Structural Requirements for Interaction of Sodium Channel β1 Subunits with Ankyrin*

Received for publication, March 11, 2002, and in revised form, May 6, 2002
Published, JBC Papers in Press, May 7, 2002, DOI 10.1074/jbc.M202354200

Jyoti D. Malhotra‡§, Matthew C. Koopmann‡, Kristin A. Kazen-Gillespie‡, Nicholas Fettman‡, Michael Hortsch§, and Lori L. Isom‡‡

From the Departments of ‡Pharmacology and §Cell and Developmental Biology, the University of Michigan, Ann Arbor, Michigan 48109

Sodium channel β subunits modulate channel kinetic properties and cell surface expression levels and function as cell adhesion molecules. β1 and β2 participate in homophilic cell adhesion resulting in ankyrin recruitment to cell contact sites. We hypothesized that a tyrosine residue in the cytoplasmic domain of β1 may be important for ankyrin recruitment and tested our hypothesis using β1 mutants replacing Tyr181 with alanine (β1Y181A), phenylalanine (β1Y181F), or glutamate (β1Y181E), or a truncated construct deleting all residues beyond Tyr181 (β1L182STOP). Ankyrin recruitment was observed in β1L182STOP, showing that residues Ile186, Tyr181 contain the major ankyrin recruiting activity of β1. Ankyrin recruitment was abolished in β1Y181E, suggesting that tyrosine phosphorylation of β1 may inhibit β1-ankyrin interactions. AnkyrinG and β1 associate in rat brain membranes and in transfected cells expressing β1 and ankyrinG in the absence of sodium channel α subunits. β1 subunits are recognized by anti-phosphotyrosine antibodies following treatment of these cell lines with fibroblast growth factor. β1 and ankyrinG association is not detectable in cells following treatment with fibroblast growth factor. AnkyrinG and β1Y181E do not associate even in the absence of fibroblast growth factor treatment. β1 subunit-mediated cell adhesion and ankyrin recruitment may contribute to sodium channel placement at nodes of Ranvier. The phosphorylation state of β1Y181 may be a critical regulatory step in these developmental processes.

Sodium channels are unique among voltage- and ligand-gated ion channels in that they contain auxiliary subunits that not only modulate channel kinetics, but also function as cell adhesion molecules (CAMs) that direct channel insertion into the plasma membrane and channel interaction with other signaling proteins. We are exploring the novel idea that the cell adhesive functions of sodium channel β subunits may be as important or even more important than modulation of channel gating in excitable cells. We propose that, as CAMs, sodium channel β subunits act as critical communication links between extra- and intracellular signaling molecules.

Sodium channel auxiliary β subunits are multifunctional CAMs of the Ig superfamily (1). β subunits participate in homophilic cell adhesion (2), heterophilic adhesion with contactin and neurofascin (3, 4), interactions with extracellular matrix molecules (5, 6), and recruitment of ankyrin to the plasma membrane at sites of cell-cell contact in response to homophilic cell adhesion (2). Interestingly, β subunit cell adhesive interactions are independent of participation in the ion conduction complex, suggesting that the β subunits may be bifunctional molecules (2, 3, 6). We have proposed that the cell adhesive functions of β subunits play critical roles in the regulation of sodium channel density and localization.

What is the molecular basis for the interaction of β1 with the cytoskeleton? We showed previously (2) that β1-mediated homophilic cell adhesion results in recruitment of ankyrin to sites of cell-cell contact in Drosophila S2 cells. Removal of the intracellular cytoplasmic domain of β1 had no obvious effects on homophilic adhesion but completely abolished ankyrin recruitment, demonstrating that the intracellular domain is required for cytoskeletal interactions. The purpose of the present study was to identify residues in the cytoplasmic domain of β1 that are required for ankyrin recruitment. Previous experiments have shown that deletion of a 5-amino acid sequence from the intracellular carboxyl-terminal domain of the L1-CAM family member neurofascin (FIGQY) abolished ankyrin binding activity (7, 8). Phosphorylation or deletion of just the tyrosine residue in this sequence abolished ankyrin binding and significantly reduced neurofascin-mediated cell adhesion. Similar experiments were performed to investigate the structural basis for signaling by Drosophila neuroglian, another member of the L1-CAM family (9). The results of this study identified a conserved intracellular 36-amino acid sequence that is responsible for ankyrin binding. Mutation of the conserved FIGQY tyrosine residue in this region reduced the extent of ankyrin recruitment as well as cell adhesion. A missense mutation of the FIGQY tyrosine in human L1 (Y1229H) results in clinical disease and confirms this residue as critical for the proper function of L1 in neuronal development (10).

