GRIF-1–kinesin-1 interactions: a confocal microscopy study

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Abstract
GRIF-1 [GABA$_A$ (γ-aminobutyric acid$_A$) receptor interacting factor-1] is a member of a coiled-coil family of proteins thought to function as adaptors in the anterograde trafficking of organelles utilizing the kinesin-1 motor proteins to synapses. To study in more detail the molecular interaction between GRIF-1 and the kinesin-1 family member KIF5C, fluorescent yellow- and fluorescent cyan-tagged GRIF-1, KIF5C, the KIF5C MD (motor domain) and the KIF5C NMD (non-motor domain) fusion proteins were generated. Each was characterized with respect to size and ability to co-associate by immunoprecipitation following expression in HEK-293 (human embryonic kidney 293) cells. Further, their distribution in transfected HEK-293 and transformed African green monkey kidney (COS-7) cells was analysed by confocal microscopy. The fluorescent GRIF-1 and KIF5C fusion proteins were all found to behave as wild-type. Double GRIF-1/KIF5C transfectants revealed co-localization. The GRIF-1/KIF5C and GRIF-1/KIF5C NMD double transfectants showed different subcellular distributions compared with single GRIF-1, KIF5C or KIF5C NMD transfections. These studies confirm the association between GRIF-1 and kinesin-1 NMDs. Fluorescence resonance energy transfer studies are ongoing to characterize this interaction in more detail.

Introduction
GRIF-1 [GABA$_A$ (γ-aminobutyric acid$_A$) receptor interacting factor-1] was initially identified from rat brain by a yeast two-hybrid screen searching for GABA$_A$ receptor acting factor-1] was initially identified from rat brain by a 'YinOYang' relationship [6]. Cytoplasmic O-glycosylation and regulation via phosphorylation, the so-called to have a reciprocal relationship with post-translational modification occurs in the human protein, OIP98 [OGT (β-N-linked N-acetylglucosamine transferase) interacting protein 98], and it is the homologue of the protein OIP106. 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 [2]. GRIF-1, OIP106 and Milton belong to a newly identified family of coiled-coil proteins. Although their function is not definitively established, GRIF-1 interacting proteins have been identified, which gives insight into their possible role in neurons. These proteins include the enzymes OGT [3,4] and kinesin [2,5].

OGT catalyses the addition of N-acetylglucosamine on to serine and threonine residues of protein substrates. This O-glycosylation post-translational modification occurs in the cell cytoplasm. 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 [6]. Cytoplasmic O-glycosylated proteins are particularly abundant in the central nervous system [7].

Kinesin-1 proteins are plus-end-directed microtubule motors. They transport specific cargoes, e.g. synaptic vesicle precursors, lysosomes and mitochondria, anterogradely along the microtubules. The kinesin-1 family members are conventional kinesins that include KIF5A, KIF5B and KIF5C. KIF5B is ubiquitously expressed, whereas KIF5A and KIF5C are only present in neurons [8]. Kinesin-1 proteins are tetramers formed by the association of two KHCs (kinesin heavy chains) and two KLCs (kinesin light chains). The KHC is formed from an N-terminal MD (motor domain) that contains the microtubule- and ATP-binding sites, and a C-terminal NMD (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 the KHC of kinesin-1 proteins. 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 [9,10]. JIP-3, a c-Jun N-terminal kinase signalling pathway protein, binds to a six-tetratricopeptide motif in KLCs to transport the cargo, APP (amyloid precursor protein) [11]. Defects in these transport mechanisms may contribute to the pathology of neurodegenerative diseases such as Alzheimer’s disease, amyotrophic lateral sclerosis and hereditary spastic paraplegia (for a review, see [12]).

In the present study, we provide supporting evidence suggesting that GRIF-1 is another example of an adaptor protein involved in motor-dependent trafficking of proteins.
Confocal microscopy imaging of GRIF-1–kinesin-1 interactions

The following fluorescent constructs were generated by subcloning: ECFP (enhanced cyan fluorescent protein) GRIF-1; EYFP (enhanced yellow fluorescent protein) KIF5C-(1–957) (N- and C-terminally tagged); the EYFP KIF5C MD, KIF5C-(1–335) (referred to as EYFP KIF5C-MD); and the KIF5C-NMD EYFP, KIF5C-(336–957) (referred to as KIF5C-NMD EYFP). Each construct was expressed alone or in GRIF-1/KIF5C pairwise combinations in either HEK-293 (human embryonic kidney 293) cells or COS-7 cells, and the localization of the fluorescent proteins was imaged by confocal microscopy. Representative results are shown in Figure 1.

In COS-7 cells, ECFP GRIF-1 expressed alone was found to be localized in cytoplasmic perinuclear regions (Figure 1A), whereas EYFP KIF5C or KIF5C ECYP expressed alone in these cells was distributed throughout the cell cytoplasm (Figure 1A). When ECFP GRIF-1 and EYFP KIF5C constructs were co-expressed, the distribution of both proteins was distinct from that observed for single-protein transfections. ECFP GRIF-1 and EYFP KIF5C (or KIF5C EYFP) were found to co-localize, forming small clusters in the cell cytoplasm and also in cell processes (Figure 1B, top panels). KIF5C-NMD EYFP expressed alone was localized to filamentous, tubular structures in the cell cytoplasm (Figure 1A). In the presence of ECFP GRIF-1, however, the distribution pattern was changed such that it was recruited to EYFP GRIF-1-rich regions where co-localization of the two proteins was observed (Figure 1B, middle panels). EYFP KIF5C-MD expressed alone showed a diffuse distribution throughout the whole cell (Figure 1A). This distribution pattern was not changed by co-expression of EYFP KIF5C-MD with ECFP GRIF-1 (Figure 1B, bottom panels).

To test whether ECFP GRIF-1 associated directly with fluorescently tagged KIF5C, FRET (fluorescence resonance energy transfer) studies were carried out in transfected HEK-293 cells. FRET efficiencies were measured by acceptor photobleaching using appropriate ECFP and EYFP positive and negative controls [13,14]. A significant FRET efficiency (~10%) was measured only for the ECFP GRIF-1/C-terminal-tagged KIF5C EYFP and ECFP GRIF-1/KIF5C-NMD EYFP combinations.
**Concluding remarks**

Functional fluorescent GRIF-1 and KIF5C chimaeras were generated and used in immunofluorescence imaging studies to show that GRIF-1 associated directly with KIF5C and, more specifically, the KIF5C-NMD. This is in agreement with [15], in which the GRIF-1-binding site was mapped to the KIF5C cargo-binding site by yeast two-hybrid interaction assays and co-immunoprecipitation experiments. These findings substantiate a role for GRIF-1 as an adaptor protein linking kinesin-1 to its cargo in anterograde trafficking mechanisms in neurons (Figure 2).

![Figure 2](image)

**Figure 2 | Schematic diagram showing the proposed function of GRIF-1 as an adaptor protein linking kinesin-1 to its cargo**

GRIF-1 dimers attach a GABA receptor-containing vesicle to kinesin. GRIF-1 is also attached to OGT trimers that have also been shown to be part of the transport complex [3,4].

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**References**


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