The Motor Protein Kinesin-1 Links Neurofibromin and Merlin in a Common Cellular Pathway of Neurofibromatosis*

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Mutations in either of the two tumor suppressor genes NF1 (neurofibromin) and NF2 (merlin) result in Neurofibromatosis type 1 and 2, a condition predisposing individuals to developing a variety of benign and malignant tumors of the central and peripheral nervous systems. Here we report the identification of two distinct NF1-containing complexes, one in the soluble and the other in the particulate fraction of HeLa extract. We show that the soluble NF1 complex delineates a large holo-NF1 complex (2 MDa) encompassing the components of a smaller particulate core-NF1 complex (400 kDa). Purification of the core-NF1 complex followed by mass spectrometric analysis revealed the motor protein, kinesin-1 heavy chain (HsKHC/KIF5β), as a catalytic subunit of both NF1-containing complexes. Importantly, although NF1 and NF2 are not in a stable association, NF2 is also a component of a distinct kinesin-1-containing complex. These results point to kinesin-1 as a common denominator between NF1 and NF2.

Neurofibromatosis type 1 (NF1)3 or von Recklinghausen disease is a common neurological genetic disease that affects 1 in 3500 individuals world wide (1, 2). Mutations in the human NF1 gene lead to a common neurocutaneous disorder characterized by benign tumors (neurofibromas and gliomas), abnormal distribution of melanocytes (cafe-au-lait spots), and malignant tumors, including neurofibrosarcomas, pheochromocytomas, rhabdomyosarcomas, astrocytomas, and juvenile myeloid leukemias. NF1 patients also exhibit cognitive deficits and other symptoms unrelated to cancer, affecting neural crest-derived tissues outside of the nervous system reflective of a role for NF1 in developmental control (2, 3). NF1 encodes a large protein of 2818 amino acids designated neurofibromin (4–6). The protein is highly conserved from yeast to human. Neurofibromin is expressed ubiquitously in human, with the highest expression in adult peripheral and central nervous systems (7). The protein contains a GAP-related domain (GRD) that shares homology to known GTPase-activating proteins (GAPs). NF1-GRD has been shown to act as a GAP for the Ras family of small GTPases (8–10). Thus, several studies suggest that the tumor suppressor activity of neurofibromin depends on its ability to negatively regulate the ras-mediated signaling pathway that regulate cell growth and differentiation in a variety of cell types (11). Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder implicated in the development of sporadic schwannomas, meningiomas, ependymomas, and astrocytomas (12–14). The NF2 gene encodes a 595-amino acid protein termed merlin belonging to the ERM (ezrin, radixin and moesin) family that link the actin cytoskeleton to cell surface glycoproteins (15).

We have initiated the biochemical characterization of NF1- and NF2-containing complexes from mammalian cells. These experiments led to the identification of two distinct NF1-containing complexes. We show that while NF1 purified from the soluble fraction reside in a large complex of ~2MDa, NF1 in the particulate fraction is a component of a smaller complex of 400 kDa. To gain insights into the functions of these complexes, we used a combination of conventional and affinity chromatography to purify the smaller core-NF1 complex from the particulate fraction. We have identified the catalytic subunit of this complex as the motor protein kinesin-1. Importantly, we show that although NF1 and NF2 proteins are not stably associated, NF2 is also a component of a distinct kinesin-1-containing complex.

MATERIALS AND METHODS

Western Blot Analysis

For detection of the NF1 protein, affinity-purified polyclonal antibodies sc-68 (NF1GRP-D) raised against synthetic peptides corresponding to the carboxyl terminal domain of the human NF1 gene product were used (Santa Cruz Biotechnology). For detection of the NF2 protein, affinity-purified polyclonal antibodies sc331 (A-19) and sc332 (C-18) raised against synthetic peptides corresponding to the NH2 terminus and the COOH terminus of the NF2 protein were used (Santa Cruz Biotechnology). For detection of the kinesin-1 protein, one polyclonal antibody raised against the insert 1 region of the head of human KHC (KIF5B) (gift from Ronald D. Vale’s laboratory) and two monoclonal antibodies H1 and H2 raised against bovine brain kinesin (Chemicon International, Inc.) were used.