We hypothesized that an intracellular tyrosine residue in β1 may play a role in ankyrin recruitment similar to that observed for the conserved FIGQY tyrosine residue in members of the L1-CAM family. Interestingly, abolishment of the entire cytoplasmic domain of sodium channel β1 or β2 subunits does not have any significant effects on homophilic cell adhesion (2). Like the β subunits, the intracellular domains of L1-CAMs, such as neurofascin and neuroglian, are not essential for cellular aggregation (11, 12). However, unlike the β subunits,
mutations reducing ankyrin binding result in a reduction in both the rate and extent of cellular aggregation (7–9). The extracellular Ig domain of β1 is homologous to the Ig loop of the CAM myelin Pα (13, 14). Unlike β1, however, deletion of the intracellular domain of myelin Pα results in abolishment of homophilic adhesion (15). Thus, the sodium channel β subunits appear to diverge from L1-CAMs and myelin Pα in this respect.

We tested our hypothesis that an intracellular tyrosine residue is important for ankyrin recruitment by expressing a series of truncated and site-directed β1 subunit mutant constructs in Drosophila S2 cells. Our results show that the sequence Ile166>Tyr181 contains the major ankyrin recruiting activity of β1. Ankyrin recruitment was retained in β1Y181F, but was abolished in β1Y181E and impaired in β1Y181A. The data suggest the possibility that tyrosine phosphorylation, mimicked by introduction of a negative charge in β1Y181E, may regulate ankyrin-β1 interactions. β1 subunits are recognized by two different anti-phosphotyrosine antibodies, PY20 and PY100, in transfected mammalian cells following treatment with fibroblast growth factor (FGF). Tyr181 mutant β1 subunits are not recognized by anti-phosphotyrosine antibodies, indicating that Tyr181 is the only intracellular tyrosine residue that is phosphorylated, even though two additional tyrosine residues (Tyr161 and Tyr163) are present near the intracellular end of the transmembrane segment. Tyr161 and Tyr163 are present in β1STOP, a truncation mutant ending with Lys162 that was previously shown to be deficient in ankyrin recruiting activity (2). Ankyrinαs and β1 are co-immunoprecipitated from rat brain membranes and from transfected cells expressing β1 and GFP-tagged ankyrin (G) in the absence of sodium channel α subunits. Treatment of this cell line with FGF abolishes β1-ankyrinαs interactions. As predicted by the S2 cell experiments, GFP-ankyrinαs and β1Y181E do not associate in transfected cells. Our results suggest that reversible, receptor-mediated changes in tyrosine phosphorylation modulate the association of sodium channels with ankyrin during axonal fasciculation or at specialized domains such as nodes of Ranvier. We conclude that the association of ankyrin and sodium channel β1 subunits is mediated through a 16-amino acid segment of the intracellular domain of β1 containing a tyrosine residue and that this event occurs independently of β1 subunit association with the ion-conducting pore.

**Experimental Procedures**

**Materials—Drosophila** S2 cells were obtained from the American Type Culture Collection. Rabbit polyclonal antisera to an extracellular domain of β1 (KRRSETTAETTFTWTR), β1STOP was described previously (2). Polyclonal antiserum to an intracellular domain of β1 (LAITTSESKENCTGCVQA), β1DN, was generated and affinity purified by Research Genetics (Huntsville, AL). Mouse anti-phosphorylated, even though two additional tyrosine residues are not recognized by anti-phosphotyrosine antibodies, indicating that Tyr181 is the only intracellular tyrosine residue that is phosphorylated, even though two additional tyrosine residues (Tyr161 and Tyr163) are present near the intracellular end of the transmembrane segment. Tyr161 and Tyr163 are present in β1STOP, a truncation mutant ending with Lys162 that was previously shown to be deficient in ankyrin recruiting activity (2). Ankyrinαs and β1 are co-immunoprecipitated from rat brain membranes and from transfected cells expressing β1 and GFP-tagged ankyrin in the absence of sodium channel α subunits. Treatment of this cell line with FGF abolishes β1-ankyrinαs interactions. As predicted by the S2 cell experiments, GFP-ankyrinαs and β1Y181E do not associate in transfected cells. Our results suggest that reversible, receptor-mediated changes in tyrosine phosphorylation modulate the association of sodium channels with ankyrin during axonal fasciculation or at specialized domains such as nodes of Ranvier. We conclude that the association of ankyrin and sodium channel β1 subunits is mediated through a 16-amino acid segment of the intracellular domain of β1 containing a tyrosine residue and that this event occurs independently of β1 subunit association with the ion-conducting pore.

