Fibroblast Growth Factor Homologous Factor 1B Binds to the C Terminus of the Tetrodotoxin-resistant Sodium Channel rNa\textsubscript{v}1.9a (NaN)\textsuperscript{*}

In this study we demonstrate a direct interaction between a cytosolic fibroblast growth factor family member and a sodium channel. A yeast two-hybrid screen for proteins that associate with the cytoplasmic domains of the tetrodotoxin-resistant sodium channel rNa\textsubscript{v}1.9a (NaN) led to the identification of fibroblast growth factor homologous factor 1B (FHF1B), a member of the fibroblast growth factor family, as an interacting partner of rNa\textsubscript{v}1.9a. FHF1B selectively interacts with the C-terminal region but not the other four intracellular segments of rNa\textsubscript{v}1.9a. FHF1B binds directly to the C-terminal polypeptide of rNa\textsubscript{v}1.9a both in vitro and in mammalian cell lines. The N-terminal 5–77 amino acid residues of FHF1B are essential for binding to rNa\textsubscript{v}1.9a. FHF1B did not interact with C termini of two other sodium channels hNa\textsubscript{v}1.7a (hNaNE) and rNa\textsubscript{v}1.8a (SNS), which share 50% similarity to the C-terminal polypeptide of rNa\textsubscript{v}1.9a. FHF1B is the first growth factor found to bind specifically to a sodium channel. Although the functional significance of this interaction is not clear, FHF1B may affect the rNa\textsubscript{v}1.9a channel directly or by recruiting other proteins to the channel complex. Alternatively, it is possible that rNa\textsubscript{v}1.9a may help deliver this factor to the cell membrane, where it exerts its function.

Ten distinct pore-forming α-subunits of sodium channels have been identified in the rat, and homologues have been cloned from various mammalian species including humans (1). Various α-subunits are expressed in a tissue- and developmentally specific manner (2). Aberrant expression patterns or mutations of voltage-gated sodium channel α-subunits underlie a number of human and animal disorders (3–7), including neuropathic pain (8, 9). The α-subunits of sodium channels have been shown to interact with a number of proteins in addition to the auxiliary β subunits of the channel that affect their subcellular localization, their amplitude, and/or their kinetic properties. The C terminus of skeletal muscle and cardiac muscle α-subunits, SkM1 and SkM2, respectively, bind to syntrophin via their PDZ domain, thus linking these channels to the cortical actin network and extracellular matrix through the association of syntrophin with the dystrophin-associated protein complex (10). Brain α-subunits are phosphorylated at specific serine/threonine sites by protein kinase A that is anchored to the α-subunit via AKAP15 (11, 12). Receptor protein-tyrosine phosphatase (RPTPβ) interacts with brain α-subunits via its carbonic anhydrase homology extracellular domain and its intracellular phosphate domain and phosphorylates tyrosine residues of the channel (13).

Peripheral sensory neurons in the dorsal root ganglia (DRG)\textsuperscript{1} and trigeminal ganglia produce pharmacologically and physiologically distinguishable Na\textsuperscript{+} currents, some of which are sensitive to the neurotoxin tetrodotoxin (TTX-S), and others which are resistant to this toxin (TTX-R). The TTX-R Na\textsubscript{v}1.9a (NaN) sodium channel is preferentially expressed in nociceptive neurons (14), and its expression is modulated in response to axotomy (14) and inflammation (15). The Na\textsubscript{v}1.9a channel produces a persistent TTX-R Na\textsuperscript{+} current that activates at potentials of −60 to −70 mV and is predicted to contribute to setting the resting membrane potential and modulating sub-threshold electrogensis in these neurons (16–18).

The yeast two-hybrid (Y2H) screening system (19) has proven to be an effective tool in identifying cytosolic partners of ion channels including voltage-gated K\textsuperscript{+} channels (20), AMPA receptors (21), the ε2 glutamate receptor (22), and voltage-gated Na\textsuperscript{+} channels (23). To identify proteins that interact with the rNa\textsubscript{v}1.9a C terminus, we screened brain Y2H cDNA library using a construct that encodes the C-terminal 198 residues of rNa\textsubscript{v}1.9a as bait. These experiments identified fibroblast growth factor homologous factor 1B (FHF1B), which belongs to a subset of the FGF family (24), as a binding partner of rNa\textsubscript{v}1.9a. It is possible that this interaction may help in exporting FHF1B to the exterior face of the cell membrane, or it may modulate the properties of rNa\textsubscript{v}1.9a directly or indirectly by recruiting other factors to the channel complex.

