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14. ABSTRACT

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder, characterized by the development of bilateral vestibular and spinal schwamomas, meningiomas, and ependymomas. The hF2 gene encodes a 595 amino acid polypeptide known as NF2 protein or Merlin or Schwanomin. The primary structure of the NF2 protein is homologous to the ERM family of peripheral membrane proteins, which includes Ezrin, Radixin, and Moesin. The founding member of the ERM superfamily is the erythrocyte membrane protein 4.1, which cross-link's spectrin-actin complexes and attaches them to the plasma membrane. We have established that p55, a palmitoylated peripheral membrane phosphoprotein, forms a ternary complex with protein 4.1 and glycophorin C. Notably, the Drosophila homologue of p55 functions as a tumor suppressor in epithelial and neuronal tissues. In the 2nd year of the funding period, we demonstrated binding between p55 and the NF2 protein and established the existence of this complex in human erythrocyte plasma membrane. This unexpected finding revealed a new paradigm, integrating the known functions of the p55 family of proteins with the pathophysiology of the NF2 protein.

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INTRODUCTION: Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder, characterized by the development of bilateral vestibular and spinal schwannomas, meningiomas, and ependymomas. The NF2 gene encodes a 595 amino acid polypeptide known as NF2 protein or Merlin or Schwanomin. The primary structure of the NF2 protein is homologous to the ERM family of peripheral membrane proteins, which includes Ezrin, Radixin, and Moesin. The founding member of the ERM superfamily is the erythrocyte membrane protein 4.1, which cross-link's spectrin-actin complexes and attaches them to the plasma membrane. The present proposal originated from our findings that the NF2 protein is present in the human erythrocyte plasma membrane. The main objective of the proposed studies was to devise a method that would yield purified native NF2 protein from human erythrocytes. In addition, we proposed that our extensive knowledge of the erythrocyte membrane biochemistry will enable us to identify novel binding partners of NF2 protein using human erythrocyte plasma membrane as a model system. Since homologues of erythrocyte membrane proteins exist in a wide variety of cells, our findings will be of considerable significance in defining the molecular mechanism of NF2 function in the Schwann cells.

BODY: Technical summary of progress report-Year 1

Neurofibromatosis type 2 (NF2) is an autosomal dominantly inherited disorder predisposing individuals to develop tumors of neuronal origin. The NF2 tumor suppressor protein, termed Merlin/Schwanomin, is a member of the protein 4.1 superfamily that functions as a linker of the plasma membrane to the cortical cytoskeleton. A major limitation in understanding the biochemical properties of NF2 protein is the unavailability of purified native protein from human tissues. This limitation is further exacerbated by the difficulty in expressing functionally active NF2 protein using recombinant cDNA approaches. Our discovery of the presence of NF2 protein in the erythrocytes allowed us to develop a purification procedure that affords homogenous NF2 protein in its native state. Western blot analysis revealed the presence of a single ~70 kDa polypeptide in the erythrocyte ghosts. Selective extraction of ghosts in the low ionic strength buffer indicated that the bulk of NF2 protein remains associated with spectrin-actin depleted inside-out-vesicles (IOVs). Quantitative removal of NF2 protein by extraction of (IOVs) in 1.0 M potassium iodide shows that it is tightly associated with the membrane. Interestingly, the NF2 protein was completely solubilized from ghosts in 0.5% Triton X-100 at high ionic strength (0.5 M KCl) but not at low ionic strength. These results suggest a novel mode of NF2 protein association with the erythrocyte membrane that is distinct from protein 4.1. Based on these biochemical properties, we have devised a purification strategy that allows isolation of cytoskeleton-associated NF2 protein from human erythrocyte ghosts. Identification of the NF2 protein as a constituent of erythrocyte membrane provides a unique opportunity to study the biochemical properties of native protein, and identify proteins that bind to the NF2 protein in the mammalian plasma membrane.

EXPERIMENTAL PROCEDURES AND RESULTS

Preparation of Ghosts: Human red blood cells were washed four times with 5.0 mM sodium phosphate, pH 8.0; 150 mM NaCl, and 0.1 mM EGTA. The buffy coat was carefully removed manually as well as through the use of mixed cellulose chromatography and erythrocytes were lysed in 20 volumes of 5.0 mM sodium phosphate; pH 8.0, 0.1 mM EGTA; 1 mM PMSF. Lysed cells were pelleted by

centrifugation (20 minutes at 20,000 x g) and the membranes (ghosts) were washed several times with the lysis buffer to remove hemoglobin.

SDS-PAGE and Immunoblot Analysis: Proteins were resolved by SDS-PAGE (10% acrylamide) and electro-transferred onto the nitrocellulose membrane. The nitrocellulose blot were blocked with the blocking buffer (6% casein; 1% polyvinyl pyrrolidone-40; 10 mM EDTA in PBS, pH 7.4) and incubated with anti-NF2 antibodies at 1:1000 dilution in the blocking buffer for 2 hours at room temperature. The NF2 antibody was raised by injecting the synthetic peptide of human NF2 protein into rabbits. This antibody is specific for the NF2 protein isoform 1 (SCH1). Following incubation, blots were washed with the TBS-T buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl and 0.1% Tween-20), and incubated for 1 hour with goat anti-rabbit IgG-horseradish peroxidase at 1: 1500 in blocking buffer. After washing with the TBS-T, the NF2 protein was visualized using the ECL Western blotting detection system.

Triton X-100 mediated solubilization of human erythrocyte ghosts: All samples were analyzed in duplicate. Solubilization buffer (SB) consists of 50 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM EGTA, protease inhibitors. It is noteworthy that the NF2 protein remains associated with the cytoskeleton after extraction with Triton X-100. However, extraction of ghosts with 0.5% Triton X-100 containing 500 mM KCl completely solubilized the NF2 protein.

Extraction of cytoskeleton-associated NF2 protein by detergent- free salt solution: Ghosts were extracted with 0.5% Triton X-100 containing 150 mM KCl. Cytoskeletons were recovered by centrifugation on a cushion of sucrose. Detergent-free cytoskeletons were then extracted with an increasing concentration of KCl. The rationale of this experiment was to test whether the NF2 protein could be extracted by high salt without the use of detergent. If so, then does it co-extract with erythrocyte proteins such as ankyrin and adducin. The NF2 protein was not extracted from the detergent-extracted cytoskeleton by simple salt solution. In contrast, a significant amount of ankyrin was eluted under similar extraction conditions.

Purification of NF2 protein from the Triton extracted cytoskeleton: Triton extracted cytoskeleton was disrupted by incubation in 10 volumes of low ionic strength buffer (3 mM Tris-HCl, pH 8.5, 0.5 mM EDTA, 2 mM DTT) for 45 minutes at 37°C followed by centrifugation at 40,000 rpm for 30 minutes. The supernatant containing cytoskeletal proteins was chromatographed on DEAE-Sephacel column (2.5 x 50 cm) that had been previously equilibrated with the column buffer (20 mM Tris-HCl, pH 8.3, 1 mM EGTA, 1 mM DTT and 20 mM KCl). The bound proteins were eluted with 800 ml linear gradient of 20-500 mM KCl in column buffer. The fractions were analyzed by SDS-PAGE, followed by Coomassie blue staining and western blot analysis. The fractions rich in NF2 protein were pooled and dialyzed, and further chromatographed on a Mono Q column. The column was developed with a 50 ml linear gradient of 20-400 mM KCl. The fractions rich in NF2 protein were pooled, dialyzed, and concentrated using a membrane concentrator. The concentrated preparation was analyzed by SDS-PAGE. Purity of NF2 protein was checked by gel electrophoresis and immunoblotting. Note that the purified preparation shows only a single band corresponding to NF2 protein.

Triton X-100 extracts NF2 protein from ghosts only in the presence of 0.5 M potassium chloride.

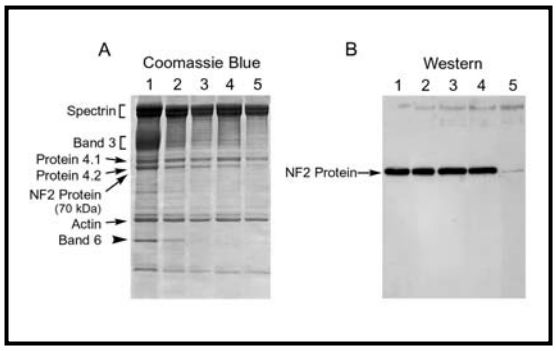

Figure 1: Solubilization buffer consists of 50 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM EGTA, protease inhibitors. Lane 1 (ghosts); 2 (ghosts extracted with 0.5% Triton in SB); 3 (ghosts extracted with 0.5% Triton + 50 mM KCl in SB); 4 (ghosts extracted with 0.5% Triton + 150 mM KCl in SB); 5 (ghosts extracted with 0.5% Triton + 500 mM KCl in SB).

Cytoskeleton-associated NF2 protein is not extracted by simple salt solution.

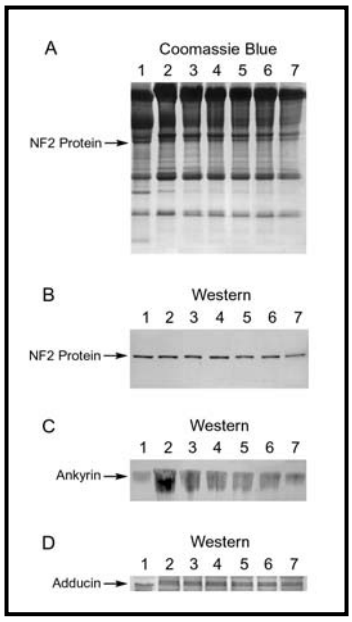

Figure 2: Ghosts were extracted with 0.5% Triton X-100 containing 150 mM KCl. Cytoskeletons were recovered by centrifugation on a cushion of sucrose. Detergent-free cytoskeletons were then extracted with an increasing concentration of KCl. Lane 1 (ghosts); 2 (cytoskeletons); 3 (200 mM KCl); 4 (400 mM KCl); 5 (600 mM KCl); 6 (800 mM KCl); 7 (1000 mM KCl). The NF2 protein is not extracted from the detergent-extracted cytoskeleton by simple salt solution. In contrast, a significant amount of ankyrin and adducin was eluted under similar extraction conditions (panels C and D).

NF2 protein from Triton X-100 extracted cytoskeleton is purified to near homogeneity.