**Materials—Drosophila** S2 cells were obtained from the American Type Culture Collection. Rabbit polyclonal antisera to an extracellular domain of β1 (KRRSETTAETTFTWTR), β1STOP was described previously (2). Polyclonal antiserum to an intracellular domain of β1 (LAITTSESKENCTGCVQA), β1DN, was generated and affinity purified by Research Genetics (Huntsville, AL). Mouse anti-phosphorylated, even though two additional tyrosine residues are not recognized by anti-phosphotyrosine antibodies, indicating that Tyr181 is the only intracellular tyrosine residue that is phosphorylated, even though two additional tyrosine residues (Tyr161 and Tyr163) are present near the intracellular end of the transmembrane segment. Tyr161 and Tyr163 are present in β1STOP, a truncation mutant ending with Lys162 that was previously shown to be deficient in ankyrin recruiting activity (2). Ankyrinαs and β1 are co-immunoprecipitated from rat brain membranes and from transfected cells expressing β1 and GFP-tagged ankyrin in the absence of sodium channel α subunits. Treatment of this cell line with FGF abolishes β1-ankyrinαs interactions. As predicted by the S2 cell experiments, GFP-ankyrinαs and β1Y181E do not associate in transfected cells. Our results suggest that reversible, receptor-mediated changes in tyrosine phosphorylation modulate the association of sodium channels with ankyrin during axonal fasciculation or at specialized domains such as nodes of Ranvier. We conclude that the association of ankyrin and sodium channel β1 subunits is mediated through a 16-amino acid segment of the intracellular domain of β1 containing a tyrosine residue and that this event occurs independently of β1 subunit association with the ion-conducting pore.
buffer and separated on 5 or 10% acrylamide SDS-PAGE gels as indicated in the figure legends. Proteins were then transferred to nitrocellulose and probed with anti-β1 subunit cDNAs in S2 cells. Each mutant-transfected S2 cells were solubilized in 1% Triton X-100 containing 1 mM sodium orthovanadate and the soluble fraction was incubated overnight at 4 °C with anti-β1 subunit antibody in the presence of 1 mM sodium orthovanadate. Protein A-Sepharose (50 μl of a 1:1 suspension) was then added and the incubation was continued for 2 h at 4 °C. The Protein A-Sepharose beads were precipitated in a microcentrifuge and washed with 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100. Immunoprecipitates were eluted from the Protein A-Sepharose with SDS-PAGE sample buffer and separated on 10% acrylamide SDS-PAGE gels. Proteins were then transferred to nitrocellulose and probed with anti-phosphotyrosine antibody, as indicated in the figure legends. Whereas both anti-PY20 and anti-PY100 recognized β1 subunits equally well, only the experiments with anti-PY20 are shown. Chemiluminescent detection of immunoreactive bands was accomplished with WestDura reagent.

Immunoprecipitation of β1 Subunits with Anti-phosphotyrosine Antibodies—Immunoprecipitations were performed from solubilized transfected cell pellets following stimulation of cell monolayers with FGF or vehicle (50 ng/ml for 30 min at 37 °C). Membranes or cells were solubilized in 1.25% Triton X-100 containing 1 mM sodium orthovanadate and the soluble fraction was incubated overnight at 4 °C with anti-β1 subunit antibody in the presence of 1 mM sodium orthovanadate. Protein A-Sepharose (50 μl of a 1:1 suspension) was then added and the incubation was continued for 2 h at 4 °C. The Protein A-Sepharose beads were precipitated in a microcentrifuge and washed with 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100. Immunoprecipitates were eluted from the Protein A-Sepharose with SDS-PAGE sample buffer and separated on 10% acrylamide SDS-PAGE gels. Proteins were then transferred to nitrocellulose and probed with anti-phosphotyrosine antibody, as indicated in the figure legends. Whereas both anti-PY20 and anti-PY100 recognized β1 subunits equally well, only the experiments with anti-PY20 are shown. Chemiluminescent detection of immunoreactive bands was accomplished with WestDura reagent.

Immunocytochemical Localization of Ankyrin in 1610 Fibroblasts—Chinese hamster lung 1610 and 1610Δ1 (6) cell lines were used to analyze ankyrin localization. Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. Mouse anti-ankyrin B (Zymed Laboratories Inc.) was used as the primary antibody (1:200), followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (1:200). Slides were then viewed with a Zeiss LSM 510 confocal microscope mounted on a Zeiss Axiovert 150M inverted microscope in the Microscopy and Image Analysis Laboratory Core Facility at the University of Michigan.

RESULTS

An intracellular tyrosine residue (Tyr181) is essential for β1-ankyrin interactions but is not required for cell adhesion—To test our hypothesis that the single intracellular tyrosine residue downstream of Lys185 in β1, Tyr181, is critical for ankyrin recruitment, we constructed a number of mutant β1 cDNAs in pRmHa3, including β1L182STOP, β1Y181F, β1Y181E, and β1Y181A (Fig. 1A) and co-transfected them with a plasmid conferring a-amanitin resistance into Drosophila S2 cells. Each cell line was then cloned using the previously described agar method (19). Fig. 1B demonstrates that each of these mutant constructs was efficiently expressed. S2 cells transfected with each of the β1Y181 mutants formed aggregates following induction with CuSO4 and mechanical shaking (Fig. 2). Untransfected S2 cells treated similarly did not aggregate. The percent of non-aggregated cells was quantified for each cell line following 20, 40, 60, and 80 min of induction. In contrast to studies with L1 family CAMs, the extent of cellular aggregation after 80 min was similar for wild type β1 and the β1 mutants. These results are consistent with our previous studies showing that the intracellular domain of β1 beyond Ile186 is not necessary for cell adhesion (2). Interestingly, there appears to be subtle differences in the rate of aggregation in the Tyr181 mutant constructs compared with wild type.