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\textsuperscript{1} The abbreviations used are: DRG, dorsal root ganglia; TTX, tetrodotoxin; TTX-R, tetrodotoxin-resistant; Y2H, yeast two-hybrid screening; FGF, fibroblast growth factor; FHF, FGF homologous factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; DBD, DNA binding domain; AP, activation domain; UR, unique region; CR, conserved region; SD, synthetic dropout base.

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EXPERIMENTAL PROCEDURES

Antibodies and Mammalian Cell Lines—The preparation and purification of an antisera to rNav1.9 (NaN) has been previously described (25). Anti-GFP and anti-Myc polyclonal antibodies were purchased from CLONTech. Human embryonic kidney (HEK) 293 cells and mouse NIH3T3 fibroblasts were used.

Plasmids and Clones—Yeast expression vectors pDBleu (Life Technologies) and pDBblu (Life Technologies) are fusion vectors for the linkage of proteins to the Gal4 DNA binding domain and to the VP16 transactivation domain, respectively. The segment encoding the C terminus of NaN (aa 1588–1765; GenBank™ accession number AF059030) was amplified by PCR and cloned in-frame into the SalI/NotI sites of pDBleu: CR1+CR2 (aa 1588–1727; pDB-NaNCR1CR2, CR1 (aa 1588–1658; pDB-NaNCR1), CR2 (aa 1658–1727; pDB- NaNCR2), and UR (aa 1727–1765; pDB-NaNUR). Fragments encoding other cytoplasmic components of this channel were also cloned into the pDBleu vector: N terminus (aa 1–124; pDB-NANL1, L1 (aa 398–564); pDB-NANL2, L2 (aa 802–1029; pDB-NANL2), and L3 (aa 1288–1340; pDB-NANL3). Similarly, cdna fragments encoding the C termini of NaN, 1.8 (SNS) (aa 1725–1957; GenBank™ accession number X92184) and hNaN (1.7 (hNaN) (aa 1753–1977; GenBank™ accession number X82835) sodium channel were cloned in-frame into the SalI/NotI sites of pDBleu vector to produce plasmids pDB-SNSC and pDB-hNEC, respectively. The full coding sequence of rat FHF1B was cloned in-frame into the SalI/NotI sites of pCP66 vector to generate plasmid pCP66-FHF1B.

The bacterial expression vector pGEX-3X (Life Technologies) was used to produce recombinant proteins in E. coli. Plasmid pGEX-NaNN (N terminus, aa 1–124); pGEX-NaNL1 (L1, aa 398–564); pGEX-NaNL2 (L2, aa 802–1029); pGEX-NaNC (C terminus, aa 1588–1765); pGEX-NaCR1CR2, pGEX-NaCR1, pGEX-NaCR2, and pGEX-NaNUR (see above for coordinates) were transformed into E. coli DH5α. The fusion proteins were purified on glutathione-agarose beads as previously described (30).

Expression and Purification of Glutathione S-Transferase (GST) Fusion Proteins—For expressing GST fusion proteins, the appropriate plasmids (pGEX-NaNN (N terminus, aa 1–124); pGEX-NaNL1 (L1, aa 398–564); pGEX-NaNL2 (L2, aa 802–1029); pGEX-NaNC (C terminus, aa 1588–1765); pGEX-NaCR1CR2, pGEX-NaCR1, pGEX-NaCR2, and pGEX-NaNUR (see above for coordinates)) were transformed into E. coli BL21 (DE3) competent cells. The fusion proteins were purified on glutathione-agarose beads as previously described (30).