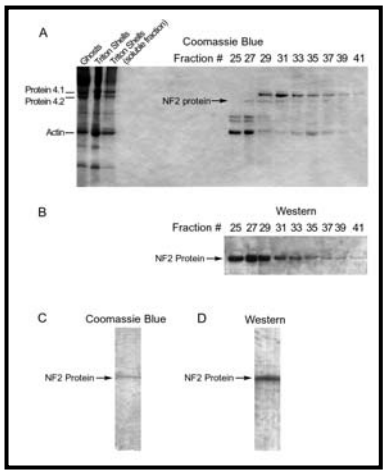

Figure 3: Triton extracted cytoskeleton was disrupted and the supernatant containing cytoskeletal proteins was chromatographed on DEAE-Sephacel column. The bound proteins were eluted with a linear gradient of 20-500 mM KCl in the column buffer. The fractions were analyzed by SDS-PAGE and Western blot analysis. The fractions rich in NF2 protein were pooled, dialyzed, and chromatographed on a Mono Q column. The concentrated protein was analyzed by gel electrophoresis (C) and Western blotting (D).

Technical summary of progress report-Year 2

During the purification of NF2 protein from human erythrocyte plasma membrane (ghosts), we noticed striking biochemical similarities between NF2 protein and p55. We have previously established that p55, a palmitoylated peripheral membrane phosphoprotein, forms a ternary complex with protein 4.1 and glycophorin C. Notably, the *Drosophila* homologue of p55 functions as a tumor suppressor in epithelial and neuronal tissues. To test the hypothesis that p55 is a major binding partner for NF2 protein in the RBC membrane, we generated maltose binding protein (MBP) fusions of recombinant NF2 protein and tested their binding to p55. Both amino and carboxyl halves of NF2 protein were tested in the binding assay. Our results indicate a direct interaction between the FERM domain of NF2 protein and p55. The binding between p55 and NF2 protein was quantified, and existence of this complex was demonstrated in human erythrocyte plasma membrane. This unexpected finding reveals a new paradigm integrating the known functions of p55 family of proteins with the pathophysiology of NF2 protein.

EXPERIMENTAL PROCEDURES AND RESULTS

Production of recombinant NF2: NF2 N-terminus (aa 1-311) and C-terminus (aa 312-595) halves were amplified by the polymerase chain reaction (PCR) using human fetal brain cDNA pool (Clontech). The cDNA fragments were cloned into pMAL-c2X vectors (NE BioLabs), and the recombinant proteins were expressed in *Escherichia coli* strain, DH 5α.

Production of recombinant Protein 4.1: The FERM domain of human protein 4.1R, which starts from the second ATG (nucleotide 801) in exon 4 to phenyalanine in exon 12 (nucleotide 1694), was PCR-amplified from the protein 4.1 cDNA and cloned into pMAL-c2X vector (NE BioLabs). The recombinant protein was expressed in *Escherichia coli* strain, DH 5α.

Production of Recombinant p55: cDNA of full-lengh p55 of human erythroid was obtained by PCR. The PCR-amplified product was cloned into pQE9 vector (Qiagen), and the recombinant protein was

expressed in *Escherichia coli* strain, DH 5α. In addition, the cDNA was also cloned into pFastBac expression vector (GIBCO-BRL). The recombinant baculoviruses were generated by using the Bac-to-Bac baculovirus expression system (Gibco) according to the manufacturer's protocol. Recombinant baculoviruses were amplified twice prior to infection of Sf9 cells, and two clones for each recombinant baculovirus stock were confirmed for recombinant full-lengh p55 DNA sequence by PCR with the same primers used for the cloning procedure. For each construct, four recombinants were amplified.

Site-directed Mutagenesis and expression of p55: The point mutation on amino acid 94 (cys to ala) in full-length p55 construct was created by site-directed mutagenesis.
The primers used were 5'-TGAAAAACAGTCCGCTACGGTGGCCAGAATT-3' (sense) and 5'-AATTCTGGCCACCGTAGCGGACTGTTTTTCA-3' (antisense). The product was cloned into pFastBac vector. Also, mutant baculoviruses were generated using the Bac-to-Bac baculovirus expression system.

Bacterial expression of NF2 N- and C-terminus halves and full-length p55: A single bacterial colony containing the desired plasmid was grown overnight at 37 °C and diluted in 300 ml of culture media, and grown to mid-log phase before induction with 0.2 mM IPTG. The cell pellet was resuspended in 20 ml of lysis buffer containing 8.0 mM sodium phosphate, pH 7.3, 1.0 mM EDTA, 1% Triton X-100, and 72 mM 2-mercaptoethanol, and was lysed by brief sonication. The supernatants of NF2 N- and C-terminus halves were purified on amylase resin. Beads bearing fusion proteins were stored on ice. In addition, the supernatant of full-length p55 was purified on the Ni^{++} column (Invitrogen).

Expression of wild-type and mutant recombinant p55s in insect cells: Expression of recombinant p55 was evaluated in cell lysates and the medium of Sf9 cultures. The Sf9 cells, grown in monolayers, were infected at a multiplicity of infection (MOI) of 10 with the appropriate recombinant baculovirus, and the cells were collected 36 h postinfection (hpi). The expression of the recombinant p55 protein in the medium was analyzed by infection of a 200-ml insect cell culture at a density of 2×10^6 cells/ml (4×10^8 total cells per spinner flask) with an MOI of 5. Cells were harvested and recombinant p55 was purified from the cell lysate using Ni^{++} column (Invitrogen).

In Vitro NF2 N- and C-terminus halves Binding Assay with Full-length p55 Expressed in Bacteria and in Insect cells: NF2 N- and C-terminus halves were immobilized to amylase resin in TBS-Tween20 for 2 hours in 4°C. Lysate containing full length p55 was diluted 10-fold in TBS-T, and the respective MBP fusion proteins were added to each tube followed by incubation for 2 hours at 4°C. Binding of MBP fusion NF2 N- and C-terminus halves to full-length p55 were assessed by maltose binding protein (MBP) pull down assay. Beads were recovered by centrifugation, washed, and bound protein was analyzed by SDS-PAGE. The gel was stained by Coomassie Blue.

SDS-PAGE and Western blot: Proteins were resolved by SDS-PAGE (10% acrylamide) and electro-transferred onto the nitrocellulose membrane (Hybond-C). The nitrocellulose blot was blocked with the blocking buffer (5% milk; 0.1% Tween 20, 10 mM Tris-HCl, and 150mM Nacl in TBS, pH 7.5) and incubated with anti-p55 monoclonal antibodies at 1: 5000 dilution in the blocking buffer for 2 hours at room temperature. Following incubation, blots were washed with the TBS-T buffer (0.1% Tween 20, 10 mM Tris-HCl, and 150mM Nacl in TBS, pH 7.5), and incubated for 1 hour with goat anti-mouse IgG HRP at 1: 5000 in blocking buffer. After washing with the TBS-T, the p55 protein was visualized using the ECL Western blotting detection system.

Surface Plasmon Resonance Measurements: Protein interactions were quantified using BIAcore Optical Biosenser 1000 (BIAcore Inc). To analyze the interaction between MBP-NF2 N and WTP55,

MBP-NF2 C and wild type P55, MBP-NF2 N and MBP-NF2 C fusion protein were passed over the immobilized wild type P55 surface. The wild type p55 was used at a concentration of 200nM. In kinetic experiments, the concentration of MBP fusion proteins of NF2 N and NF2C ranged from 20 nM to 120 nM. The experiments were carried out at 25 °C at a flow rate of 30 µl/min for the kinetic measurements, except the immobilization and regeneration processes, which were carried out at a flow rate of 5.0 µl/min. The composition of the running buffer was 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20. The immobilization buffer for wild type P55 was at a pH of 3.5, 10 mM Sodium Acetate, and the regeneration buffer had 100 mM NaCl and 10 mM NaOH.

Our binding assays demonstrate that the N-terminal FERM domain of NF2 protein specifically interacts with the human erythrocyte p55, produced either in bacteria or in insect cells (Figs. 1-3). In contrast, the C-terminal half of the NF2 protein failed to associate with p55. Surface plasmon resonance measurements confirmed the findings made by the bead pull-down assay and quantified the affinity of the interaction in the 3-4 nM range (Figs. 4-5). Together, our results identify a new interaction of NF2 protein in the erythrocyte plasma membrane.

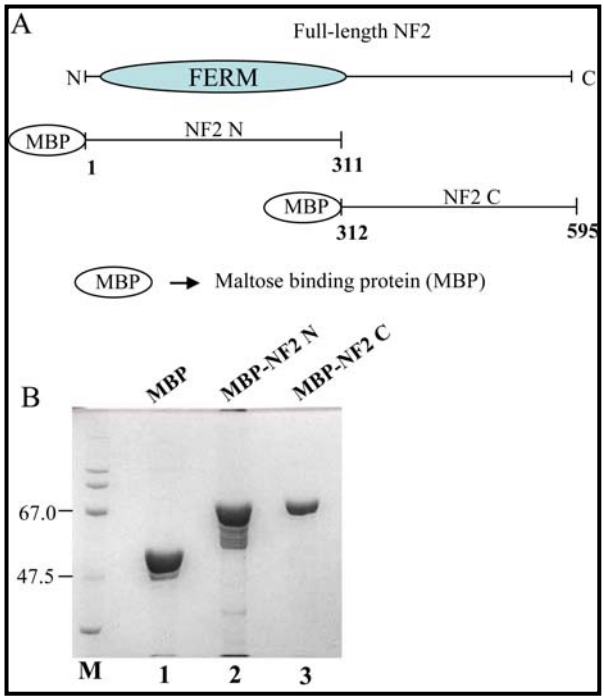

Figure 1. Recombinant proteins of NF2. A, a schematic diagram of NF N- and C-terminal constructs made for the experiments. The NF2 N corresponds to N-terminal half (311 a.a) that includes whole FERM domain, and NF2 C is C-terminal half (283 a.a). B, MBP fusion proteins expressed in bacteria were analyzed by SDS-PAGE.