We also showed previously that the intracellular domain of β1 from Ile186 to the carboxyl terminus is required for homophilic adhesion-induced ankyrin recruitment (2). Immunocytochemical analysis of β1L182STOP-induced cell aggregates using an antibody to Drosophila ankyrin showed ankyrin recruitment to sites of cell-cell contact similar to wild type β1 (72 versus 80% recruitment, Table 1), indicating that residues Ile186-Tyr181 contain the major ankyrin-recruiting activity for this molecule (Fig. 3, A and B). We next tested a series of point mutations to evaluate the importance of Tyr181 in β1-mediated ankyrin recruitment. β1Y181F-induced cell adhesion resulted in ankyrin recruitment to an extent similar to wild type β1 (75 versus 80% recruitment, Table 1) (Fig. 3, C and D). In contrast, ankyrin recruitment induced by β1Y181A was significantly impaired (15 versus 80% recruitment, Table 1) (Fig. 3, E and F). These results are similar to those reported for neurofascin (22), and suggest that the aromatic ring of Tyr181 may be critical for β1-ankyrin inter-
Summary of effects of mutations in the cytoplasmic domain of β1 on ankyrin recruitment in S2 cells

Transfected S2 cells were induced to aggregate and ankyrin was visualized by immunocytochemistry as described under “Experimental Procedures.” 100 cell contact sites for each of the cell lines were evaluated by confocal microscopy for ankyrin recruitment. The sequence of the cytoplasmic domain for each construct beginning at residue Lys 164 is given with Tyr 181 or the mutated residue at this position indicated in bold.

| construct | sequence of cytoplasmic domain | number of cell contacts evaluated | number of evaluated cell contacts showing ankyrin recruitment |
|-----------|--------------------------------|----------------------------------|-------------------------------------------------------------|
| WT        | KKAANAAAAYLAQNASEYLYTESEKDNCTQVQAE | 100                              | 0                                                           |
| Y181F     | KKAANAAAAYLAQNASEYLYTESEKDNCTQVQAE | 100                              | 75                                                          |
| Y181A     | KKAANAAAAYLAQNASEYLYTESEKDNCTQVQAE | 100                              | 15                                                          |
| Y181E     | KKAANAAAAYLAQNASEYLYTESEKDNCTQVQAE | 100                              | 0                                                           |
| L182/Eotr | KKAANAAAAYLAQNASEYLYTESEKDNCTQVQAE | 100                              | 72                                                          |

Fig. 3. Effects of β1Y181 substitution on ankyrin recruitment. Mutant β1-transfected S2 cells were induced in the presence of 0.7 mM CuSO4 and aggregation was induced by rotary shaking. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with anti-Drosophila ankyrin antibody (1:200 dilution) followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (1:100 dilution) and viewed with a Bio-Rad MRC-600 laser scanning confocal microscope attached to a Nikon Diaphot microscope in the Microscopy and Image Analysis Laboratory at the University of Michigan. Two different views of each cell line are presented. Scale bar: 10 μm. A and B, L182/Eotr; C and D, Y181F; E and F, Y181A; G and H, Y181E.

actions, although the hydroxyl group of this tyrosine residue may be less important in ankyrin recruitment.

Previous studies with neurofascin have also shown that phosphorylation of an intracellular tyrosine residue in the conserved FIGQY domain (Tyr1239 in human neurofascin) resulted in the abolishment of ankyrin recruiting activity (8). Mutation of this tyrosine residue to glutamic acid mimicked phosphorylation by introducing negative charges and greatly impaired both cell adhesion and ankyrin recruitment (22). We performed a similar experiment using the β1Y181E mutant construct. This substitution completely abolished ankyrin recruitment in S2 cells (0% recruitment, Table I) (Fig. 3, G and H). In contrast to studies using neurofascin or neurogin, the Y181E mutation had little effect on β1 subunit-induced cell adhesion following 80 min of induction compared with wild type β1 (Fig. 2). Under bright field illumination, cells in Fig. 3, panels G and H, were tightly aggregated (data not shown), yet ankyrin staining was absent from cell-cell contact sites and appeared to be completely cytoplasmic. We conclude that the major ankyrin recruiting activity of β1 is contained in residues 166–181 Tyr181 in the intracellular domain and that Tyr181 is critical for β1-ankyrin interactions. These results are summarized in Table I.