In Vitro Binding Assay—To examine the binding of FHF1B and its derivatives to the C terminus of NaN in vitro, glutathione-Sepharose beads (50 μl) preincubated with purified GST (0.5 μg), serving as control, or GST-NaN (0.5 μg) were incubated with extract (500 μg of protein) of HEK293 cells transfected with an expression plasmid encoding either full-length FHF1B-GFP or its derivatives (i.e. FHF1B-l1–1511-GFP, FHF1B-l2–1511-GFP, FHF1B-l7–1511-GFP, and FHF1B-l143–1511-GFP, as indicated, in 150 μl of buffer AM (10 mm Tris, pH 7.9, 10% glycerol, 1 mm MgCl2) (31) supplemented with 100 mm KCl and 0.5 mg/ml bovine serum albumin (30). The bound proteins were denatured in sample buffer and separated by 12% SDS-PAGE, and GFP fusion proteins were detected by immunoblotting with anti-GFP antibodies (CLONTECH). Similarly, GST fusion proteins of C terminus derivatives (0.5 μg) were analyzed in this binding assay to further delineate the sequence in the C terminus of NaN that is responsible for the interaction with FHF1B.

Expression of FHF1B and its derivatives in HEK293 cells was also performed. The HEK293 cell lines were transfected with plasmids pEGFP-N1 and pEGFP-N1-C1 to produce plasmid pEGFP-N1-C1. A C-terminal tag of NaN was also expressed with a His6 tag. The His6-tagged NaN fusion protein was purified from cell lysates by Ni-NTA affinity chromatography.

Two-hybrid Assay for Interacting Proteins in Mammalian Cells—Components of the mammalian two-hybrid assay (Promega) were used to study the interaction of FHF1B and the C terminus of NaN in NIH3T3 cells. NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 unit/ml antibiotics. When cultures reached about 50% confluence, the cells were transfected with 1 μg each of the indicated plasmids using Lipofectin (Life Technologies) according to the manufacturer’s recommendations. Plasmid pUC19 was used as a carrier to bring the total amount of DNA in the transfection solution to 3 μg. The cultures were harvested 48 h after transfection and lysed in buffer containing 0.2 mm Tris/SDS (pH 7.9). Protein extracts were prepared and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were probed with primary antibody diluted 1:1000 in blocking solution and then detected using either chemiluminescent or colorimetric detection methods as described.

Yeast Two-hybrid (Y2H) Library Screen—Plasmid pDBNaNC (see above) was used as bait to screen rat brain cDNA library (pC86) by colony hybridization. The rat brain cDNA library in pC86 was then transformed into the resultant Leu+ yeast strain and plated on medium lacking tryptophan, leucine, histidine, and uracil but containing 25 mg 3-aminotriazole (SD/3AT) to select for clones that interacted with the Gal4 DNA binding domain and to the other Yeast Two-hybrid system.

Asymmetric two-hybrid assays were performed by co-transfecting NIH3T3 cells with the mammalian expression plasmids pEFB1GP and pDBNaNC, which expresses a bait plasmid and a target plasmid, respectively. All yeast two-hybrid plasmids were further verified for interaction with bait by repeating the Y2H assay.

Expression of Proteins Produced by Yeast—The yeast strains by which the reporter gene assay was performed are listed in Table II. The yeast strains were grown in synthetic complete (SC) medium lacking uracil (SD/uracil), leucine (SD/leucine), his, and tryptophan (SD/tryptophan) supplemented with 200 μg/ml adenine sulfate (SD/ara/200 μg/ml adenine sulfate). Two-hybrid interactions were scored using the yeast strain MAV203. A yeast strain containing an intact lacZ reporter gene and the HIS3 reporter gene at 20°C. Yeast strain MAV203 was transformed with the plasmids pDBleu-FHF1B and pDBleu-NaNUR. The cultures were grown at 30°C for 7 days at 30 °C, colonies were screened for β-galactosidase by a filter lift assay (28). Individual pCP66 recombinant plasmids were further verified for interaction with yeast by repeating the Y2H assay.
vested 48 h later, and cell extracts were prepared according to Liu et al. (33). After a 1-h incubation with either anti-Myc (25 μg/ml) (CLONTECH) or anti-NaN (20 μg/ml) antibodies, aliquots (200 μl) from this mixture were incubated with 30 μl of protein A-agarose (Life Technologies) at 4 °C overnight. After washing five times with immunoprecipitation buffer (34), the bound proteins were released by boiling in 20 μl of 2× SDS loading buffer for 3 min (30). The released proteins were examined by Western blotting with anti-GFP antibodies (CLONTECH), and the signal was detected using the ECL chemiluminescent system (Amersham Pharmacia Biotech).