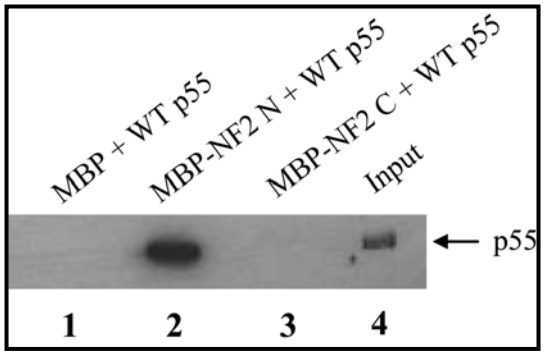

Figure 2. Western blot of i*n vitro* NF2 N- and C-terminus halves binding assay with full-length p55 expressed in bacteria. Full-length p55 was detected by anti-p55 monoclonal antibody.

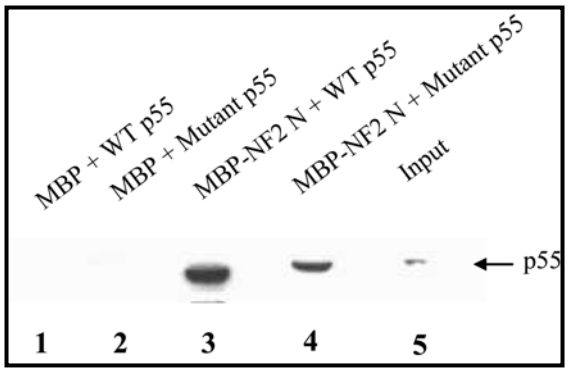

Figure 3. Western blot of *in vitro*. NF2 (N-terminal half) binding assay with full-length p55 expressed in bacteria and in insect cells.

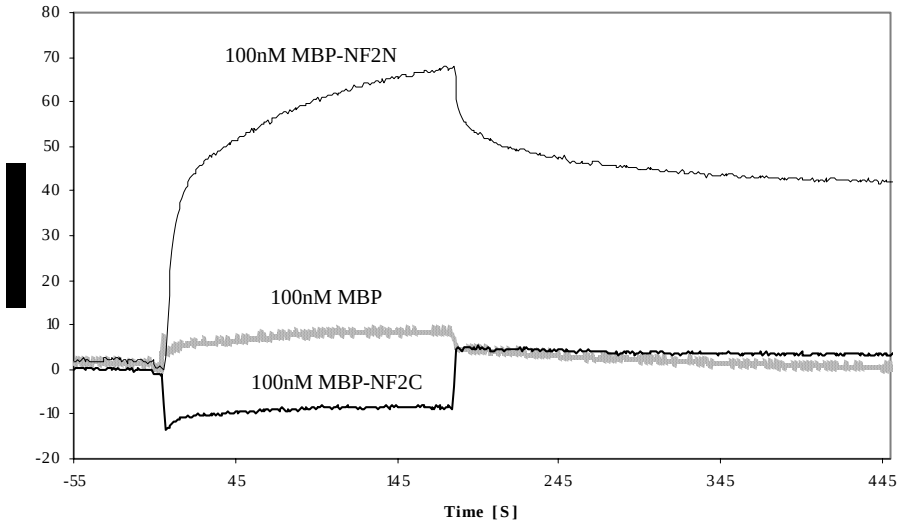

Figure 4. Measurements of the interaction between wild type p55 and MBP-NF2 N fusion protein, wild type p55 and MBP-NF2 C fusion protein, and wild type p55 and MBP protein. Responses in RU are plotted as a function of time (sensorgrams). Wild type p55 were injected at a concentration of 200 nM over immobilized and a total of 280 RU of p55 was immobilized on the CM5 sensor chip (BIAcore Inc.). Binding was performed at a flow rate of 30 µl/min, 25°C. No interactions were observed in wild type p55 and MBP-NF2 C fusion protein, wild type p55 and MBP protein.

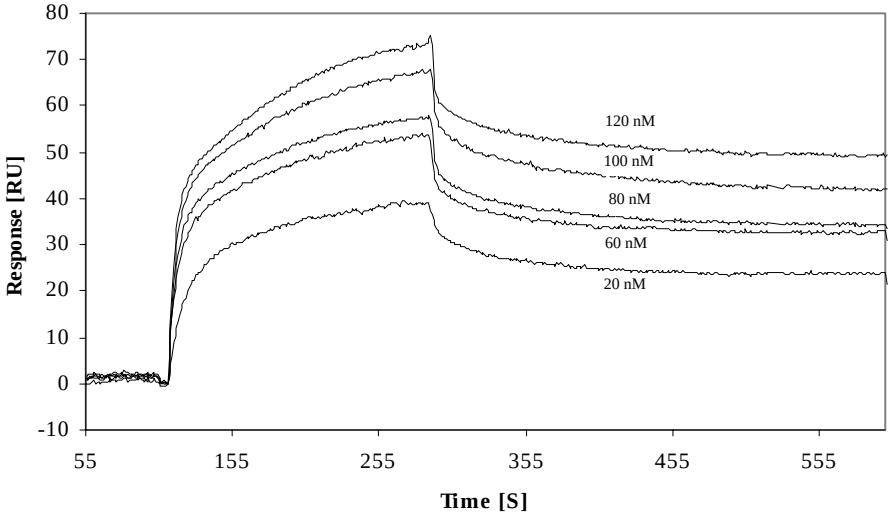

Figure 5. The interaction between MBP-NF2 N fusion protein with wild type p55 at different concentrations for the kinetic analysis. A total of 280 RU of p55 was immobilized on the CM5 sensor chip, and binding was carried out at a flow rate of 30 µl/min at 25 °C. The data were analyzed with BIAevaluation 3.0 software and a conformation change model was chosen because it is the most appropriate compared with other models. The conformation change model is: A+B → AB → AB* (analyte (A) binds to ligand (B); complex AB changes to AB*, which cannot dissociate directly to A+B). In the first step A+B→AB, association rate constant (k_{a1}) obtained was (86.9 ± 6) x 10^4 M^{-1} s^{-1} and dissociation rate constant (k_{d1}) obtained was (55.8 ± 5) × 10^3 s^{-1}. In the second step AB → AB*, association rate constant (k_{a2}) obtained was (122 ± 7) x 10^{-4} s^{-1}, and dissociation rate constant (k_{d2}) obtained was (69.1 ± 4) × 10^5 s^{-1}. An equation: (k_{a1}/ k_{d1})* (k_{a2}/ k_{d2}) was used to calculate the association equilibrium constant (K_A). The association equilibrium constant (K_A) obtained was 2.74 x 10^8 M^{-1}, and the dissociation equilibrium constant (K_D) was 3.7 nM.

Technical summary of progress report-Year 3

We have established that p55, a palmitoylated peripheral membrane phosphoprotein, forms a ternary complex with protein 4.1 and glycophorin C. Notably, the *Drosophila* homologue of p55 functions as a tumor suppressor in epithelial and neuronal tissues. In the 2nd year of the funding period, we demonstrated binding between p55 and the NF2 protein and established the existence of this complex in human erythrocyte plasma membrane. This unexpected finding revealed a new paradigm, integrating the known functions of the p55 family of proteins with the pathophysiology of the NF2 protein. In the 3rd year, we organized all the data for the purification of the NF2 protein from human erythrocyte membrane and assembled a detailed manuscript for publication. This manuscript did not include our recent findings showing the binding of the FERM domain of NF2 with p55. To extend these findings, a critical requirement was to demonstrate the presence of p55 in neuronal cells where the NF2 protein is abundantly expressed. Since the existing polyclonal antibodies do not generally recognize non-erythroid p55 antigens, we set out to generate a monoclonal antibody that can detect the p55 antigen in all tissues. Using this newly developed monoclonal antibody, we demonstrated the presence of p55 in non-myelin forming Schwann cells. Importantly, the co-localization studies showed a perfect co-localization of p55 and NF2 proteins in Schwann cells. These results suggest that the biochemical interaction between p55 and NF2 protein might play a physiological role in Schwann cells. Currently, we are investigating the physiological significance of this work by using the p55 null mouse model recently developed in our laboratory.

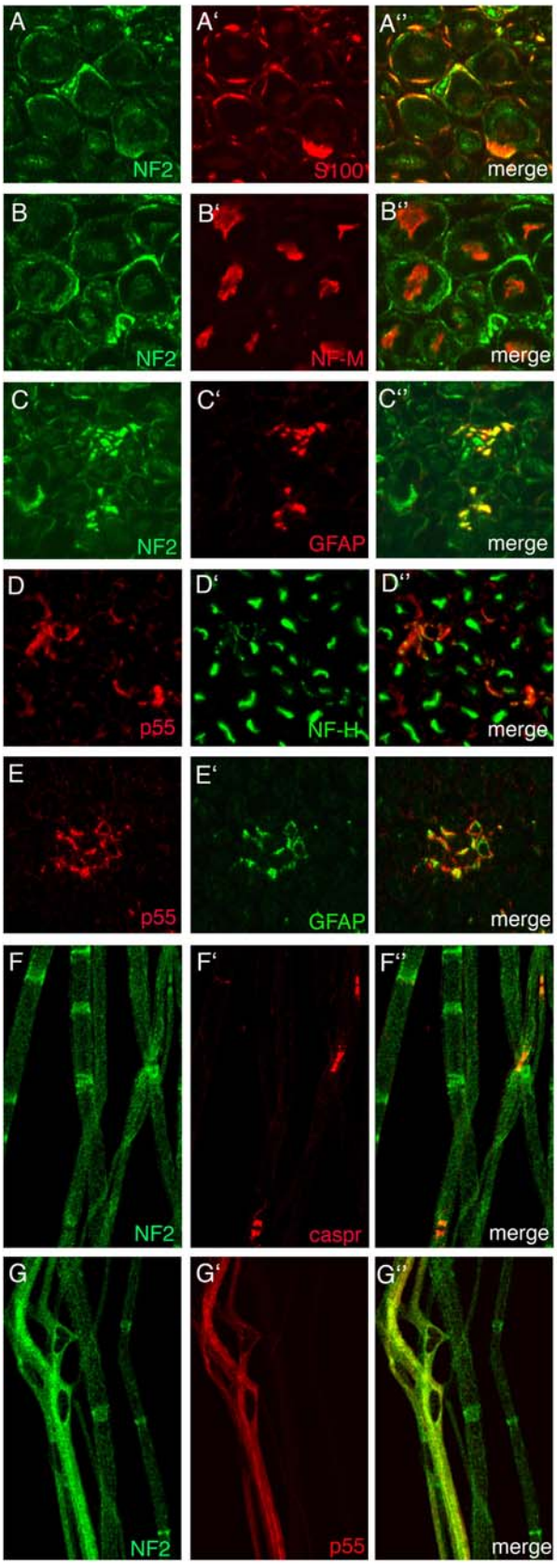

Figure 1. Immunohistocytochemistry of p55 and NF2 proteins in nerve fibers.

