apparent at the end of cellular extensions. In cells where no processes were evident, co-localization was seen adjacent to the cell membrane (supplemental Fig. S1). In some co-transfected cells (19 out of 35 cells; 54%), some ECFP-GRIF-1 was still found in the cell cytoplasm where it was not associated with EYFP-KIF5C (Fig. 6, G and H). This may be a result of over-expression of ECFP-GRIF-1 in those particular cells. Fig. 6 also shows the distribution of mitochondria in COS-7 cells transfected with either ECFP-GRIF-1 (L–P), ECFP-KIF5C (Q–U), or ECFP-GRIF-1 plus EYFP-KIF5C (V–AA). Aggregated mitochondria are only seen in cells when ECFP-GRIF-1 was overexpressed; i.e. compare Fig. 6N (ECFP-GRIF-1 transfectant) with Fig. 6S (EYFP-KIF5C transfectant).

EYFP-KIF5C-(1–335) expressed alone showed a diffuse distribution throughout the whole cell, including in 55% of transfected cells (16 of 29 cells), and the cell nucleus (Fig. 7B). In addition, in ~24% of transfected cells (7 of 29 cells), KIF5C-(1–335) was associated with filamentous structures thought to be the microtubules, consistent with the existence of a microtubule binding site within the KIF5C-(1–335) (supplemental Fig. S2). This distribution pattern was not changed by co-expression of EYFP-KIF5C-(1–335) with ECFP-GRIF-1 (Fig. 7, E–G). EYFP-KIF5C-(336–957) expressed alone was localized as filamentous structures in the cell cytoplasm (Fig. 7F). This distribution was similar to that reported by Navone et al. (18) following overexpression in CV-1 monkey kidney endothelial cells of a vesicular stomatitis virus-tagged KHC construct that contained the KHC C-terminal portion of the α-helical coiled-coil rod and the C-terminal tail. The filamentous labeling found by Navone et al. (18) co-localized with microtubules and a micro-
tubule binding site within the non-motor domain was identified (18). In the presence of ECFP-GRIF-1 the distribution pattern of EYFP-KIF5C-(336–957) was changed. It was always co-localized with ECFP-GRIF-1-rich regions close to the nucleus (Fig. 7, M and O). This pattern is reminiscent of the localization of GRIF-1 with aggregated mitochondria as reported for GRIF-1-transfected HEK 293 cells (5). Indeed, when COS-7 cells were transfected with pECFP-GRIF-1,
pEYFP-KIF5C-(336–957), and pDsRed1Mito to label mitochondria, all three fluorescent moieties were co-localized close to the cell nucleus (Fig. 7, Q–V).

**FRET Measurements**—To test whether ECFP-GRIF-1 associated directly with fluorescently tagged KIF5C, FRET studies were carried out in transfected HEK 293 cells. FRET efficiencies were measured by acceptor photobleaching. It was necessary to carry out these measurements in HEK 293 cells rather than COS-7 cells, because in the latter, the expression level of the fluorescent constructs was relatively low thus exposure to the appropriate laser for cell imaging resulted in the photobleaching of either EYFP or ECFP. In HEK 293 cells, the expression of the constructs was higher due to both a higher transfection efficiency and greater protein expression thus circumventing problems due to the photobleaching. Note that similar subcellular distribution profiles were found for the expression of single and pairwise GRIF-1/KIF5C combinations for both COS-7 cells and HEK 293 cells, *i.e.* GRIF-1/KIF5C and GRIF-1/KIF5C-(336–957) both co-localized.

In initial studies, optimum conditions were established for the measurement of FRET efficiency by acceptor photobleaching.
using positive and negative ECFP/EYFP controls (Fig. 8). A FRET efficiency of 24.6% ± 2.0 (n = 22 cells) was determined for the negative control (ECFP-EYPF tandem construct) and a FRET efficiency of 3.8% ± 0.5 (n = 18 cells) was measured for the positive control (pECFP and pEYPF). The percentages of FRET efficiency for the GRIF-1/KIF5C constructs were: 5.6% ± 0.5 for ECFP-GRIF-1/EYFP-KIF5C (n = 15 cells); 10.3% ± 0.7 for ECFP-GRIF-1/KIF5C-EYFP (n = 20 cells); 4.0% ± 0.4 for ECFP-GRIF-1/EYFP-KIF5C-(1–335) (n = 11 cells); and 12.5% ± 0.5 for ECFP-GRIF-1/EYFP-KIF5C-(336–957) (n = 16 cells). Thus, a significant FRET value was only found for the ECFP-GRIF-1/KIF5C-EYFP and ECFP-GRIF-1/EYFP-KIF5C-(336–957) pairs showing a direct association between GRIF-1 and the CT-terminal, non-motor domain of the kinesin-1 molecule, KIF5C.