Protein Identification Using Liquid Chromatography-MS/MS

Gel bands were excised from colloidal Coomassie-stained gels, and bands were destained, alkylated with iodoacetamide, and digested using modified trypsin (Promega) for 16 h at 37 °C essentially. A portion of the extracted peptides were loaded to a nanocapillary reverse-phase 75-μm column terminating in a nanospray 15-μm tip (New Objective) packed with Porous R2 resin (Applied Biosystems). The nanocolumn was directly coupled to a ThermoFinnigan LCQ quadrupole ion trap mass spectrometer, and peptides were eluted into the mass spectrometer using an acetic acid-acetonitrile gradient. Data were acquired using triple play mode to automatically obtain peptide masses, peptide charge states, and MS/MS spectra. The resulting data were searched against the non-redundant NCBI using the TurboSEQUENT Browser to identify proteins.

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The abbreviations used are: NF1 and NF2, neurofibromatosis types 1 and 2; GAP, GTPase-activating protein; GRD, GAP-related domain; MS, mass spectrometry; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; βME, 2-mercaptoethanol; KHC, 120-kDa heavy chain; KLC, 64-kDa light chain; APP, amyloid precursor protein.

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Materials and Methods.

Preparation of the Soluble and Particulate Fractions from HeLa Cells or Calf Brain

The method of Dignam et al. (16) was used to prepare soluble or particulate fractions from HeLa cells and Calf brain. First, viable cells are prepared and collected in a conical test tube by centrifuging. Next, cells are resuspended in hypotonic buffer A (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10 mM NaCl, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) that causes them to swell, thus making them easy to lyse. The outer membranes are disrupted by homogenization, and the soluble fraction is then collected after pelleting membrane debris. The particulate fraction is carefully resuspended in buffer B (20 mM Tris-HCl, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). Following further homogenization and pelleting of the nuclear membranes, the particulate fraction is collected.

Chromatographic Purification of NF1 Complex from HeLa Cells or Calf Brain

HeLa Particulate Fraction—HeLa particulate extract (3 g) was loaded on a 500-ml column of phosphocellulose (P11, Whatman) and fractionated stepwise by the indicated KCl concentration in buffer A (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10 mM βME, 10% glycerol, 0.2 mM PMSF). The P11 0.3 M KCl fraction (700 mg) was loaded on a 80-ml DEAE-Sephacel column (Amersham Biosciences) and eluted with 0.5 M KCl. 60 mg of the 0.5 M KCl elution was dialyzed to 700 mM NH₄SO₄ in buffer HB (20 mM Hepes, pH 7.6, 4 mM DTT, 0.5 mM EDTA, 10% glycerol, 0.5 mM PMSF) and loaded on a butyl-Sepharose (Amersham Biosciences). The column was resolved using a linear 10-column volume gradient of 700 to 0 mM NH₄SO₄ in buffer HB. NF1-containing fractions 10–14 were dialyzed to 100 mM KCl in buffer A and loaded on Heparin-SPW (Tosohaas). The column was resolved using a linear 20-column volume gradient of 100–500 mM KCl in buffer A. The fractions 10–16 were used for the immunoaffinity purification of the NF1-containing complex.

Immunoaffinity Purification of the NF1-containing Complex

Anti-NF1 antibodies (500 µg, COOH-terminal, Santa Cruz Biotechnology, sc-68) were cross-linked to protein A-Sepharose (1 ml, Repligen) using standard techniques for affinity purification. The heparin fractions from HeLa cells and calf brain were incubated with 1 ml of antibody-protein A beads for 4–6 h at 4 °C in buffer A. The beads were then washed with 1 M KCl and 1% Nonidet P-40 in buffer A. The beads were then washed with 100 mM KCl in buffer A, and the proteins were eluted with 0.1 M glycine, pH 2.5, and neutralized with 0.10 volume 1.0 M Tris-HCl, pH 8.0.

RESULTS

Identification of Two Distinct NF1-containing Complexes, a Soluble Holo-NF1 and a Particulate Core NF1—To gain insight into the biochemical properties of NF1, we fractionated HeLa soluble and particulate extracts (Fig. 1a and see “Materials and Methods”). This procedure was required to enrich for the NF1-containing complexes. Surprisingly, the fractions containing NF1 derived from the soluble or particulate extract behaved differently following phosphocellulose (P11) chromatography.