Sodium Channel β1 Subunits Are Tyrosine-phosphorylated—The results shown in Fig. 3 suggest that tyrosine phosphorylation may be a key regulatory event in β1-mediated ankyrin recruitment in Drosophila S2 cells. Substitution of β1Y181 with glutamic acid to mimic phosphorylation by introducing negative charges resulted in the abolishment of ankyrin recruitment. Are β1 subunits phosphorylated in mammalian cells? Fig. 4 shows that β1 subunits expressed in 1610/β1 fibroblasts are recognized by anti-PY20 antibodies following treatment of the cells with FGF in the presence of orthovanadate. Prior to Western blot analysis, β1 subunits were immunoprecipitated with anti-β1N antibody. Both panels represent the identical blot probed with 2 different antibodies following immunoprecipitation with anti-β1 antibodies. In Fig. 4, left panel, the blot was probed with anti-β1 antibody to show approximately equal expression of β1 subunits in 1610/β1 cells with and without FGF treatment. The blot was then stripped and reprobed with anti-PY20 (Fig. 4, right panel). Arrows indicate the position of the immunoreactive β1 band in both panels. It is clear from this experiment that β1 subunits are directly phosphorylated on tyrosine residues following FGF stimulation. Longer exposures of co-immunoprecipitation experiments in the absence of FGF treatment revealed a low level of basal phosphorylation of β1 subunits in the presence of orthovanadate alone (data not shown). We were also able to detect low levels of basal phosphorylation of β1 subunits in rat brain membranes in the presence of orthovanadate (data not shown). Two different anti-phosphotyrosine antibodies, anti-PY20 and anti-PY100, recognized β1 subunits in rat brain membranes. Substitution of β1Y181 with glutamic acid to mimic phosphorylation by introducing negative charges resulted in the abolishment of ankyrin recruitment (22). We performed a similar experiment using the β1Y181E mutant construct. This substitution completely abolished ankyrin recruitment in S2 cells (0% recruitment, Table I) (Fig. 3, G and H). In contrast to studies using neurofascin or neurogin, the Y181E mutation had little effect on β1 subunit-induced cell adhesion following 80 min of induction compared with wild type β1 (Fig. 2). Under bright field illumination, cells in Fig. 3, panels G and H, were tightly aggregated (data not shown), yet ankyrin staining was absent from cell-cell contact sites and appeared to be completely cytoplasmic. We conclude that the major ankyrin recruiting activity of β1 is contained in residues 166–181 Tyr181 in the intracellular domain and that Tyr181 is critical for β1-ankyrin interactions. These results are summarized in Table I.

Sodium Channel β1 Subunits Are Tyrosine-phosphorylated—The results shown in Fig. 3 suggest that tyrosine phosphorylation may be a key regulatory event in β1-mediated ankyrin recruitment in Drosophila S2 cells. Substitution of β1Y181 with glutamic acid to mimic phosphorylation by introducing negative charges resulted in the abolishment of ankyrin recruitment. Are β1 subunits phosphorylated in mammalian cells? Fig. 4 shows that β1 subunits expressed in 1610/β1 fibroblasts are recognized by anti-PY20 antibodies following treatment of the cells with FGF in the presence of orthovanadate. Prior to Western blot analysis, β1 subunits were immunoprecipitated with anti-β1N antibody. Both panels represent the identical blot probed with 2 different antibodies following immunoprecipitation with anti-β1 antibodies. In Fig. 4, left panel, the blot was probed with anti-β1 antibody to show approximately equal expression of β1 subunits in 1610/β1 cells with and without FGF treatment. The blot was then stripped and reprobed with anti-PY20 (Fig. 4, right panel). Arrows indicate the position of the immunoreactive β1 band in both panels. It is clear from this experiment that β1 subunits are directly phosphorylated on tyrosine residues following FGF stimulation. Longer exposures of co-immunoprecipitation experiments in the absence of FGF treatment revealed a low level of basal phosphorylation of β1 subunits in the presence of orthovanadate alone (data not shown). We were also able to detect low levels of basal phosphorylation of β1 subunits in rat brain membranes in the presence of orthovanadate (data not shown). Two different anti-phosphotyrosine antibodies, anti-PY20 and anti-PY100, recognized β1 subunits equally well. However, only the experiments with anti-PY20 are shown. Tyr181 mutant β1 subunits expressed in FGF-treated 1610 cells are not recognized by anti-phosphotyrosine antibodies, indicating that Tyr181 is the only intracellular tyrosine residue that is phos-
β1 subunits interact with ankyrin in mammalian cells? To answer these questions, we first performed co-immunoprecipitation experiments to demonstrate the association of β1 and ankyrinG in solubilized rat brain membranes (Fig. 6A). This result, whereas positive, could have been indirect because of direct interaction of ankyrinG with sodium channel α subunits, as has been suggested previously (23, 24). To investigate this question in greater detail, we transfected Chinese hamster lung 1610β1 fibroblasts with a GFP-tagged ankyrinG cDNA construct and then tested for β1 subunit-ankyrinG interactions. Immunoprecipitation of solubilized 1610β1 cells with anti-β1 antibody followed by Western blot analysis with anti-GFP showed that β1 and ankyrinG interact in these cells in the absence of α subunits (Fig. 6B). Immunocytochemistry experiments demonstrate that endogenous ankyrinG is localized diffusely throughout the cytoplasm in untransfected 1610 cells and becomes concentrated at the plasma membrane at sites of cell-cell contact in cells stably transfected with β1 (Fig. 7).