EXPERIMENTAL PROCEDURES AND RESULTS

Development of new monoclonal antibody against human erythrocyte p55. Recombinant GST fusion protein containing the SH3 and GUK domain construct of human erythrocyte p55 was injected into mice. The immune response was monitored using a His-tagged fusion of SH3-GUK domain construct.

The 2G4 monoclonal works fine in immunoprecipitation and immunocytochemistry applications. The isotype of this monoclonal is IgG2b. The epitope of this antibody is located within the guanylate kinase-like (GUK) domain of p55.

Immunohistochemistry of transverse section of rat sciatic nerves. Samples of rat sciatic nerve transverse section were stained with anti-p55 monoclonal antibody, anti-NF2 polyclonal (A-19, Santa Cruz Biotechnology), along with markers for both myelin forming and non-myelin forming Schwann cells (S100), for axon (NF-M), and for non-myelin forming Schwann cells (GFAP). The NF2 protein co-localized with S100 and GFAP, but not with NF-M (Fig. 1, A, B, C), confirming its expression in Schwann cells. P55 did not co-localize with NF-M (Fig. 1, D), and co-localized with GFAP (Fig. 1, E). These results suggest that p55 is preferentially expressed in non-myelin forming Schwann cells in rat sciatic nerve, and not in axons.

Immunohistochemistry of mouse nerves teased fibers. NF2 was stained along with Caspr, which is the marker for paranode, in teased fibers of mouse nerves. Prominent staining of NF2 was observed at Schmidt-Lanterman incisures as well as paranode (Fig. 1, F) as previously reported. Double staining of p55 and NF2 shows extensive co-localization of two proteins in non-myelin forming Schwann cells (Fig. 1, G). In myelin-forming Schwann cells, the expression of p55 was not detected. In conclusion, p55 and NF2 co-localize in non-myelinated fibers in mouse nerves.

Progress report-Year 4: This was a no-cost extension period. We completed some final immuno-localization experiments, started characterization of the p55 knockout mice, and currently writing up the new data for publications.

KEY RESEARCH ACCOMPLISHMENTS

- NF2 protein migrates as a single polypeptide of ~ 70 kDa as detected by SDS-PAGE and Western blotting.

- Complete solubilization of the NF2 protein from cytoskeletons with 0.5% Triton X-100 in the presence of 500 mM KCl but not in the absence of Triton X-100 suggests that the NF2 protein may directly interact with membrane lipids in the erythrocyte plasma membrane.

- Release of the bulk of NF2 protein by extraction of IOVs in 1.0 M potassium iodide suggests that the NF2 protein is a peripheral protein that is tightly associated with the plasma membrane.

- Based on the biochemical properties of the NF2 protein, two purification strategies are designed. Approximately, 30,000 copies of NF2 protein are present in each erythrocyte.

- The presence of NF2 protein in the membranes of mutant red cells suggests that the major skeletal membrane proteins such as protein 4.1, glycophorin C, ankyrin, alpha spectrin, band 3, and protein 4.2 are not required for the binding of NF2 protein to the plasma membrane. This result implies that novel binding partner(s) for NF2 protein may exist in the human erythrocytes.

- The MBP fusion proteins of N- and C- halves of NF2 protein were expressed in bacteria and purified in soluble form.

- NF2 protein binds to erythrocyte membrane protein p55 by *in vitro* binding assay and BIAcore Surface Plasmon Resonance measurements. The amino-terminal FERM domain of NF2 protein encodes the binding site for p55.

- The affinity (K_D) of the interaction between NF2 N and recombinant p55 is 3.7 nM as measured by the surface plasmon resonance assay.

- The development of the p55 monoclonal antibody permitted us to demonstrate the preferential expression of p55 in non-myelin forming Schwann cells in rat sciatic nerves, but not in axons.

- The p55 and NF2 proteins co-localize in non-myelinated fibers in mouse nerves but not in myelin-forming Schwann cells.

REPORTABLE OUTCOMES

(1) Jindal, H.K., Yoshinaga, K., Seo, P.S., Lutchman, M., Dion, P.A., Rouleau, G.A., Hanada, T., and Chishti, A.H. (2006). Purification of the NF2 tumor suppressor protein from human erythrocytes. *The Canadian Journal of Neurological Sciences 33:394-402.*

(2) Pil-Soo Seo, Jong-Jin Jeong, Lixiao Zeng, Christos G. Takoudis, Brendan J. Quinn, Toshihiko Hanada, and Athar H. Chishti (2007). Alternatively spliced exon 5 of the FERM domain of Protein 4.1R encodes a second binding site for p55 and is required for membrane targeting in epithelial cells. Journal of Biological Chemistry (revised manuscript under review).

(3) A new manuscript is under preparation that will report the interaction of p55 with NF2 protein.

CONCLUSION

During the course of this investigation, we identified and purified NF2 protein from human erythrocyte membranes. We demonstrated direct biochemical interaction between p55 and NF2 protein and showed their co-localization in rat Schwann cells. Currently, we are characterizing the phenotype of p55 knockout mice recently generated in our laboratory. These mice show defects in cell polarity and a subset of animals develop the tumorigenic phenotype. We anticipate that further characterization of this new mouse model will provide novel insights into the mechanism of NF2 function in Schwann cells.

REFERENCES

None

APPENDICES

A pdf version of the published manuscript is attached.

ORIGINAL ARTICLE

Purification of the NF2 Tumor Suppressor Protein from Human Erythrocytes

Hitesh K. Jindal, Kazumi Yoshinaga, Pil-Soo Seo, Mohini Lutchman, Patrick A. Dion, Guy A. Rouleau, Toshihiko Hanada, Athar H. Chishti

ABSTRACT: ***Background:*** Neurofibromatosis type 2 (NF2) is an autosomal dominant disease predisposing individuals to the risk of developing tumors of cranial and spinal nerves. The NF2 tumor suppressor protein, known as Merlin/Schwanomin, is a member of the protein 4.1 superfamily that function as links between the cytoskeleton and the plasma membrane. ***Methods:*** Upon selective extraction of membrane-associated proteins from erythrocyte plasma membrane (ghosts) using low ionic strength solution, the bulk of NF2 protein remains associated with the spectrin-actin depleted inside-out-vesicles. Western blot analysis showed a ~70 kDa polypeptide in the erythrocyte plasma membrane. Furthermore, quantitative removal of NF2 protein from the inside-out-vesicles was achieved using 1.0 M potassium iodide, a treatment known to remove tightly-bound peripheral membrane proteins. ***Results:*** These results suggest a novel mode of NF2 protein association with the erythrocyte membrane that is distinct from the known membrane interactions of protein 4.1. Based on these biochemical properties, several purification strategies were devised to isolate native NF2 protein from human erythrocyte ghosts. Using purified and recombinant NF2 protein as internal standards, we quantified approximately ~41-65,000 molecules of NF2 protein per erythrocyte. ***Conclusion:*** We provide evidence for the presence of NF2 protein in the human erythrocyte membrane. The identification of NF2 protein in the human erythrocyte membrane will make it feasible to discover novel interactions of NF2 protein utilizing powerful techniques of erythrocyte biochemistry and genetics in mammalian cells.

RÉSUMÉ: Purification de la protéine codée par le gène suppresseur de tumeur NF2 à partir d'érythrocytes humains. Contexte : La neurofibromatose de type 2 (NF2) est une maladie dominante autosomique qui prédispose au développement de tumeurs au niveau des nerfs crâniens et des nerfs spinaux. La protéine codée par le gène suppresseur de tumeurs NF2, connue sous le nom de Merlin/Schwannomin, fait partie de la superfamille des protéines 4,1 impliquées dans l'interface entre le cytosquelette et la membrane plasmatique. Méthodes : Lors de l'extraction sélective des protéines associées à la membrane cellulaire d'érythrocytes plasmatiques (fantômes d'hématies) au moyen d'une solution dont la force ionique est faible, la majeure partie de la protéine NF2 demeure associée aux vésicules inversées dépourvues de spectrine-actine. L'analyse par buvardage Western a démontré la présence d'un polypeptide de ~70 kDa dans la membrane plasmatique erythrocytaire. L'élimination quantitative de la protéine NF2 des vésicules inversées a été effectuée au moyen d'iodure de potassium 1,0 M, un traitement qui extrait les protéines membranaires périphériques fortement liées. Résultats : Ces résultats sont compatibles avec un nouveau mode d'association de la protéine NF2 à la membrane érythrocytaire qui est distinct des interactions membranaires connues au sujet des protéines 4,1. Plusieurs stratégies de purification fondées sur ces propriétés biochimiques ont été élaborées pour isoler la protéine NF2 native des fantômes d'hématies humaines. Nous avons quantifié approximativement ~41-65 000 molécules de protéine NF2 par érythrocyte en utilisant la protéine NF2 purifiée et la protéine NF2 recombinante comme standards internes. Conclusion : Ces données sont compatibles avec la présence de la protéine NF2 dans la membrane des érythrocytes humains. L'identification de la protéine NF2 dans la membrane des érythrocytes humains permettra de découvrir de nouveaux modes d'interactions de la protéine NF2 dans les cellules de mammifères au moyen de techniques puissantes de biochimie et de génétique érythrocytaire.