RESULTS

Identification of FHF1B as a Binding Partner of rNav1.9a—The tetrodotoxin-resistant (TTX-R) sodium channel rNav1.9a (NaN) is expressed preferentially in peripheral sensory neurons and is down-regulated after axotomy (14). This channel is also expressed at low levels in central nervous system tissues (14, 35). A screen based on the yeast two-hybrid (Y2H) system (19) was performed to identify proteins that interact with FHF1B and other members of the FHF1 subfamily. Stars represent residues that are identical to those of rFHF1B. The bipartite components of the nuclear localization signal in FHF1A are underlined. h, human; m, mouse; g, chicken; and r, rat.

A

| 1 | MESKEPQLKQIVVTRLSQSGYFLQGMFDDGTDGDNTDNYLTFLNIFVGLRVVAVQGVK |
| 61 | ASLYVAMNQEGYLYSSDVFTEPCKFKESVFENYYVIYSSLYRQESGRANFLGLNKEGQ |
| 121 | IMGNRIKTHPSSIEFVPIEVMREPLSHEIGEKQGRSKSSGTTMMGKVNQDS |
| 181 | T |

B

Fig. 1. Sequence of rat FHF1B. A, deduced amino acid sequence of rFHF1B. The sequence of rFHF1B was deposited into GenBank (accession number AF348446). The FGF family signature motif is in boldface type. Recognition sites of multiple protein kinases were identified in this sequence: protein kinase A site (RKSS, aa 182–185); PKC sites (SGR, aa 107–109; SRK, aa 161–163); casein kinase 2 (DKDE, aa 34–37; SVFE, aa 88–91; SLHE, aa 150–153). B, amino acid sequence alignment of rFHF1B and other members of the FHF1 subfamily. Stars represent residues that are identical to those of rFHF1B. The bipartite components of the nuclear localization signal in FHF1A are underlined. h, human; m, mouse; g, chicken; and r, rat.

A Y2H rat brain cDNA library (Life Technologies) was screened with the construct encoding the cytosolic C terminus of rNav1.9a (aa 1588–1765). We screened about 5 million clones and identified 23 clones that activated the three reporter genes. Further tests involved the re-transformation of yeast with the purified target plasmids and bait. Only 2 of the original 23 yeast clones expressed full-length proteins that interact with the C-terminal bait (not shown). The plasmids in these clones contained 2.96-kilobase pair identical inserts.

Sequence Analysis of Full-length rFHF1B—The sequence of the two inserts matched that of the chicken fibroblast growth factor homologous factor gFHF1B (36) and represents the rat homologue of this factor (Fig. 1). This factor belongs to the FGF family of growth factors (24). An open reading frame of 546 base pairs encodes a polypeptide of 181 amino acids with a predicted mass of 20 kDa (Fig. 1A). The rat factor, rFHF1B, is highly conserved compared with the chicken counterpart with 87% identity at the nucleotide level (not shown) and 97% identity at the amino acid level (Fig. 1B). FHF1B lacks a canonical signal peptide, like the other members of this subfamily (24). Unlike FHF1A, which possesses a nuclear targeting signal as a result of alternative splicing, rFHF1B lacks such a signal (Fig. 1B).

Multiplex PCR of FHF1B and GAPDH was used to investigate tissue distribution of FHF1B in adult rat tissue. FHF1B is...
readily detectable in central and peripheral nervous tissues including cerebrum, cerebellum, spinal cord, DRG, and trigeminal ganglia (Fig. 2). The rFHF1B fragment that is amplified from DRG tissues using these rFHF1B-specific primers shows identical sequence to that obtained from the rat brain cDNA library. This factor is expressed, however, at lower levels in other neural tissues such as superior cervical ganglia and retina, in the cardiac and skeletal muscles, whereas it is undetectable under these amplification conditions in liver, and in the cardiac and skeletal muscles, whereas it is undetectable under these amplification conditions in liver, and kidney (Fig. 2). The tissue distribution of rFHF1B shows an overlap with the expression of rNav1.9a in DRG and trigeminal ganglia (14).