**GRIF-1, KIF5C, and KLC Interactions**

To investigate whether GRIF-1 associates with the assembled heavy chain plus light chain tetrameric kinesin complex, immunoprecipitation and confocal imaging experiments were carried out on cells co-transfected with GRIF-1, KIF5C, and KLC clones. The results are shown in Fig. 9.

**Co-immunoprecipitation Studies**—In the following experiments, it was necessary to use the EYFP-tagged form of KIF5C, because pcDNAHisMaxKIF5C generates a His-tagged protein that is recognized by anti-GRIF-18–633 antibodies (anti-GRIF-18–633 antibodies were raised to poly-His tagged GRIF-18–633 (5)). In GRIF-1/EYFP-KIF5C double transfectants, GRIF-1 and EYFP-KIF5C co-immunoprecipitated (Fig. 4E); in KIF5C and KLC double transfectants, KIF5C and KLC co-immunoprecipitated (Fig. 9B), but in GRIF-1 and KLC double transfectants, no association between the two proteins was detected (Figs. 1E, 1F, and 9A). When HEK 293 cells were transfected with GRIF-1 plus EYFP-KIF5C plus KLC clones, detergent extracts were prepared and samples were immunoprecipitated with anti-GRIF-18–633 antibodies, and GRIF-1, KIF5C, and KLC immunoreactivities were then all found in the test but not control pellets (Fig. 9C). Because KLC did not associate with GRIF-1 in the absence of KIF5C, this must mean that GRIF-1 associates with the KHC, KLC tetrameric complex.

**Co-localization Studies**—The same combinations of clones as described for the co-immunoprecipitation studies were used for the transfection of COS-7 cells. Representative confocal microscopy images are shown in Fig. 9. Points to note are: (i) overexpression of GRIF-1 did not change the distribution pattern of KLC and no areas of co-localization were found (Fig. 9, D–H); (ii) EYFP-KIF5C and KLC mostly co-localized within the cell cytoplasm (Fig. 9, I–M); (iii) it was noted that in the majority of the EYFP-KIF5C plus KLC double transfectants, no fluorescence was detected at the tips of cell extensions such as seen in Fig. 6I; (iv) in the triple transfections, co-localization of EYFP-KIF5C, KLC, and ECFP-GRIF-1 was seen both in the cell cytoplasm and later, as for both EYFP-KIF5C alone and EYFP-KIF5C plus ECFP-GRIF-1, concentrated at focal points at the tips of cell processes (Fig. 9, N–S).

**DISCUSSION**

We have previously shown that GRIF-1 associates with the molecular motor, kinesin (5). In this report, we have used four experimental approaches to map the GRIF-1 binding domain of the prototypic kinesin-1, KIF5C. Immunoprecipitations and yeast two-hybrid studies both mapped the GRIF-1 binding region of KIF5C to the C3 cargo binding domain. Further, in the yeast two-hybrid interaction assays it was found that the C3 cargo binding domain specifically associated with GRIF-1 (124–283) in agreement with the previous mapping of the kinesin binding domain of GRIF-1 (5). Overexpression of EYFP- and ECFP-tagged KIF5C and GRIF-1, respectively, in either HEK 293 cells or COS-7 cells showed that GRIF-1 co-localized with KIF5C (Fig. 6, H–J) and in refined studies, with the KIF5C non-motor domain, KIF5C-(336–957). FRET experiments established that the interaction between the two proteins was direct. The fact that a significant FRET value was found between the C-terminal-tagged KIF5C and ECFP-GRIF-1, the C-terminal-tagged KIF5C-(336–957) and GRIF-1, but not between the N-terminal-tagged KIF5C nor the motor domain, EYFP-KIF5C-(1–335), confirmed the direct association between the two proteins at the KIF5C C-terminal and GRIF-1 N-term
GRIF-1 was shown to form a ternary complex with KHC and KLC. These findings substantiate a role for GRIF-1 as an adaptor protein linking kinesin to its cargo in the anterograde trafficking processes in neurons. A schematic model summarizing the possible interactions between GRIF-1 and kinesin is shown in Fig. 10.