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Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder, characterized by the development of bilateral vestibular and spinal schwannomas, meningiomas, and ependymomas.[1,2] The NF2 gene encodes a 595 amino acid polypeptide known as NF2 protein or Merlin or Schwanomin. The primary structure of NF2 protein is homologous to the ERM family of peripheral membrane proteins, which includes Ezrin, Radixin, and Moesin.[3,4] The founding member of the ERM superfamily is the erythrocyte membrane protein 4.1, which cross-link's spectrin-actin complexes and attaches them to the plasma membrane.[5]

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The ERM family members, including the distant homologues such as talin and some tyrosine phosphatases, are believed to regulate cell surface dynamics by linking the underlying cytoskeleton to membrane receptors. Several mechanisms have been proposed to explain the role of ERM proteins in signaling pathways that regulate cell growth and differentiation.[3,6] More recently, extensive evidence supports a functional role of NF2 protein in the regulation of cell proliferation, motility, and adhesion pathways.[7-12] Despite this progress, the precise molecular function of NF2 protein in the regulation of tumor suppression pathways remains poorly understood. For example, it is not known whether the NF2 protein exists as a monomer, dimer, or self-associates into an oligomer in order to act as a tumor suppressor protein. In addition, it remains unclear whether the NF2 protein undergoes any lipid modifications *in vivo*. Self-association and lipid modifications are common characteristics of many tumor suppressor proteins with important regulatory implications.[13,14] Understanding the biochemical basis of NF2 protein interaction with the plasma membrane and identifying the respective modulators for such interactions will help elucidate the regulatory implications of the NF2 protein in tumor suppression pathways.

However, there are limitations in carrying out biochemical studies of NF2 protein. For instance, since the primary structure of NF2 protein does not encode any known enzymatic domains, most efforts have focused on the identification of binding proteins that interact either with its amino-terminal FERM domain or the carboxyl terminal regulatory domain.[15,16] Nevertheless, genetic manipulations using transfected cells and lysates have demonstrated the existence of an intramolecular "close" and "open" regulatory switch in the NF2 protein.[3,17,18] Additionally, phosphorylation of the NF2 protein by several kinases and degradation of NF2 protein by calpain *in vivo* suggest the importance of NF2 in cellular processes.[19-22] Also, the unavailability of purified NF2 protein from mammalian cells, largely due to the presence of NF2 in Schwann cells has limited the opportunity to obtain biochemically pure NF2 protein in its native form. This is perhaps one reason why the NF2 protein has never been isolated and purified from mammalian cells.

We identified NF2 protein by accident while investigating the syntenin-NF2 protein interactions by Western blot analysis of samples that included purified erythrocyte plasma membrane (ghosts) as a negative control. The identification of NF2 protein in the erythrocyte ghosts gave us an opportunity to purify human NF2 protein in its native state. Also, initial characterization studies suggested a novel association of NF2 protein with the plasma membrane. Combining established knowledge and powerful techniques of erythrocyte membrane biochemistry with the finding of NF2 tumor suppressor in the erythrocyte membrane could provide an unexpected opportunity to develop a simple diagnostic blood test for detecting truncating mutations within the Neurofibromatosis type 2 gene in order to monitor the progression of the disease in clinical settings. Some of these possibilities will be discussed.

EXPERIMENTAL

Antibodies

The anti-NF2 polyclonal antibody was raised in rabbit against the C-terminus peptide of NF2 protein isoform-1.[23] This polyclonal antibody does not detect NF2 protein isoform-2. The anti-NF2 polyclonal antibody (A-19), which can detect an epitope located within the N-terminal segment of NF2 protein, was purchased from Santa Cruz Biotechnology.

Blood samples

Blood samples from protein 4.1 and glycophorin C deficient patients were used as described before.[24] Ankyrin-deficient nb/nb mice, spectrin-deficient sph/sph mice, and band 4.2 null mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, with the assistance of Dr. Luanne Peters. Band 3 knock out mice were generated in our laboratory as previously described.[25]

Recombinant protein expression

The recombinant C-terminal half (amino acids 312-595) of human NF2 isoform-1 was generated by the polymerase chain reaction using human fetal brain cDNA pool (CLONTECH) as template. The cDNA fragment was cloned into pMAL-c2X vector (New England BioLabs) to express the C-terminal NF2 protein segment as a fusion with the maltose-binding protein in *Escherichia coli*.

Preparation of erythrocyte plasma membrane (ghosts)

Freshly obtained erythrocytes from normal human subjects were washed four times with the wash buffer (5.0 mM sodium phosphate, pH 8.0, 150 mM NaCl, and 0.1 mM EGTA). No samples were taken from NF2 patients in order to perform the experiments described here. The buffy coat was removed, and erythrocytes were filtered through the mixed cellulose column packed with 1:1 mixture of alpha-cellulose and Sigma Cell Type 50 to remove the residual leukocytes. The purity of gel-filtered erythrocytes was established by the Coulter counter (CELL DYN 4000) to confirm the removal of white blood cells in the final preparation. Purified erythrocytes were lysed by 20 volumes of the lysis buffer (5.0 mM sodium phosphate, pH 8.0, 0.1 mM EGTA, and 1.0 mM PMSF), and the lysate was centrifuged for 20 minutes at 20,000 x g (Sorvall SS-34 rotor). The resultant membrane pellet (ghosts) was washed several times with the lysis buffer to remove hemoglobin, and ghosts were used the same day to avoid any detrimental effects of the freezing and thawing cycle on the membrane-protein interactions.

Preparation of inside-out vesicles (IOVs)

Erythrocyte ghosts were incubated in 30 volumes of 0.1 mM EGTA, pH 8.5, at 37ºC for 30 minutes. This incubation step results in the extraction of spectrin, actin, and spectrin-actin-4.1 complexes from the plasma membrane. The membrane pellet containing sealed inside-out vesicles was washed with 5.0 mM sodium phosphate pH 8.0, 0.5 mM EGTA, and vesicles were collected by centrifugation at 17,000 rpm (Sorvall SS-34 rotor) for 30 minutes. IOVs were stripped of remaining peripheral membrane proteins by incubation with 30 volumes of 0.1 mM EGTA, pH 11.0, at 25ºC for 20 minutes. The stripped vesicles were collected by centrifugation at 17,000 rpm (Sorvall SS-34 rotor) for 30 minutes, and washed twice with 5.0 mM sodium phosphate, pH 8.0, 0.5 mM EGTA. The alkaline-stripped vesicles essentially contained the transmembrane proteins such

as band 3 and glycophorins, but may have also retained some tightly associated peripheral membrane proteins.

Extraction of NF2 protein from ghosts and IOVs

Erythrocyte ghosts were incubated in the solubilization buffer (50 mM Tris-HCl, pH 7.5, 1.0 mM EGTA, 1.0 mM EDTA, and protease inhibitors) containing either 0.5% Triton X-100 or no detergent in the presence of increasing concentration of KCl. Incubations were carried out on ice for 60 minutes, and pellet was collected by centrifugation at 35,000 rpm (Beckman Type 42.2 Ti rotor) for 60 minutes. The supernatant was discarded and the membrane pellet was analyzed by 10% SDS/PAGE. Alternatively, the NF2 protein was extracted from IOVs by incubation with a solution of 1.0 M potassium iodide containing 0.5 mM EGTA, pH 8.0, for 60 minutes at 23ºC.

Purification of NF2 protein

Ghosts were extracted in the solubilization buffer (as described above) containing 0.5% Triton X-100 on ice for 60 minutes. The detergent-extracted skeletal fraction (Triton shells) was dissociated by incubation with 10 volumes of low ionic strength buffer (3.0 mM Tris-HCl, pH 8.5, 0.5 mM EDTA, 2.0 mM DTT) for 45 minutes at 37ºC followed by centrifugation at 40,000 rpm for 30 minutes (Beckman Type 42.2 Ti rotor). The supernatant, containing NF2 protein and other cytoskeletal proteins, was fractionated on a DEAE-Sephacel column (2.5 x 50 cm) pre-equilibrated with the column buffer (20 mM Tris-HCl, pH 8.3, 1.0 mM EGTA, 1.0 mM DTT, and 20 mM KCl). Proteins that bound to the anion exchange column were eluted using a linear gradient of 20-500 mM KCl (800 ml total volume) in the column buffer. Fractions enriched in NF2 protein, as assessed by Western blot analysis, were pooled, dialyzed against the column buffer, and further purified on a Mono Q column (FPLC). The Mono Q column was developed with a linear gradient of 20-400 mM KCl (50 ml total volume) in the column buffer and fractions containing purified NF2 protein were pooled, dialyzed, and concentrated using an Amicon membrane concentrator.

Quantification of NF2 protein in human erythrocytes

Two complementary approaches were used to determine the amount of NF2 protein in human erythrocytes. In the first approach, quantitative Western blotting was performed using ^{125}I-Protein A to detect antibodies bound to NF2 protein. Ghosts were isolated from a precise number of human erythrocytes, and analyzed by SDS-PAGE and Western blotting assays. Bands corresponding to NF2 protein were quantified using scintillation counting, as well as densitometric scanning of the Western blots. Purified NF2 protein, isolated from human erythrocyte ghosts, was used as a positive standard to calculate the amount of NF2 protein in ghosts. Increasing amount of purified NF2 protein was loaded, and the intensity of each band was compared with the NF2 protein signal from a pre-determined number of human erythrocytes. Alternatively, a recombinant C-terminal segment of NF2 protein was generated and purified, and used as a positive control to quantify the amount of NF2 protein in human erythrocytes. Experimentally, 6.0 ml of freshly obtained human blood was collected in the ACD anticoagulant solution and erythrocytes free of leukocytes were obtained as described above. Ghosts were washed several times to remove hemoglobin, and used immediately for protein quantification. Protein concentration was determined either using Bradford protein assay (BioRad) or by densitometric scanning of the Coomassie-stained gels using BSA as control. Gel images were recorded using the BioRAD Gel Doc System and processed with the Quantity One Software.

SDS-PAGE and Western blotting

Proteins were resolved by 10% SDS-PAGE and blotted onto the nitrocellulose membrane. The nitrocellulose membrane was blocked with the blocking buffer (6% casein, 1% polyvinyl pyrrolidone-40, 10 mM EDTA in phosphate buffered saline, pH 7.4), and incubated with an anti-NF2 antibody at 1:1,000 dilution in the blocking buffer for two hours at room temperature. Blots were washed with the TBS-T buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20), and incubated for one hour with goat anti-rabbit IgG-horseradish peroxidase at 1: 1,500 in the blocking buffer. After repeated washings with TBS-T, the NF2 protein on the membrane was visualized using the ECL blot detection system (GE Healthcare).