The Y2H assay was repeated to verify the interaction between rFHF1B and the C terminus of rNav1.9a. For this purpose, the plasmid encoding the C terminus of rNav1.9a linked to the Gal4DBD and the plasmid encoding rFHF1B fused to the VP16AD were used to co-transform the yeast strain MAV203. Plasmid pairs encoding c-Jun/c-Fos and Rb/lamin were used as positive and negative protein-protein interaction controls, respectively (Fig. 3A). The interaction between the C terminus polypeptide of rNav1.9a and rFHF1B was verified by β-galactosidase assay and growth phenotype on selective media (Fig. 3A). Like the c-Jun/c-Fos pair, which is known to interact, our assays indicate that rFHF1B interacts with the C terminus of rNav1.9a in yeast, based on the activation of the LacZ reporter gene (Fig. 3A, left panel), growth on the plates lacking histidine and uracil but containing 3-amino-1,2,4-triazole (Fig. 3A, middle panel), and inhibition of growth on plates containing 0.2% 5-fluoroorotic acid (Fig. 3A, right panel).

The other cytoplasmic polypeptides of rNav1.9a (Fig. 3B) were tested for their ability to interact with rFHF1B. Filter-based β-galactosidase assays were used to determine if the co-expression of the various rNav1.9a/Gal4DBD and rFHF1B/VP16AD fusion proteins activated the reporter LacZ gene. As shown in Fig. 3C, rFHF1B selectively interacts with the C-terminal polypeptide of rNav1.9a, among the five intracellular polypeptides tested in this assay.

rFHF-1B Binds Directly to the C-terminal Polypeptide of rNav1.9a.
rNav1.9a in Vitro—To test whether rFHF1B binds to the C-terminal polypeptide of rNav1.9a in vitro, we expressed rFHF1B-GFP fusion protein in HEK293 cells and expressed the C-terminal polypeptide of rNav1.9a as a GST fusion protein (GST-NaNC). Affinity-purified GST and GST-NaNC immobilized on glutathione-Sepharose beads were incubated with cell lysates expressing either GFP or FHF1B-GFP. Purified GST or GST-NaNC did not pull down GFP protein in this assay (Fig. 4A, lanes 1–3). Purified GST did not pull down rFHF1B-GFP fusion protein (Fig. 4A, lane 5), whereas GST-NaNC efficiently pulled down the rFHF1B-GFP fusion protein (Fig. 4A, lane 6).

We used an overlay binding assay to show that rFHF1B binds directly to the C-terminal polypeptide of rNav1.9a. GFP or rFHF1B-GFP proteins were immunoprecipitated with anti-GFP antibodies from lysates of HEK293 cells transfected with the corresponding expression plasmid. These immunoprecipitated proteins were separated by SDS-PAGE electrophoresis, electrotransferred and immobilized onto nitrocellulose membrane, and incubated with affinity-purified GST or GST-NaNC. Binding was then detected using anti-GFP antibodies. Fig. 4B shows that the purified GST-NaNC fusion protein binds to the purified rFHF1B-GFP (lane 2) but not to GFP alone (lane 1); GST did not bind to either rFHF1B-GFP or GFP (not shown).

**FHF-1B binds to the C-terminal polypeptide of rNav1.9a in Vivo**—To test whether rFHF1B binds to the C-terminal polypeptide of rNav1.9a in vivo, we performed a mammalian two-hybrid assay in NIH3T3 cells containing a reporter plasmid in which the expression of the firefly luciferase gene was driven by five Gal4-specific enhancer elements (Fig. 5A). By analogy to the Y2H system, the interaction between the C-terminal polypeptide of rNav1.9a and rFHF1B brings together the Gal4DBD and the VP16AD of the fusion proteins and activates the reporter gene in NIH3T3 cells (Fig. 5A). Fig. 5B shows that these two proteins interact in mammalian cells as well as in yeast. Therefore, any post-translational modifications that may occur in mammalian cells, but that are absent in yeast, do not interfere with the interaction of rFHF1B and the C-terminal polypeptide of rNav1.9a.

The result of the mammalian two-hybrid assay was confirmed by a co-immunoprecipitation assay (Fig. 5C). In this assay, the extracts of cells expressing both rFHF1B-GFP fusion protein (lane 1) and Myc-tagged C-terminal polypeptide of rNav1.9a were first incubated with either anti-Myc or anti-NaNC antibodies, and the immunoprecipitated complexes were counter-tested with anti-GFP antibodies. A clear rFHF1B-specific band was present in the immunoprecipitated complexes brought down by either anti-Myc (lane 2) or anti-NaNC (lane 3) antibodies, demonstrating that rFHF1B binds to the C-terminal polypeptide of rNav1.9a in vivo.