The concept of the selective transport of proteins and/or organelles via adaptor proteins linking kinesin motor proteins to their cargoes is an emerging feature of trafficking mechanisms in neurons. As described in the introduction, there are now several examples of such kinesin-associated adaptors and their cargoes, i.e. mitochondria and syntaxin-1-containing vesicles are attached to the KHC cargo binding domain by the adaptor protein, syntabulin, for their transport to synapses (9);
JIP-3, a JNK signaling pathway protein, binds to a six tetratricopeptide motif in KLCs to transport the cargo, APP (10); KIF17 forms a complex with mLIn10 in the trans- 
portation of N-methyl-D-aspartate receptor NR2B subunits to the syn- 
apse (11, 12); and GRIP1 is an adap- 
tor protein linking the AMPA subtype of glutamate receptor-con- 
taining vesicles to KIF5 (13). In all 
these examples, the KHC or the 
KLC were studied in isolation, whereas, here it is clearly shown that 
GRIF-1 binds to the KHC when 
asssembled with the KLC. Further, it 
was noted that the distribution pat- 
tern of EYFP-KIF5C is affected by 
co-expression of KLCs. This observation agrees with that 
reported by Verhey et al. (19). Here it was shown that associa- 
tion of KHCs with KLCs inhibits the binding of KHC to micro- 
tubules by inducing the formation of a folded KHC confor- 
mation. In the presence of GRIF-1, KHCs and KLCs are 
co-associated with GRIF-1 in the cell cytoplasm and 
at the tips of transsected COS-7 cell processes as observed for cells 
transfected with EYFP-KIF5C alone but not for the majority of cells 
for EYFP-KIF5C plus KLC transfectants. This suggests that, 
perhaps, GRIF-1 mediates conformational changes of KHC/ 
KLC complexes such as unfolding. This then permits binding of 
the complex to microtubules, and active motor transport can 
now occur as has been shown in kinesin motility assays in vitro 
(20, 21).

A further example of kinesin adaptor proteins are the α and β 
isforms of dystrobrevin (22). Like GRIP1 (13) and now, 
GRIF-1, β-dystrobrevin was shown to bind to the C-terminal 
KHC cargo binding domain (23). Both GRIP-1 and α- and 
β-dystrobrevins bind to the cargo binding domain with similar 
affinities as determined by surface plasmon resonance analy- 
sis, i.e. GRIP-1, Kd = 1.9 x 10^-8 M (13); β-dystrobrevin, 
Kd = 4 x 10^-8 M (23). Moreover and interestingly, Ceccarini et al. (23) showed that two distinct regions of β-dystrobrevin 
contribute to the binding to KHC and that in vitro phospho- 
rylation of a glutathione-β-dystrobrevin construct resulted in 
a decreased binding to KHC (23). This suggests that both 
tertiary structure and post-translational modification may 
modulate β-dystrophin-KHC or, more generally, adaptor- 
kinesin interactions.

The one or more cargoes transported by GRIF-1 (and the 
hoemologous protein, OIP106) have yet to be ascertained. 
The first description of GRIF-1 identified it as a GABA_α 
receptor β2 subunit-associated protein (1). This suggested that 
the cargo may be assembled, β2 subunit-containing 
GABA_α receptors, but in vivo evidence to support this func- 
tion is still lacking. However, Gilbert et al. (6) recently 
showed that OIP106 (TRAK1) co-associated with GABA_α 
receptor α1 subunits, thus corroborating a role for the GRIF- 
1/OIP106 family in transporting GABA_α receptors. Addi- 
tionally, both GRIF-1 and OIP106, like the Drosophila ortho-
logue Milton, have been shown to aggregate mitochondria 
following their respective overexpression in mammalian 
cells (3, 5). Further, without Milton, Drosophila are blind and 
mitochondria accumulated in neuronal cell bodies, whereas 
synaptic terminals and axons were depleted of mitochondria 
(3). However, if the cargo is a mitochondriod, it is unclear 
with which mitochondrial protein GRIF-1 or OIP106 associ- 
ates. It is unlikely that GABA_α receptors are expressed in 
mitochondria. GRIF-1 and OIP106 have, however, both been 
shown to associate with the enzyme, OGT (2). There are two 
forms of OGT, a mitochondrial (m) and a nucleocytoplasmic (nc) variant. The GRIF-1 or OIP106 binding domain of OGT 
is conserved between both forms mapping to mOGT-(51– 
100) and ncOGT-(167–283) (2, 5). mOGT has been 
reported to localize within the mitochondrial inner mem- 
brane (24) and would not therefore be accessible for binding to 
soluble GRIF-1-KHC or OIP106-KHC complexes. It is thers 
usely likely that GRIF-1 and OIP106 associate with 
ncOGT.