Immunocytochemistry

Leukocyte-free erythrocytes were centrifuged at 800 rpm onto Probe-On microscope glass slides (Baxter) using a Cytospin 3 (Shandon USA) for 5.0 minutes. Erythrocytes were washed once in the phosphate buffered saline (PBS), and then fixed with 4% formaldehyde for 5.0 minutes. This step was followed by three 5.0 minute washes in PBS. Immobilized erythrocytes were permeabilized with 0.1% Triton X-100 in PBS for 5.0 minutes and washed again with PBS for 5 minutes. Slides were then rinsed twice in PBS for 5.0 minutes at room temperature, blocked for one hour at room temperature in PBS/1% BSA/1% normal goat serum, and washed in PBS for 5.0 minutes. The anti-NF2 polyclonal antibody (1:100 dilution in PBS) was placed on the slides and incubated for one hour at room temperature. Slides were then washed in PBS prior to incubation with the goat anti-rabbit FITC (Sigma). Slides were mounted in antifade solution (BioRad) and viewed with a Zeiss inverted Microscope (Magnification X 1000). Photomicrographs were generated using a CCD camera (Cooke Corporation, USA), and digital images were processed using Phase 3 Imaging Software (Phase 3 Imaging USA).

RESULTS

Expression of NF2 protein in mature erythrocytes

We identified NF2 protein in mature erythrocytes, while using erythrocyte plasma membrane as a negative control during our previous studies of NF2 protein interactions with the PDZ domains of syntenin.[16] To rule out the possibility of contamination from other white blood cells, we purified erythrocytes using a mixed cellulose resin to remove traces of leukocytes. The absence of white blood cells was confirmed by assessing the purity of erythrocytes by Coulter counter measurements, as described in the Experimental section. Human erythrocyte ghosts were prepared from freshly drawn blood and analyzed by Western blotting using an anti-NF2 polyclonal

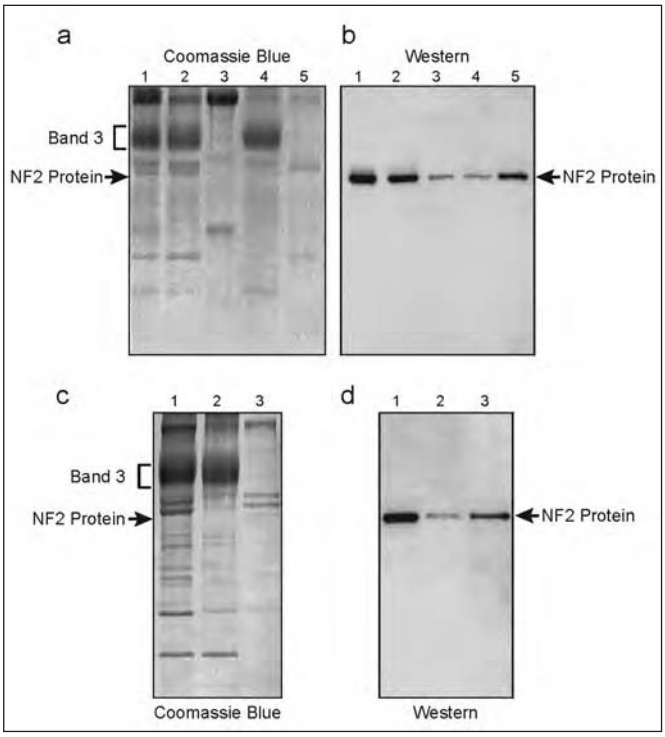

Figure 1: *Detection of NF2 protein in human erythrocytes. (a) Coomassie blue staining of human erythrocyte membrane fractions. 10% SDS-PAGE. (b) Western blot analysis using anti-NF2 C-terminus polyclonal antibody. Lanes 1 (ghosts), 2 (IOVs), 3 (supernatant of IOVs enriched in spectrin and actin), 4 (IOVs stripped at pH 11), 5 (supernatant of pH 11 extraction). The bulk of NF2 protein remains associated with IOVs (lane 2). A significant amount of NF2 protein is extracted after incubation of IOVs at pH 11 (lane 5). (c) Coomassie blue staining of IOVs extracted with 1.0 M potassium iodide. (d) Western blot analysis. Lanes 1 (IOVs), 2 (IOVs extracted with 1.0 M KI), 3 (1.0 M KI supernatant). The bulk of NF2 protein was released from the IOVs after extraction with 1.0 M potassium iodide.*

antibody directed against the C-terminus of NF2 protein.[23] A single polypeptide of 70 kDa was detected in the human erythrocyte ghosts (Figure 1a & b). Although a corresponding band at ~70 kDa is not discernible by Coomassie staining of the ghosts with moderate protein loading, as shown in Figure 1a (lane 1), a detectable band of ~70 kDa is visible in overloaded protein gels (data not shown). We confirmed the presence of NF2 protein by Western blotting using a second antibody directed against the N-terminus of NF2 protein. Moreover, the anti-peptide polyclonal antibody directed against the C-terminus of NF2 protein recognizes only the isoform-1 of NF2 protein and not isoform-2. At this stage, it remains unknown whether the NF2 protein isoform-2 exists in erythrocytes. It is relevant to mention here that only NF2 protein isoform-1 contains the binding sequence for the PDZ domains of syntenin, and has been shown to be a tumor suppressor in the transformation assays.[16, 26] To further confirm the presence of NF2 protein in mature erythrocytes, we examined intact erythrocytes by an immunofluorescence assay using a polyclonal antibody against the C-terminus of NF2 protein. Specific staining of NF2 protein was restricted to the plasma membrane compartment of permeabilized erythrocytes with no detectable signal in the cytosol (data not shown). Together, these results demonstrate that the mature erythrocytes contain NF2 protein isoform-1 as a single polypeptide of 70 kDa associated with the plasma membrane.

NF2 protein is tightly associated with the erythrocyte plasma membrane

Intact erythrocytes were lysed in the hypotonic solution to separate cytosol fraction from the plasma membrane by high speed centrifugation. Western blotting failed to detect any trace of NF2 protein in the cytosol fraction, whereas the anti-NF2 reactive polypeptide was exclusively associated with the plasma membrane. To further investigate the NF2 protein-membrane interactions, inside-out-vesicles (IOVs) were prepared from erythrocyte ghosts under low ionic strength solution conditions. This step removed the bulk of spectrin, actin, and spectrin-actin-protein 4.1-protein 4.9 complex from erythrocyte ghosts (Figure 1a, lanes 2, 3). Western blotting indicated that the majority of NF2 protein remains associated with the IOVs (Figure 1b, lane 2). Since the IOVs still contain some peripheral membrane proteins, which could be dissociated by further extraction with an alkaline solution of pH 11, we examined the elution of NF2 protein under these conditions (Figure 1a, lanes 4, 5). Western blotting indicates that ~90% of NF2 protein is released from IOVs by extraction with a solution of pH 11 (Figure 1b, lane 5). It is of interest to note that a relatively small pool (~5-10%) of NF2 protein still remained associated with the IOVs even after extraction with the pH 11 solution (Figure 1b, lane 4). In addition, the IOVs were extracted with a solution of 1.0 M potassium iodide, a treatment that is known to remove tightly-bound peripheral membrane proteins (Figure 1c, lanes 2, 3). Indeed, extraction of IOVs with potassium iodide released ~85% NF2 protein from the vesicles, but again could not remove a small pool of tightly associated NF2 protein (Figure 1d, lane 2). Together, these results indicate that the NF2 protein is tightly associated with the cytoplasmic face of the erythrocyte membrane.

Association of NF2 protein with the erythrocyte membrane skeleton

To investigate the nature of NF2 protein association with the erythrocyte membrane skeleton, ghosts were extracted with 0.5% Triton X-100, a non-ionic detergent, and the skeleton-associated NF2 protein was quantified by Western blotting. Sequential extraction of ghosts with Triton X-100 was carried out in the presence of increasing concentration of potassium chloride, as shown in Figure 2a. Interestingly, the NF2 protein remains associated with detergent-extracted skeletal fraction until the salt concentration is raised to 500 mM (Figure 2b, lane 5). Solubilization of ghosts with 0.5% Triton X-100 and 500 mM KCl or NaCl quantitatively released NF2 protein from the detergent-insoluble pellet, a feature akin to the known biochemical properties of lipid modified membrane proteins such as p55 and protein 4.2.[27,28] Since the NF2 protein remains associated with the detergent-extracted skeletal fraction prepared in the presence of 150 mM salt (Figure 2b, lane 4), we attempted to solubilize the NF2 protein by high salt in the absence of Triton