The N-terminal segment (aa 5–77) of rFHF1B is required for association with the C-terminal polypeptide of rNav1.9a—A series of N-terminal deletions of rFHF1B were linked to GFP (Fig. 6A). Pull-down assays involving the immobilized GST (Fig. 6B, lanes 2, 5, 8, 11, and 14) and GST-NaNC (Fig. 6B, lanes 3, 6, 9, 12, and 15) and cell extracts prepared from HEK293 cells transfected with rFHF1B mutants fused to GFP (Fig. 6B, lanes 1, 4, 7, 10, 13) were performed to determine the ability of the various rFHF1B mutants to associate with the C-terminal polypeptide of rNav1.9a. Immunoblotting using anti-GFP antibodies shows that rFHF1B lacking the N-terminal pentapeptide binds GST-NaNC as efficiently as the full-length factor (compare lanes 3 and 6). Deletion of the N-terminal 42 residues significantly reduced the interaction of the two polypeptides (lane 9), whereas removal of 77 residues totally disrupted this interaction (lane 12). Not surprisingly, rFHF1B lacking the N-terminal 143 residues does not show any interaction with GST-NaNC (lane 15). As a negative control, GST did not pull down either of the rFHF1B derivative polypeptides (Fig. 6B, lanes 2, 5, 8, 11, 14). These data show that the N-terminal segment (aa 5–77) contains the molecular determinants for the interaction of rFHF1B with the C-terminal polypeptide of rNav1.9a.

Conserved segments of the C-terminal polypeptide of rNav1.9a are important for rFHF1B binding—Alignment of
the C-terminal polypeptides of all mammalian α-subunits (not shown) allowed us to divide this part of rNa,1.9a polypeptide into two conserved regions (CR1 and CR2) of about 70 amino acids each and a terminal unique region (UR) of 38 amino acids. We subcloned fragments encoding the individual conserved and unique regions to identify the molecular determinants of rFHF1B binding. We first carried out Y2H assays using CR1, CR2, CR1+CR2, and UR GAL4DBD baits and full-length rFHF1B fused to VP16AD. These experiments demonstrate that conserved region 1 (CR1, aa 1588–1657) alone or together with conserved region 2 (CR1+CR2, aa 1588–1726) of the C-terminal polypeptide interact with rFHF1B, whereas the
unique region (UR, aa 1727–1765) did not interact with this factor (data not shown). We also used a GST pull-down assay to determine quantitatively the efficiency of the interaction of the various constructs with rFHF1B (Fig. 7A). In these assays, comparable amounts of the various purified GST fusion proteins (Fig. 7C) were preincubated with glutathione-Sepharose beads (50 μl) followed by incubation with cell extracts from HEK293 cells expressing rFHF1B-GFP fusion protein (Fig. 7B, lane 1). The results confirm that the UR was neither capable of binding to this factor (Fig. 7B, lane 7) nor did its removal affect the interaction between rFHF1B and the conserved regions of the polypeptide (Fig. 7B, lane 4). Like the unique region, conserved region 2 alone did not interact with rFHF1B (Fig. 7B, lane 6). The removal of CR2, however, significantly reduced the strength of the interaction of CR1 with rFHF1B (compare lanes 4 and 5 of Fig. 7B). These results suggest that the binding site of rFHF1B straddles the CR1-CR2 junction or that CR2 influences the folding of CR1 such that the molecular determinants of rFHF1B binding are optimally accessible when CR2 is present. Further experiments are needed to distinguish between these two possibilities.

**rFHF1B Binds Specifically to rNav1.9a**—The length of the C-terminal polypeptides of the Na+ channel α-subunits from rat tissues varies from 177 for rNav1.9a to 251 for rNav1.1a (brain type III). Alignment of these polypeptides (not shown) shows that the similarity of the C-terminal polypeptide of rNav1.9a ranges from 50% for rNav1.1a (brain type I) to 56.7% for rNav1.5a (rSkM2). This similarity is 51.1 and 53.4% to rNav1.8a (SNS) and hNav1.7a (hNaNE), respectively, both of which, like Na1.9a, are expressed preferentially in DRG and trigeminal neurons (37, 38). Therefore, we tested whether the C-terminal polypeptides of these two channels are capable of binding the rFHF1B protein in Y2H assays. The data in Fig. 8 shows that rFHF1B selectively binds to the C-terminal polypeptide of rNav1.9a but not to those of rNav1.8a or hNav1.7a.