Other candidate proteins that mediate the association 
between GRIF-1 and mitochondria include syntabin (25) and 
the mitochondrial GTPase, Miro (26). Both have both been 
implicated in anterograde mitochondrial trafficking in neu- 
rons. Indeed, it was recently demonstrated that Miro forms 
complexes with both GRIF-1 and OIP106 (27) and in Drosoph- 
ila, Milton with Miro (28).

GRIF-1 is found within the brain in both neurons and glial 
cells. Furthermore, it is also expressed in the heart and at lower 
levels in skeletal muscle (1). It is possible that the cargo trans- 
ported by GRIF-1-KHC complexes may be cell type-specific 
depending on the repertoire of KHCs expressed by the host cell 
and that the formation of such complexes may possibly be regul- 
ated by post-translational modifications such as that recently 
described for α- and β-dystrobrevin. The enzyme, OGT, with 
which GRIF-1 and OIP106 are known to be associated, cata- 
lizes the addition of N-acetylglucosamine onto serine and 

\[ \text{Mapping of the GRIF-1 Binding Domain of KIF5C} \]

\[ \text{FIGURE 10. A schematic diagram depicting putative GRIF-1-kinesin-cargo interactions for the anterograde trafficking of defined cargoes to synapses. Kinesin is depicted as a tetramer with two copies of the KHC and two copies of the KLC. For the heavy chain, the motor domain is shown associated with microtubules and contains, additionally, the stalk and cargo binding regions. GRIF-1 is depicted as a dimer (G. Ojla, M. Beck, K. Brickley, and F. A. Stephenson, manuscript in preparation) forming a ternary complex with the cargo binding domain of the KHC and the cargo. Yeast two-hybrid interaction assays using GRIF-1 deletion constructs previously showed that GABA_α receptor β2 subunits and KIF5C share the same coiled-coil binding domain of GRIF-1, i.e. GRIF-1-(124–283) (1 and 5) that is depicted in the figure by the lighter shading. A is a model where the KHC and the cargo bind separately to each of the GRIF-1 polypeptides; B is an alternative model whereby the KHC, GRIF-1, and the cargo associate with the same GRIF-1 subunit.} \]
Mapping of the GRIF-1 Binding Domain of KIF5C

threonine residues of protein substrates. This O-glycosylation post-translational modification occurs in the cell cytoplasm, and it is thought to regulate protein function and to have a reciprocal relationship with post-translational modification and regulation via phosphorylation, the so-called “YinOYang” relationship (29). The YinOYang neural network predictions for O-β-GlcNAc attachment sites in eukaryotic protein sequences (www.cbs.dtu.dk/services/YinOYang) predicts several sites for O-glycosylation within the non-kinesin binding GRIF-1 C-terminal domain. It is known that GRIF-1 and OIP106 are O-glycosylated in vivo (2), although the residues that are actually O-glycosylated have not been identified. Thus perhaps the association of GRIF-1 and OIP106 with OGT is to regulate GRIF-1-KHC interactions rather than OGT per se being a cargo.

In summary, the work described herein maps the GRIF-1 binding domain of KIF5C and shows that GRIF-1 forms a ternary complex with KIF5C and KLC that results in the aggregation of mitochondria. These findings consolidate the role of GRIF-1 as an adaptor protein involved in motor-dependent anterograde transport. Defects in these transport mechanisms, especially in the transport of mitochondria and GABA_A receptors, purported to be GRIF-1 cargoes, may contribute to the pathology of neurodegenerative diseases such as Alzheimer disease, amyotrophic lateral sclerosis, hypertonia, and hereditary spastic paraplegia (30).

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