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

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Abstract

Mutations in either of the two tumor suppressor genes NF1 (neurofibromin) and NF2 (merlin) result in Neurofibromatosis, 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 (HsuKHC/KIF5B), as a catalytic subunit of both NF-1-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.
Introduction

Neurofibromatosis type 1 (NF1) or von Recklinghausen disease is a common neurological genetic disease that affects 1 in 3500 individuals worldwide (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 depend on its ability to negatively regulate the *ras*-mediated signalling 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). 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 approximately 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.

Material 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 carboxy 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 N-terminus and the C-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 uKHC (KIF5B) (gift from Vale R.’s lab) and two monoclonal antibodies H1 and H2 raised against bovine brain kinesin (Chemicon International, Inc) were used.
Protein Identification using LC-MS/MS

Gel bands were excised from Colloidal Coomassie stained gels, bands were destained, alkylated with iodoacetamide, and digested using modified Trypsin (Promega) for 16 hr at 37°C essentially. A portion of the extracted peptides were loaded to a nanocapillary reverse-phase 75••m column terminating in a nanospray 15••m 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 was 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 TurboSEQUEST Browser to identify proteins.

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 a hypotonic buffer A (10 mM Tris Hcl pH 7.9, 1.5 mM MgCl_, 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 MgCl_, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). Following further homogenization and pelleting of the nuclear membrane debris, the particulate fraction is collected.
Chromatographic purification of NF1 complex from HeLa cells or Calf brain.

**From 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 (Pharmacia) and eluted with 0.5 M KCl in buffer A. The 0.5 M KCl elution (500 mg) was dialyzed to 10 mM K₂PO₄ in buffer B (5 mM Hepes, pH 7.6, 1 mM DTT, 0.5 mM PMSF, 10 μM CaCl₂, 10% glycerol, 40 mM KCl) and loaded on a 70 ml BioGel HT column (Hydroxyapatite, Bio-Rad). The column was resolved by using a linear 10-column volume gradient of 50 to 500 mM K₂PO₄. A pool of the fractions 11-13 were 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 (Pharmacia). The column was resolved using a linear 10 column volume gradient of 700 to 0 mM NH₄SO₄ in Buffer HB. NF1-containing fractions 11-15 were dialyzed to 100 mM KCl in Buffer A, and loaded on Heparine-5PW (TosoHaas). The column was resolved using a linear 20 column volume gradient of 100 to 500 mM KCl in Buffer A. The fractions 12 to 14 were used for the Immunoaffinity-Purification of the NF1-containing Complex.

**From Calf brain particulate fraction.** Calf brain particulate fraction (1 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 0.5 M KCl fraction (700 mg) was loaded on a 80 ml DEAE-Sephacel column (Pharmacia) 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 (Pharmacia). 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 Heparine-5PW (TosoHaas). The column was resolved using a linear 20 column volume gradient of 100 to 500 mM KCl in Buffer A. The fractions 10 to 16 were used for the Immunoaffinity-Purification of the NF1 containing Complex.

**Immunoaffinity-Purification of the NF1-containing Complex**

Anti-NF1 antibodies (500 µg, C-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 cell and Calf brain were incubated with 1 ml of antibody-Protein A beads for 4-5 h at 4°C in buffer A. The beads were washed with 1M KCl and 1 % NP40 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 1/10 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, see Material 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. 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).

**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 SDS-PAGE and colloidal blue staining revealed the association of NF1 with three polypeptides of 150, 110 and 55 kDa (Fig. 2a 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. 2a 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 causes a similar disease manifestation as that of NF1 (12-14). We therefore asked whether NF1 and NF2 are stably associated. Immunoprecipitation experiments using either anti-NF1 or anti-NF2 antibodies did not support 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 N- and the C-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 through 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 a fraction of NF2 and NF1 are stably associated but that antibodies to each protein disrupts 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 sub-cellular 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 are 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 \( \beta \)-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 adenylyl 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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Figure Legends

Figure 1. NF1-containing complexes derived from the soluble or particulate fractions. (a) Particulate and soluble fractions from HeLa cell line were fractionated by chromatography as described in 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.

**Figure 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 in 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. Western analysis of NF1 and kinesin-1. 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.

**Figure 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. Western
blot analysis using α-KIF5B and α-NF1(sc-68). Antibodies against KIF5B also detected a heterodimeric complex formed by the endogenous KIF5B and FLAG-KIF5B.

Figure 4. NF2 is a component of a distinct kinesin1-containing complex. (a) Western analysis using α-NF1 (sc-68) and α-NF2(A19) antibodies. Immunoprecipitation was performed using affinity-purified α-NF1(sc-68), α-NF2(C18) and α-TRAP220 antibodies from HeLa soluble fraction. (b) Immunoprecipitation using α-NF2(C18), α-NF2(A19) and α-TRAP220 from HeLa soluble fraction was analyzed by western blotting using antibodies shown to the left of the panel. (c) After transfection of HeLa cells with either FLAG-KIF5B or pFLAG-CMV2, anti-FLAG antibodies were used to immunoprecipitate complexes associated with KIF5B. Western blot analysis using α-KIF5B and α-NF2(C18). (d) Western blot analysis of Superose 6 column fractions using antibodies to the left of the figure.
a

HeLa

Soluble

P11

0.1 0.3 0.5 1.0

NF1

DEAE-Sephacel

0.1 0.5

Superose 6

0.5

Particulate

P11

0.1 0.3 0.5 1.0

b

Input

Soluble

14 16 18 20 22 24 26 28 30 32 34 36

NF1

Superoxide 6

VOID

670 kDa

Soluble

Western

c

Input

Particulate

14 16 18 20 22 24 26 28 30 32 34

NF1

Superoxide 6

VOID

670 kDa

Particulate

Western
The motor protein kinesin-1 links neurofibromin and merlin in a common cellular pathway of neurofibromatosis
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