β1Y181A and β1Y181F also associate with GFP-ankyrinG in 1610 cells (Fig. 6C). In this co-immunoprecipitation experiment, β1Y181A appears to associate less efficiently with ankyrin compared with wild type, although the difference is clearly not as large as that seen in the ankyrin recruitment assays (Fig. 3, Table I). We attribute this apparent discrepancy to the non-quantitative nature of chemiluminescent detection of Western blots as well as the possibility of signal saturation in this assay. As predicted from the ankyrin recruitment experiments, we were not able to co-immunoprecipitate β1Y181E subunits and GFP-ankyrinG from co-transfected 1610 cells (Fig. 6C). Western blot analysis of cell lysates showed that β1Y181E was efficiently expressed in this cell line (Fig. 6C). Finally, treatment of 1610β1 cells with FGF abolished β1-ankyrinG interactions (Fig. 6D). Thus, β1 subunits appear to bind ankyrinG and ankyrinB directly. The sodium channel signaling complex at nodes of Ranvier may include both α-ankyrin and β1-ankyrin linkages. The present data suggest that β1-ankyrin interactions are negatively regulated by phosphorylation of β1 subunits at residue Tyr181. We propose that this regulatory process plays a critical role in sodium channel localization and clustering at mammalian nodes of Ranvier.

**DISCUSSION**

We demonstrated previously that sodium channel β1 and β2 subunits are members of the Ig superfamily and mediate homophilic cell adhesion leading to recruitment of ankyrin to points of cell-cell contact in transfected *Drosophila* S2 cells (2). Deletion of the intracellular carboxyl-terminal domain of β1 or β2 abolished ankyrin binding but retained homophilic cell adhesion, indicating that the intracellular domains of β subunits are critical for signal transduction events leading to cytoskeletal communication (2). In the present study we focused on the mechanism of β1 subunit interactions with ankyrin. β1 subunits contain a single intracellular tyrosine residue downstream of Lys165, identified as Tyr161 (25). We hypothesized that this tyrosine may play a role in signal transduction leading to cytoskeletal communication following cell adhesion. To test our hypothesis, we generated mutant β1 subunit constructs, focusing on Tyr161. We found that β1 subunit-mediated cellular aggregation was retained in all of the mutant constructs. Although subtle differences in the relative rates of aggregation were observed, the extent of aggregation following 80 min of induction was unchanged. Ankyrin recruitment was retained in β1L182→TOP, indicating that the sequence Ile166-Tyr181 contains the major ankyrin recruiting activity of β1. Ankyrin recruitment was retained in β1Y181F, impaired in β1Y181A, and abolished in β1Y181E, denoting the importance of an aromatic residue at this position and suggesting the
with anti-GFP antibody (1:500 dilution) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:100,000 dilution). Chemiluminescent detection of immunoreactive bands was accomplished with WestDura reagent. IgG, nonimmune serum; GFP, anti-GFP antibody; β1, anti-β1k antibody. The arrow indicates the position of the β1 immunoreactive band. Molecular mass markers are indicated in kDa. C, β1Y181F and GFP-ankyrin G, solubilized in 1.25% Triton X-100 and the soluble fraction was incubated overnight at 4 °C with 5 μg of anti-β1k antibody. Protein A-Sepharose was added and the incubation was continued for 2 h at 4 °C. The Protein A-Sepharose beads were precipitated in a microcentrifuge and washed with 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100. Immunoprecipitates were eluted from the Protein A-Sepharose beads with SDS-PAGE sample buffer and separated on 5% acrylamide SDS-PAGE gels. Proteins were then transferred to nitrocellulose and probed with anti-GFP antibody (1:500 dilution) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:100,000 dilution). Chemiluminescent detection of immunoreactive bands was accomplished with WestDura reagent. IgG, nonimmune serum; GFP, anti-GFP antibody; β1, anti-β1k antibody. The arrow indicates the position of the β1 immunoreactive band. Molecular mass markers are indicated in kDa. D, FGF treatment abolishes β1-ankyrin interactions. 1610β1 cells were stimulated with FGF (+) or vehicle (−) as described in the legend to Fig. 4, solubilized in 1.25% Triton X-100 and the soluble fraction was incubated overnight at 4 °C with 5 μg of anti-β1k antibody. Protein A-Sepharose was added and the incubation was continued for 2 h at 4 °C. The Protein A-Sepharose beads were precipitated in a microcentrifuge and washed with 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100. Immunoprecipitates were eluted from the Protein A-Sepharose beads with SDS-PAGE sample buffer and separated on 5% acrylamide SDS-PAGE gels. Proteins were then transferred to nitrocellulose and probed with anti-GFP antibody (1:500 dilution) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:100,000 dilution).
may be a regulated event. β1 and ankyrinG, the ankyrin isoform present at axon initial segments and nodes of Ranvier, associate in transfected 1610 cells in the absence of α subunits, suggesting that these two proteins may interact in mammalian neurons in vivo. In contrast, β1Y181E and ankyrinG do not associate, and FGF treatment of cells results in inhibition of wild type β1 interactions with ankyrin, supporting our conclusion that tyrosine phosphorylation inhibits β1-mediated ankyrin recruitment.