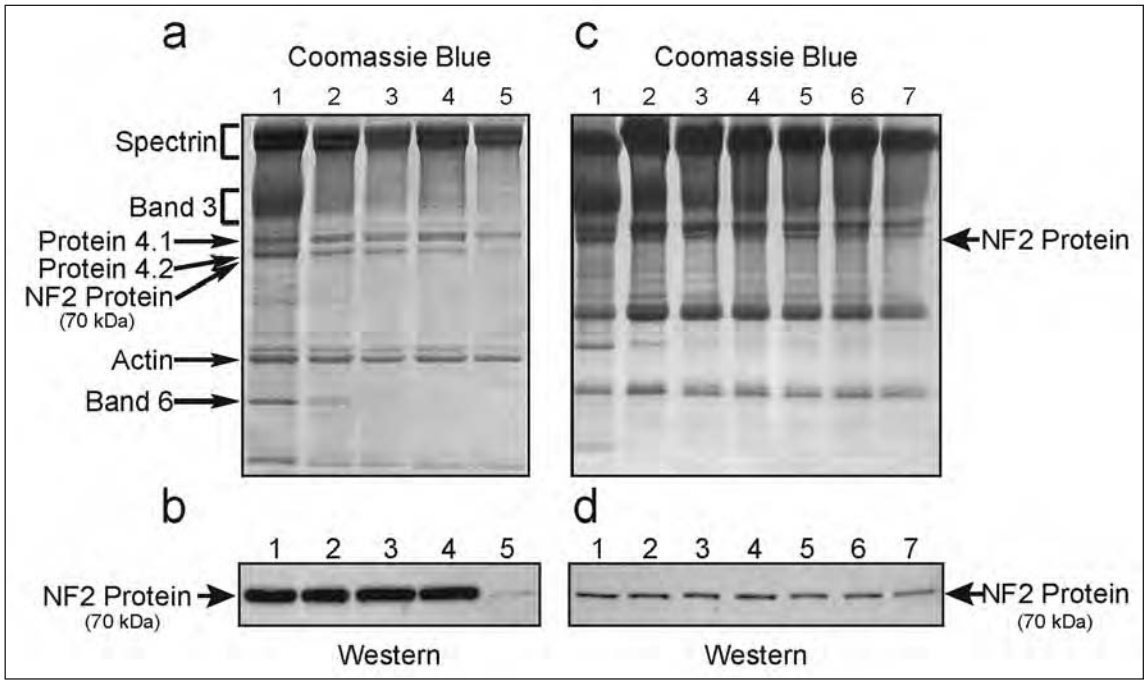

Figure 2: *Triton X-100 mediated solubilization of human erythrocyte ghosts. (a) Coomassie blue staining of ghosts. 10% SDS-PAGE. (b) Western blot analysis using anti-NF2 C-terminus polyclonal antibody. Solubilization buffer (SB) contains 50 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM EGTA, and protease inhibitors. Lanes 1 (ghosts), 2 (ghosts extracted with 0.5% Triton X-100 in SB), 3 (same as lane 2 plus 50 mM KCl), 4 (same as lane 2 plus 150 mM KCl), 5 (same as lane 2 plus 500 mM KCl). Note that the NF2 protein remains associated with the cytoskeletal fraction after extraction of ghosts with Triton X-100 in the presence of 150 mM salt. However, extraction of ghosts with 0.5% Triton X-100 containing 500 mM KCl completely solubilized the NF2 protein. (c) Coomassie blue staining of ghosts and cytoskeletal fraction. Ghosts were extracted with 0.5% Triton X-100 containing 150 mM KCl. Cytoskeletal pellet was recovered by centrifugation on a cushion of sucrose. Detergent-free cytoskeleton was then extracted with an increasing concentration of salt. Lanes 1 (ghosts), 2 (cytoskeleton pellet), 3 (pellet extracted with 200 mM salt), 4 (same as lane 2 plus 400 mM salt), 5 (same as lane 2 plus 600 mM salt), 6 (same as lane 2 plus 800 mM salt, 7 (same as lane 2 plus 1.0 M salt). (d) Western blot analysis using anti-NF2 C-terminus polyclonal antibody. Note that the NF2 protein is not released from the cytoskeletal fraction even at 1.0 M salt in the absence of Triton X-100.*

X-100. Ghosts were extracted with 0.5% Triton X-100 containing 150 mM salt and the skeletal pellet was recovered by centrifugation on a cushion of sucrose. Detergent-free (or reduced) skeletal pellet was then extracted with high concentration of salt. The NF2 protein remained tightly associated with the skeletal fraction, even in the presence of 1.0 M salt (Figure 2c, d). A parallel Western blot analysis indicated that the bulk of ankyrin is released from the skeletal fraction under these conditions (data not shown). Together, these results suggest that the mode of NF2 protein association with the erythrocyte cytoskeleton is unlike proteins such as spectrin, actin, protein 4.1, dematin, and ankyrin, but appears to be similar to the properties of lipid modified p55 and protein 4.2.

Purification of native NF2 protein from human erythrocytes

Guided by the biochemical properties of erythrocyte NF2 protein, as outlined above, we devised a purification strategy to isolate native NF2 protein from human erythrocytes. Since the bulk of NF2 protein remains associated with the Triton X-100 insoluble cytoskeletal fraction, ghosts were extracted with 0.5% Triton X-100 at low ionic strength and the detergent-insoluble pellet was washed to remove excess detergent. The washed cytoskeletal pellet was dissociated at low ionic strength at 37ºC in the absence of any detergent. The dissociated soluble proteins were then fractionated by anion exchange chromatography using DEAE-Sephacel and Mono Q columns, respectively. The elution of the 70 kDa NF2 polypeptide was monitored by Western blotting. From the DEAE-Sephacel column, the 70 kDa NF2 protein begins to elute at a salt concentration that co-elutes p55 and dematin, and the NF2 protein elution continues in fractions containing protein 4.1 (Figure 3a, fractions 25-29, see arrow). Fractions enriched with NF2 protein were pooled, dialyzed, and further purified on a Mono Q column, as described in the Experimental section (Figure 3c, d). Purified NF2 protein was stored in 25% glycerol under frozen conditions. In a typical protein purification protocol, ~500-600 microgram of purified NF2 protein was obtained from one unit of packed erythrocytes. The purified NF2 protein migrated as a single band on Coomassie stained polyacrylamide gels, with an estimated purity of ~95% or greater (Figure 3c).

It is noteworthy that although we have presented only one purification scheme here (Figure 4, scheme #1), in principle the NF2 protein could be isolated from ghosts using at least two other purification schemes. In the second approach (Figure 4, scheme #2), ghosts were extracted with Triton X-100 containing 150 mM salt to remove additional cytoskeleton-associated

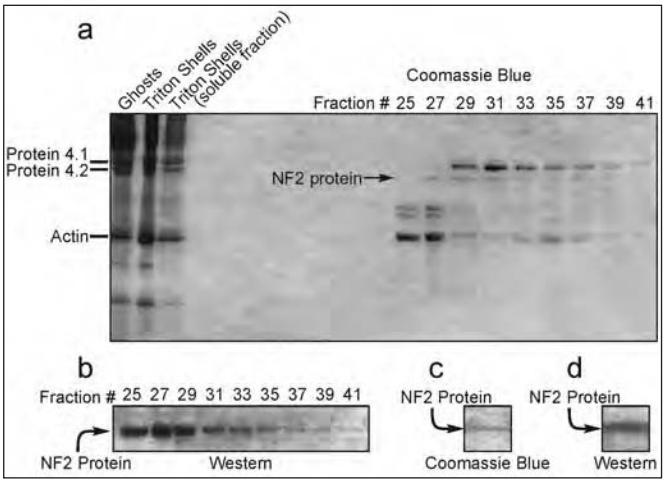

Figure 3: *Purification of NF2 protein from the Triton shells. Triton shells were prepared as described in the Experimental section and dissociated by incubation in 10 volumes of low ionic strength buffer (3.0 mM Tris-HCl, pH 8.5, 0.5 mM EDTA, 2.0 mM DTT) at 37°C. The supernatant was loaded on the DEAE-Sephacel column. (a) DEAE-Sephacel eluted fractions were analyzed by SDS-PAGE, followed by Coomassie blue staining, and (b) Western blot analysis using anti-NF2 C-terminus polyclonal antibody. The DEAE-Sephacel-eluted fractions enriched in NF2 protein were pooled, dialyzed, and loaded on a Mono Q column. Fractions containing purified NF2 protein were pooled, dialyzed, and concentrated. (c) Coomassie blue staining of purified NF2 protein. (d) Western blot analysis of purified NF2 protein.*

proteins. The NF2 protein was released from the detergent-insoluble fraction by further solubilization in a solution of Triton X-100 containing 500 mM salt. Although we did not pursue purification of this material further, the NF2 protein could be isolated from the detergent-soluble fraction using immuno-affinity and hydrophobic chromatography steps. Finally, a third purification scheme was designed to isolate detergent-free NF2 protein from erythrocyte ghosts. Biochemical extraction with 1.0 M potassium iodide released the bulk of NF2 protein from the inside-out-vesicles (Figure 4, scheme #3). Subsequent purification of salt-extracted NF2 protein could be achieved by ion exchange chromatography steps as described above. Together, these purification strategies outline a feasible experimental approach to isolate native NF2 protein of mammalian origin, suitable for various biochemical assays in the future.

Determination of NF2 protein copy number in human erythrocytes

An immunoblot assay was developed to determine the number of NF2 protein molecules in human erythrocytes. Ghosts were prepared from a precisely determined number of human erythrocytes and purified NF2 protein was used as a positive standard. The NF2 protein signal was detected using antibodies specific for both N- and C- termini of NF2 protein. Quantification of bound antibodies by ^{125}I-Protein A estimated ~40,300 molecules of NF2 protein per erythrocyte (Figure 5). Alternatively, a recombinant C-terminal segment of NF2 protein was expressed, and used as a positive control, to quantify NF2 protein in human ghosts. Quantitative immunoblot analysis estimated ~65,000 copies of NF2 protein per erythrocyte. To further confirm the accuracy of the immunoblot assay, we determined the number of p55 molecules in erythrocytes by using a recombinant GST fusion protein linked to the SH3-Guanylate kinase segment of p55.[29] A quantitative immunoblot analysis estimated ~70,000 copies of p55 per erythrocyte. This value compares reasonably well with the previously reported ~80,000 copies of p55 per erythrocyte, which was originally estimated by using native p55 as a positive control.[30]

NF2 protein expression in mutant erythrocytes

Erythrocytes with defined mutations in the membrane proteins have often served as a paradigm to discover novel

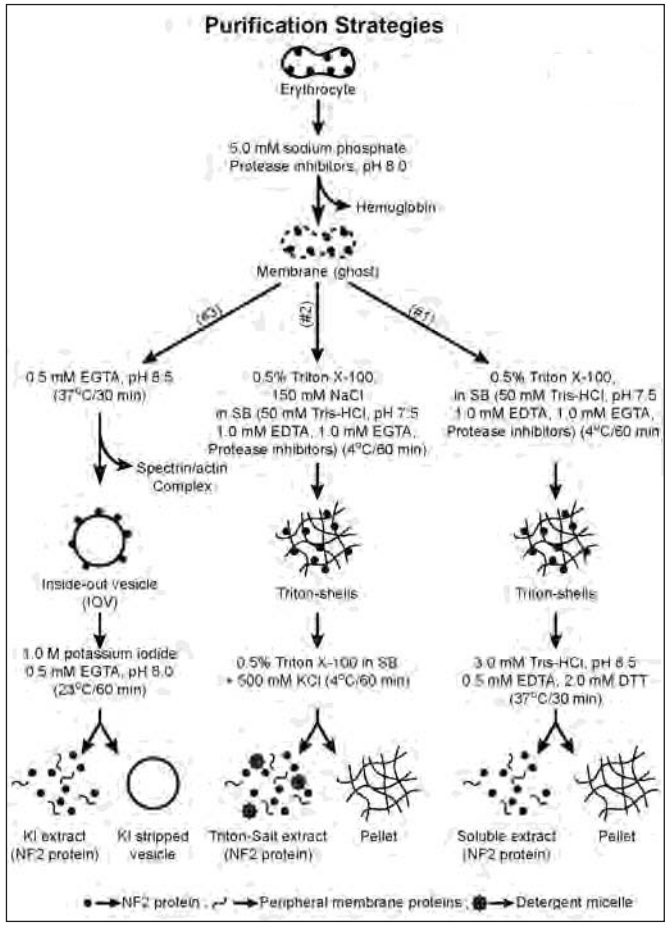

Figure 4: *Schematic flow diagram of various purification schemes for erythrocyte NF2 protein. The steps for the NF2 protein purification shown in Figure 3 are outlined in scheme #1. Scheme #2 outlines a procedure to isolate NF2 protein by releasing the protein in Triton X-100 in the presence of 500 mM salt. This approach may increase the yield of purified NF2 protein substantially. Finally, in scheme #3, an experimental approach is presented where NF2 protein could be isolated without the use of any non-ionic detergents. The yield of purified protein is generally low using this purification scheme.*