![Figure 7](http://www.jbc.org/)

**Figure 7.** rFHF1B binds to the conserved region in the C terminus of rNav1.9a. A, schematic diagrams of GST-NaNC fusion proteins used to map the sites of rFHF1B binding on the C-terminal polypeptide of rNav1.9a. The numbers refer to amino acid residues in the C terminus of rNav1.9a. CR1, CR2, and UR stand for conserved region 1, conserved region 2, and unique region, respectively. The strengths of the binding of rFHF1B to the various GST-NaNC segments as revealed in B are indicated. B, binding of rFHF1B by the C terminus of rNav1.9a and its derivatives. Glutathione-Sepharose beads carrying GST and GST-NaNC or its derivatives as indicated were incubated with extracts prepared from HEK293 cells transfected with an expression plasmid encoding rFHF1B-GFP fusion protein, and the bound FHF1B-GFP was detected by immunoblotting with anti-GFP antibodies. The FHF1B-GFP band is indicated. C, expression of free GST or GST linked to the C-terminal polypeptide of rNav1.9a or its derivatives. Samples of affinity-purified GST and GST-NaNC or its derivatives (0.5 μg) as indicated were examined by SDS-PAGE and Coomassie Blue staining. The positions of size markers in kDa are indicated.

![Figure 8](http://www.jbc.org/)

**Figure 8.** rFHF1B selectively associates with the C-terminal of rNav1.9a. The β-galactosidase assay was used to test the interaction between rFHF1B and the C termini of the sodium channels rNav1.9a (NaNC), hNav1.7a (hNEC), and rNav1.8a (SNSC). The known interaction between c-Jun and c-fos serves as a positive control, whereas the lack of interaction of Rb and lamin serves as a negative control. Three independent yeast transformants for each pair of plasmids were transferred onto the nitrocellulose membrane, and the β-galactosidase activity was determined as previously described (28, 29).
DISCUSSION

We used the yeast two-hybrid (2H) screen to identify protein partners of the TTX-R sodium channel rNa1.9a (NaN). We present evidence that rFHF1B, which is a member of the FGF family of growth factors, binds directly to the C-terminal polypeptide of rNa1.9a. This interaction is specific to rNa1.9a and is not seen in other Na+ channels that, like rNa1.9a, are preferentially expressed in DRG and trigeminal neurons. We also present evidence that localizes the binding site to specific segments of rNa1.9a C terminus polypeptide and rFHF1B, respectively. Our results provide the first evidence for a direct link between a growth factor and a voltage-gated sodium channel.

Sodium channels interact with multiple protein partners, affecting their localization and the amplitude and kinetics of the currents that they produce. The β2 subunit is proposed to increase the delivery of pore-forming α-subunits to the cell membrane (39), whereas the β1 subunit modulates the amplitude and rate of inactivation of recombinant α-subunits expressed in Xenopus oocytes (40, 41) and permits interaction of the sodium channel complex with the extracellular matrix proteins tenascin-C and L1 (42–45). Skeletal and cardiac muscle sodium channels interact directly with the cytoskeletal element syntrophin, which may determine their localization (10). The interaction of brain sodium channels with AKAP15 is required for dopaminergic modulation (11). More recently, the C-terminal polypeptide of rNa1.2 (brain type II) was shown to bind calmodulin, suggesting modulation of these channels by Ca2+ (23), and the rNa1.2-L1 was shown to interact with synaptotagmin in a Ca2+-regulated manner (46). An interaction with synaptotagmin may modulate sodium channel activity by controlling the accessibility of a protein kinase A phosphorylation site located inside the binding region of this complex or may play a role in the internalization of sodium channels (46). A more intriguing possibility is that this interaction may result in NaN+-dependent exocytosis (46).

The TTX-R sodium channel rNa1.9a is preferentially expressed in small diameter C-type sensory neurons of DRG and trigeminal ganglia (14). The expression of rNa1.9a is dynamic and is modulated by the action of neurotrophic factors. Rat Na1.9a transcripts and protein and the current attributable to rNa1.9a are significantly down-regulated in vivo after axotomy or chronic constriction injury of the sciatic nerve (14, 17, 47) and in an in vitro model of axotomy (47, 48). Expression of rNa1.9a after axotomy is restored by exposure to the neurotrophic factor glial derived neurotrophic factor, but not nerve growth factor (47, 48).