Previous studies identified a highly conserved FIGQY sequence in the cytoplasmic domain of neurofascin as the principal site of regulation of ankyrin binding (7, 8). Phosphorylation of this tyrosine residue abolished cell adhesion and ankyrin recruitment in cells expressing this CAM (7, 8). Structural requirements for the association of neurofascin with ankyrin were found to be very similar to the present results (22). Briefly, substitution of the critical tyrosine residue (Tyr1229) in neurofascin with phenylalanine retained ankyrin binding, whereas substitution with alanine or glutamic acid greatly impaired this interaction. Interestingly, manipulation of the cytoplasmic domain of neurofascin had dramatic effects on the rate and extent of cell adhesion, suggesting an additional inside-out signaling mechanism. In contrast, the cytoplasmic domain of β1 appears to have little influence on homophilic cell adhesion, indicating that whereas β1-mediated extracellular adhesion events influence cytoskeletal communications, cytoskeletal interactions have little effect on β1-mediated cell adhesion.

Neuronal sodium channel α and β1 subunits interact with receptor protein-tyrosine phosphatase β during neonatal development (26). A consequence of formation of this signaling complex is to reduce tyrosine phosphorylation of α subunits. Coexpression of receptor protein-tyrosine phosphatase β and Nav1.2 α subunits in tsA-201 cells resulted in a depolarizing shift in the voltage dependence of sodium channel inactivation and an increase in whole cell current. The present data introduce the possibility that receptor protein-tyrosine phosphatase β may also function to regulate tyrosine phosphorylation of sodium channel β1 subunits resulting in modulation of ankyrin recruitment to the plasma membrane. These events would be predicted to influence sodium channel localization and density via CAM-cytoskeletal interactions during formation of the node of Ranvier or during axonal fascilitation. In glia, where receptor protein-tyrosine phosphatase β and sodium channel β1 subunits are also expressed (27–31), modulation of β1 subunit tyrosine phosphorylation may be critical to axoglial communication during myelination and nodal formation.

Mutations in voltage-gated sodium channel α subunit genes have been shown to be linked to inherited human diseases causing paroxysmal events such as long QT syndrome in the heart and hyperkalemic periodic paralysis in skeletal muscle (32). Until recently there were no identified neuronal sodium channel mutations associated with human disease. Mutations in the human SCN1A α subunit gene have been linked to two families with idiopathic generalized epilepsy with febrile seizures plus type 2 (GEFS+2) (33–35). A mutation in SCN1B has been shown to be linked to a large family with GEFS+1 (36). This mutation, C121W (C102W using the original numbering presented in Isom et al. (25)), changes a critical cysteine residue defining the carboxyl-terminal region of the Ig loop in β1 to a tryptophan, most likely destabilizing this critical cell adhesion and α subunit association domain. Coexpression of the GEFS+1 mutant β1 subunit with rat brain Nav1.2 sodium channels in Xenopus oocytes revealed a lack of the classic β1-mediated acceleration of the inactivation rate (36). It is not yet known whether GEFS+1 mutant β1 subunits are expressed at the plasma membrane or, if so, whether they are associated with α subunits. It will be important in future studies to examine the coexpression of these mutant β1 subunits with α subunits in transfected mammalian cells and transgenic animals as well as their cell adhesion properties in S2 cells. Nevertheless, this important study showed for the first time that mutations in sodium channel β subunits result in pathophysiology of the human nervous system and predicted that cell adhesion may play a role in the epileptic phenotype.