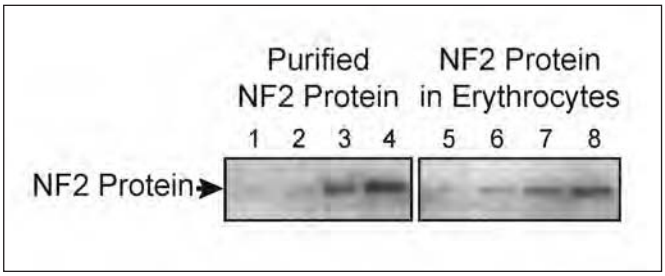

Figure 5: *Determination of the number of copies of NF2 protein in human erythrocytes. Quantitative Western blot analysis was performed using an increasing amount of NF2 protein purified from human erythrocytes. Amount of NF2 protein in lanes 1 (24 ng), 2 (48 ng), 3 (96 ng), and 4 (192 ng). Human erythrocyte ghosts isolated from a fixed number of erythrocytes were loaded in each lane. Lanes 5 (5 x 10^6), 6 (10 x 10^6), 7 (20 x 10^6), and 8 (40 x 10^6). ^{125}I-Protein A was used to detect the amount of NF2 protein-bound antibodies, and bands corresponding to NF2 protein were quantified by densitometric scanning. A similar analysis was performed using a recombinant domain of NF2 protein (data not shown).*

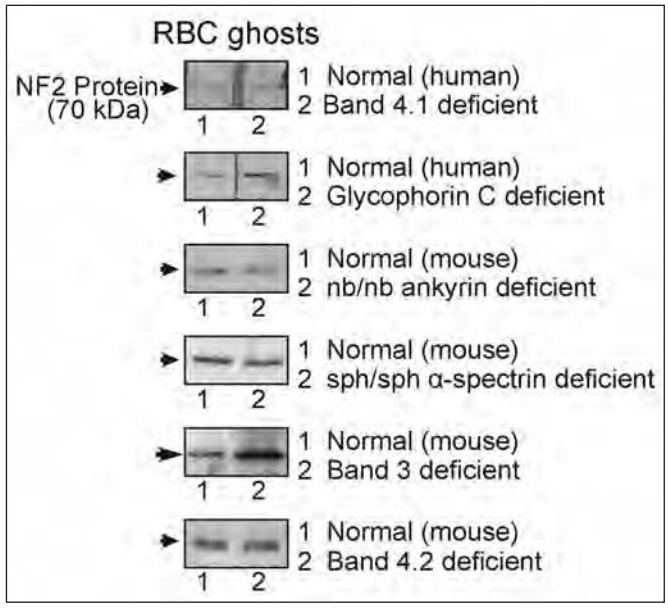

Figure 6: *Western blot analysis of NF2 protein in the mutant erythrocytes. Ghosts were isolated from human/mouse erythrocytes deficient in various proteins. After SDS-PAGE and electro-blotting, the presence of NF2 protein was examined by Western blot analysis. The presence of NF2 protein in the mutant erythrocyte plasma membrane suggests that the binding of NF2 protein to various cytoskeletal proteins is not essential for its retention on the erythrocyte plasma membrane. At this stage, we could not perform a detailed study of the membrane protein interactions of NF2 protein because of the limited quantity of mutant mouse erythrocyte ghosts obtained from various sources.*

protein-protein interactions.[24,31] For example, the loss of p55 in protein 4.1 null human erythrocytes led to the identification of the ternary complex between p55, protein 4.1, and glycophorin C.[24] Similarly, the loss of glycophorin A in band 3 null mouse erythrocytes provided further genetic evidence for the existence of band 3-glycophorin A complex in the erythrocyte plasma membrane.[32] Using a Western blot assay, we examined the expression of NF2 protein in human and mouse erythrocytes lacking protein 4.1, glycophorin C, p55, nb/nb ankyrin mutant protein, alpha spectrin, band 3, and band 4.2. No significant deficiency of NF2 protein was observed in these mutant erythrocytes (Figure 6). It is noteworthy here that the presence of NF2 protein in mutant erythrocyte ghosts does not rule out the possibility of weakened membrane interactions of NF2 protein as a consequence of defined mutations.

Discussion

The cloning of NF2 tumor suppressor gene in 1993 revealed a novel pathway for the regulation of cell proliferation and differentiation cascade presumably by factors acting at the interface of the membrane-cytoskeleton.[33,34] Since then, this paradigm has remained a guiding principle for functional characterization of NF2 protein, also known as Merlin or Schwannomin, in numerous cellular processes. The primary structure of NF2 protein includes an amino-terminal FERM domain and a carboxyl terminal tail domain, thus categorizing the tumor suppressor into the Protein 4.1 superfamily. To date, considerable efforts have been made to identify proteins that interact with NF2 protein and elucidate its function in vitro and in vivo. Despite the intense interest in the signaling pathways of NF2 protein, little is known about the biochemical properties of native NF2 protein. This gap exists mainly because the endogenous NF2 protein has never been purified from mammalian cells. Although the phosphorylation status of NF2 protein has been examined in some detail, it is not known whether its tumor suppressor activity requires regulation by other post-translational modifications such as lipids and protein-protein interactions. With this perception, the identification of NF2 protein as a constituent of the erythrocyte plasma membrane provided a unique opportunity to isolate native NF2 protein of human origin from a well characterized plasma membrane.

This report presents the first identification and purification of NF2 protein from the human erythrocyte plasma membrane. The anti-peptide polyclonal antibody raised against the carboxyl-terminus of NF2 protein recognizes only the isoform-1 of NF2 protein. The NF2 protein isoform-1, which contains a PDZ-binding sequence at the carboxyl-terminus, has been shown to function as a tumor suppressor.[16] In contrast, the NF2 protein isoform-2 lacking a PDZ-binding motif is not active as a tumor suppressor. At this stage it is not known whether the NF2 isoform-2 exists in the erythrocytes. It is of interest to note that the amino-terminus specific polyclonal antibody (A-19) against NF2 protein detected only a single polypeptide in the human erythrocyte ghosts suggesting that the isoform-1 is presumably the dominant polypeptide in the erythrocyte membrane. A single polypeptide of 70 kDa encoding the NF2 protein is associated exclusively with the erythrocyte plasma membrane, and no trace of soluble NF2 protein was observed in the cytosol. The membrane-bound NF2 polypeptide appears to be quite stable,

since no evidence of proteolysis under a variety of membrane extraction and solubilization conditions was found. The NF2 protein remains tightly associated with the plasma membrane, even after the removal of ~90% spectrin and actin from the membrane vesicles (Figure 1). Intriguingly, the NF2 protein remained bound to the detergent-insoluble skeletal fraction (Triton shells) upon extraction with Triton X-100 in the presence of low to moderate ionic strength (Figure 2). In contrast, the NF2 protein was rapidly released from the Triton shells when ghosts were extracted with Triton X-100 in the presence of 0.5 M salt (Figure 2). This biochemical property of NF2 protein is more akin to the known characteristics of erythrocyte p55 and protein 4.2, both of which are lipid conjugated peripheral membrane proteins.[28,30] At this stage, it is not known whether the NF2 protein contains any bound lipid that is required for its interaction with the erythrocyte plasma membrane.

The NF2 protein purification scheme, as outlined in Figures 3-4, was devised largely based on previous experience with the purification of erythrocyte p55 and dematin.[35,36] This experimental strategy was selected because of the observed biochemical similarities between NF2 protein and erythrocyte p55 and protein 4.2. In principle, several alternate purification schemes could be developed in the future, including the isolation of detergent-free NF2 protein, resulting in even higher protein yields (Figure 4). At this stage, we did not explore further purification of NF2 protein from the detergent extract of erythrocyte ghosts at high ionic strength because of the inefficiency of ion-exchange chromatography to achieve NF2 protein purification in the presence of Triton X-100. Quantification of NF2 protein using two independent internal standards revealed ~41,000 and ~65,000 copies of NF2 protein, respectively, in the human erythrocyte plasma membrane. Interestingly, the NF2 protein copy number of ~65,000 determined by the recombinant protein method compares well with the ~70,000 copies of p55, also calculated using the recombinant protein method, present in the erythrocyte membrane. In contrast, our previous estimate of ~80,000 copies of p55 per erythrocyte was determined using native and purified erythrocyte p55 as an internal standard.[30] In summary, the abundance of NF2 protein in human erythrocytes is stoichiometrically comparable to other significant proteins of the plasma membrane.

One rationale to further pursue biochemical characterization of NF2 protein in the erythrocytes comes from the protocol of using the erythrocyte plasma membrane from models of hemolytic anemia to uncover novel binding partners. Since the polyclonal antibody against the carboxyl-terminus of NF2 protein can detect both human and mouse forms, we tested a set of mutant erythrocytes from human and mouse models of hemolytic anemia for the loss of NF2 protein (Figure 6). Although we did not detect any significant loss of NF2 protein in the mutant erythrocytes examined to date, this does not rule out the possibility of independent interactions of NF2 protein with at least some of these proteins such as spectrin, protein 4.2, and p55. Clearly, the identification of NF2 protein in the erythrocyte membrane will now make it feasible to utilize a variety of biochemical approaches to identify novel binding partners in the future.

It is interesting to speculate that the identification of a stable 70 kDa polypeptide encoding the NF2 protein in the erythrocyte membrane may open up new avenues for the development of a simple Western blot-based assay to detect NF2 gene mutations in patients. A modified diagnostic assay could also be developed that can detect truncated NF2 polypeptides in the presence of a normal allele; this is currently not always possible by PCR techniques because truncations can occur at various position of the protein. Combining such information with genetic testing could lead to invaluable insights into the progression of NF2 disease in clinical settings.

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