FIG. 1. NF1-containing complexes derived from the soluble or particulate fractions. a, HeLa particulate and soluble fractions from the HeLa cell line were fractionated by chromatography as described under “Materials and Methods.” Western blot analysis of P11 fractions using NF1 (sc-68) antibodies. b, Western blot analysis of the soluble eluate fractionated by Superose 6 gel filtration using NF1 (sc-68) antibodies. c, Western analysis of the particulate eluate fractionated by Superose 6 gel filtration using NF1 (sc-68) antibodies.

FIG. 2. Purification of NF1-containing complexes derived from the particulate fraction of HeLa and calf brain. a, HeLa particulate extract was fractionated by chromatography as described under “Materials and Methods.” The affinity-purified α-NF1 (sc-68) complex was separated in an SDS-polyacrylamide gel (4–12%), and proteins were visualized by colloidal blue staining and Western blot analysis using anti-NF1 and anti-kinesin-1 antibodies. Molecular masses of marker proteins are indicated on the left and the proteins analyzed by ion trap mass spectrometry on the right. b, calf brain particulate extract was fractionated by chromatography and analyzed as described above.
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The soluble NF1 was enriched in the 1 M KCl elution, while the particulate NF1 peaked at 0.5 M KCl (Fig. 1a). This difference was further demonstrated once the two fractions were analyzed by gel filtration. The soluble NF1 complex eluted at an apparent molecular mass of 2 MDa (Fig. 1b), fraction 18 was the peak. In contrast, the particulate NF1 derived from either the 0.3 or 0.5 M KCl elution of P11 chromatography exhibited an elution profile consistent with a complex of 400 kDa (Fig. 1c). These sizes are estimated relative to globular protein standards and assume that the complexes are themselves globular. If the complexes are elongated they would have a smaller mass. These results suggest the existence of two distinct NF1-containing complexes, a large NF1-containing complex enriched in the soluble fraction and a smaller complex in the particulate fraction. It is possible that the NF1 complex in the particulate fraction is in association with the outer nuclear membrane. The association of NF1 with the particulate fraction was previously detected by immunofluorescence staining in neurons following detergent extraction (17).

Fig. 3. Kinesin-1 is a component of NF1 complexes derived from both the soluble and the particulate fractions. a, immunoprecipitation using three affinity-purified antibodies for kinesin-1 (one polyclonal α-KIF5B and two monoclonals α-H1 and α-H2) and α-TRAP220 (control) followed by Western analysis using α-NF1 (sc-68) and α-KIF5B antibodies. Calf brain particulate extract was used as the input. b, Western analysis using α-KIF5B antibodies following immunoprecipitation using the affinity-purified α-H2, α-NF1 (sc-68), and α-TRAP220 from HeLa soluble fraction. c, after transfection of HeLa cells with either FLAG-KIF5B or pFLAG-CMV2, anti-FLAG antibodies were used to immunoprecipitate complexes associated with KIF5B. The eluate was analyzed by Western blot using the antibodies shown to the right of the figure. Antibodies against KIF5B also detected a heterodimeric complex formed by the endogenous KIF5B and FLAG-KIF5B.

Purification of the Core-NF1 Complex from HeLa and Calf Brain Particulate Fraction Revealed the Presence of Kinesin-1—To define the polypeptide composition of NF1-containing complexes, we isolated the smaller NF1 complex. NF1 was purified from both HeLa cells and calf brain particulate extract using a combination of conventional and affinity chromatography following the scheme presented in Fig. 2a and b. Analysis of α-NF1 affinity eluate by SDSPAGE and colloidal blue staining revealed the association of NF1 with three polypeptides of 150, 110, and 55 kDa (Fig. 2, a and b). Mass spectrometric analysis established the 220-kDa band as NF1 and identified the 110-kDa band as the motor protein kinesin-1 heavy chain (HsuKHC/KIF5B) (18). Western blot analysis confirmed the association of kinesin-1 and NF1 in both HeLa cells and calf brain (Fig. 2, a and b). Furthermore, immunoprecipitation experiments using three different kinesin-1 antibodies demonstrated a stable association of NF1 and kinesin-1 from particulate extract of both calf brain and HeLa cells (Fig. 3a and data not shown). Interestingly, NF1 derived from the soluble fraction is also in a stable association with kinesin-1 (Fig. 3b). We further confirm this association by immunoprecipitating endogenous NF1 with ectopically expressed FLAG-KIF5B using anti-FLAG antibodies followed by elution of bound material with FLAG peptide (Fig. 3c). Together, these results demonstrate the stable association of NF1 and kinesin-1 in both soluble and particulate fractions.