The NaN+-channel has unique properties compared with other sodium channels. The NaN+-channel is persistent at −60 to −70 mV and shows a significant window current around these potentials (16), thus contributing to setting the resting membrane potential and to subthreshold electrogensis (18). Heterologous expression of recombinant NaN+-1a in HEK293 mammalian cells or DRG neurons in culture for 7 days after biologic transformation did not show TTX-R currents similar to those observed in vivo.2 One possibility is that these expression systems may lack a factor(s) that is important for the stability or efficient anchoring of the NaN+-1a channels in the membranes of these cells or the modulation of these channels so that they can produce the predicted NaN+- currents. The expression of the Drosophila sodium channel para in heterologous expression systems is dependent upon the co-expression of tipE factor (49).

This study presents evidence from multiple independent assays that demonstrates the direct interaction of rFHF1B with the C-terminal polypeptide of rNa1.9a both in vivo and in vitro. Despite the 50% similarity of the amino acid sequence of the C-terminal polypeptides of sodium channels, rFHF1B did not bind the C termini of hNa1.7 (hNaNE) and rNa1.8a (SNS), the two other sodium channels that are preferentially expressed in DRG neurons. Although the conserved region CR1 of rNa1.9a (aa 1588–1657) is sufficient to bind this factor, the full conserved region CR1 + CR2 (aa1588–1726) is needed for efficient binding with rFHF1B. These data clearly demonstrate the specific interaction between the C-terminal polypeptide of rNav1.9a and rFHF1B.

FHF members constitute a distinct branch of the FGF family with significant homology to other FGF members in the central domain but with divergent N and C termini (24). Alternative splicing produces variants of FHFs that contain a nuclear localization signal and others that lack such signals (24, 50–52). All FHF members discovered to date that lack a nuclear localization signal (24, 50–52), like FGF-1 and FGF-2, also lack a canonical signal peptide for secretion via the endoplasmic reticulum/Golgi pathway. Consistent with the lack of signal peptides, recombinant FHFs expressed in HEK293 cells are not secreted into the culture media (24). Although some FHF isoforms can interact with extracellular heparin sulfate proteoglycans, they do not activate FGF receptors nor have they been directly linked to a biological activity (36, 53). The ectopic expression of FHF-2 in chicken limb buds, however, leads to morphological abnormalities, suggesting that this factor play a role in pattern formation (36).

Like other members of the FGF family of growth factors, FHF1B is highly conserved among various species. The similarity in the amino acid level between full-length rat (this study) and chicken (36) FHF1B is 97%. Rat FHF1B is 100 and 97% similar, respectively, to the corresponding sequences that have been reported for mouse and human FHF1B (52). The expression of rFHF1B in adult rat tissue as determined by reverse transcription-PCR (Fig. 2) is similar to its expression pattern in the chicken (36), except for the presence of rFHF1B in adult rat DRG and trigeminal tissue. The expression of FHF1B, however, is more extensive than that of rNav1.9a. Therefore, this factor may interact with other proteins, possibly including other sodium channels. More experiments are needed to determine if rFHF1B interacts with sodium channels that are expressed in the brain.

The lack of a canonical signal peptide and nuclear localization signal in rat and chicken FHF1B suggests that FHF1B may exert its effect via interaction with cytosolic proteins either directly or by acting as a bridge to assemble a functional protein complex. Alternatively, FHF1B may be exported to the extracellular space via an endoplasmic reticulum/Golgi-independent mechanism similar to that responsible for FGF2 export (54), which is inhibited by cardenolides, suggesting a role of the α-subunit of NaN+-K+ ATPase in this pathway (55, 56). It is not unreasonable to speculate that NaN+-1a may have a function other than the generation of a persistent sodium current in DRG neurons (16) and that it may participate in the export of FHF1B to the cell surface or the exterior of the cell (55, 56), where it might exert its function. Alternatively, the interaction between rFHF1B and the C terminus of rNav1.9a may modulate this channel directly or indirectly by recruiting other proteins to the channel complex.

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Fibroblast Growth Factor Homologous Factor 1B Binds to the C Terminus of the Tetrodotoxin-resistant Sodium Channel rNa1.9a (NaN)
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