Kinesin-1 Is Also Associated with a Distinct Soluble NF2-containing Complex—Mutations in the NF2 gene also cause a similar disease manifestation as that of NF1 (12–14). We there-
fore asked whether NF1 and NF2 are stably associated. Immunoprecipitation experiments using either anti-NF1 or anti-NF2 antibodies did not show an association between NF1 and NF2 proteins from the soluble fraction of HeLa cells (NF2 was not detected in particulate fraction, Fig. 4a). We then asked whether NF2 is also a stable component of kinesin-1 containing complexes. Immunoprecipitation experiments using both the NH₂- and the COOH-terminal anti-NF2 antibodies revealed the stable association of NF2 and kinesin-1 (Fig. 4b). We confirmed the association by immunoprecipitating endogenous NF2 with ectopically expressed FLAG-KIF5B using anti-FLAG antibodies followed by elution of bound material with FLAG peptide (Fig. 4c). Indeed, fractionation of soluble HeLa extract by gel filtration revealed the coelution of NF2 and kinesin-1 in a large complex (Fig. 4d, fractions 16–20), although a fraction of NF2 was also detected at a smaller molecular mass (fractions 30–36). These results indicate that although NF2 is a component of a large kinesin-1-containing complex, this complex seems distinct from the NF1-containing complex. However, since both NF1 and NF2 are components of a large kinesin-1-containing complex, it is possible that fractions of NF2 and NF1 are stably associated but that antibodies to each protein disrupt this association.

**DISCUSSION**

Kinesin-1 is a tetramer consisting of two 120-kDa heavy chains (KHC) and two 64-kDa light chains (KLC). Kinesin-1 heavy chain HsUKHC/KIF5B belongs to the kinesin protein superfamily (KIF) (19). This family has been shown to transport protein complexes, organelles, and mRNA to specific destinations in an ATP- and microtubule-dependent manner (20, 21). Furthermore, some members of this family are also involved in chromosomal and spindle movements during mitosis and meiosis (22, 23). Although a stable association of kinesin-1 and NF1 or NF2 was an unexpected finding, it is consistent with previous microscopy studies indicating the subcellular localization of NF1 and NF2 with the cytoskeleton (17, 24–27). Taken together, the association of NF1 and NF2 with the motor protein kinesin-1 suggests a role for these proteins in microtubule-mediated intracellular signal transduction pathways.

Recent studies have shown that the axonal transport of amyloid precursor protein (APP) in neurons is mediated by the direct biochemical interaction between APP and KLC, the light chain subunit of kinesin-1 (28, 29). Considering that microtubule-dependent trafficking requires at least two entities, a cargo-bound receptor and the motor proteins, the authors proposed that APP may be a membrane cargo receptor for a kinesin-mediated axonal transport of β-secretase and presenilin-1 (29). In analogy with this model, the association between kinesin-1 and NF1 or NF2 might reflect a new function for these proteins in transport of vesicular cargoes within cells. Although NF1 has several known functions, including Ras GTPase-activating protein activity (8–10) or adenyl cyclase modulation (30, 31), this new function might explain the high incidence of learning disabilities and cognitive problems related to NF1 mutations (1, 3, 31–33). Thus aberrant kinesin-1/NF1-mediated trafficking or transport of neurotransmitter containing vesicle may affect the normal development of the cerebral cortex. Future studies are needed to test this hypothesis rigorously. In conclusion, our data through the demonstration of a stable association of NF1 and NF2 proteins with the motor protein kinesin-1 identifies a common pathway underlying the mechanism of neurofibromatosis.

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**REFERENCES**