Sodium channel β subunits play important roles in channel modulation and regulation of channel density at the plasma membrane. We have shown that β subunits also function as cell adhesion molecules in heterologous expression systems. What are the potential implications of these findings? It has been shown previously that ankyrinG, the CAMs neurofascin and NrCAM, and voltage-gated sodium channels co-localize at the axonal membrane of the adult node of Ranvier in specialized membrane domains (37–39). Early clusters of neurofascin and NrCAM are joined later by ankyrinG and sodium channels during differentiation of myelinated axons. Formation of the node of Ranvier may then result from the fusion of two cluster intermediates. An ankyrinG-mediated link between neurofascin, NrCAM, and ion channels may allow these CAMs to cluster sodium channels in the axonal membrane. It was proposed that sodium channel β2 subunits, because of their homology to F3/contactin, may interact in a lateral or cis fashion with NrCAM and thus contribute to sodium channel localization (38). Recent studies have shown that β1 also interacts with neurofascin-186 directly (4). Our present data propose a direct link between sodium channel β1 subunits and ankyrinG that is modulated by tyrosine phosphorylation of β1 subunits. The multivalent membrane-binding domain of ankyrinG (40) may allow interaction with multiple CAMs, including neurofascin, NrCAM, as well as sodium channel β1 subunits, forming a dynamic sodium channel signaling complex at the node of Ranvier that may also include the cell adhesion molecule contactin, as well as receptor protein-tyrosine phosphatase β and tyrosine kinases. As has been proposed for neurofascin (7), tyrosine phosphorylation of β1 may be receptor-mediated and reversible, resulting in strong versus weak cytoskeletal connections depending on cellular signaling. β1 subunit mRNA expression has been described in sciatic nerve Schwann cells, astrocytes from spinal cord, optic nerve, and sciatic nerve, oligodendrocytes, and B104 oligodendrocyte precursor cells in culture (27–29, 31). trans-Homophilic cell adhesion may occur between axonal and glial cell sodium channel β subunits in addition to the proposed cis interactions. This putative adhesion may also contribute to sodium channel clustering at nodes of Ranvier during the process of myelination. Our challenge now is to relate these exciting observations in heterologous expression systems to physiological events.

REFERENCES
1. Isom, L. L. (2001) The Neuroscientist 7, 42–54
2. Malhotra, J. D., Kazen-Gillespie, K., Hortsch, M., and Isom, L. L. (2000) J. Biol. Chem. 275, 11383–11388
3. Kazarinova-Noyes, K., Malhotra, J. D., McEwen, D. P., Mattei, L. N., Berglund, E. O., Ranscht, B., Levinson, S. R., Schachner, M., Shragger, P., Isom, L. L., and Xiao, Z.-C. (2001) J. Neurosci. 21, 7517–7525
4. Ratcliffe, C. F., Westenbroek, R. E., Curtis, R., and Catterall, W. A. (2001) J. Cell Biol. 154, 427–434
5. Srinivasaan, J., Schachner, M., and Catterall, W. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15753–15757
6. Xiao, Z.-C., Ragadege, D. S., Malhotra, J. D., Mattei, L. N., Braun, P. E., Schachner, M., and Isom, L. L. (1999) J. Biol. Chem. 274, 26511–26517
7. Turvay, S., Garver, T. D., and Bennett, V. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12957–12962
8. Garver, T. D., Ren, Q., Turvay, S., and Bennett, V. (1997) J. Cell Biol. 137, 703–714
9. Hortsch, M., Homer, D., Malhotra, J. D., Chang, S., Frankel, J., Jefford, G., and Dehreuil, R. R. (1998) J. Cell Biol. 142, 251–261
10. Needham, L. R., Thelen, K., and Maness, P. F. (2001) J. Neurosci. 21, 1490–1500
Interaction of Sodium Channel β1 with Ankyrin

11. Hortsch, M., Wang, Y.-M., Marikar, Y., and Bieber, A. J. (1995) J. Biol. Chem. 270, 18809–18817
12. Wong, E. V., Cheng, G., Payne, H. R., and Lemmon, V. (1995) Neurosci. Lett. 200, 155–158
13. McCormick, K. A., Isom, L. L., Ragsdale, D., Smith, D., Scheuer, T., and Catterall, W. A. (1998) J. Biol. Chem. 273, 3954–3962
14. Morgan, K., Stevens, K. B., Cox, P. J., Dixon, A. K., Lee, K., Kinnick, R. D., Hughes, J., Richardson, P. J., Mizuguchi, K., and Jackson, A. P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2308–2313
15. Wong, M. H., and Filbin, M. (1994) J. Cell Biol. 126, 1089–1097
16. Malhotra, J. D., Tsiotra, P., Karagogeos, D., and Hortsch, M. (1998) J. Biol. Chem. 273, 3954–3962
17. McCormick, K. A., Isom, L. L., Ragsdale, D., Smith, D., Scheuer, T., and Catterall, W. A. (1998) J. Biol. Chem. 273, 3954–3962
18. Morgan, K., Stevens, K. B., Cox, P. J., Dixon, A. K., Lee, K., Kinnick, R. D., Hughes, J., Richardson, P. J., Mizuguchi, K., and Jackson, A. P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2308–2313
19. Wong, M. H., and Filbin, M. (1994) J. Cell Biol. 126, 1089–1097
20. Malhotra, J. D., Tsiotra, P., Karagogeos, D., and Hortsch, M. (1998) J. Biol. Chem. 273, 33354–33359
21. West, J. W., Scheuer, T., Maechler, L., and Catterall, W. A. (1992) Neuron 8, 59–70
22. Bunch, T. A., Grinblat, Y., and Goldstein, L. S. (1988) Nucleic Acids Res. 16, 1043–1061
23. Bieger, A. J. (1994) in Drosophila melanogaster: Practical Uses in Cell Biology (Goldstein, L., and Fryberg, E., eds) Vol. 44, pp. 683–696, Academic Press, San Diego
24. Jokerst, R. S., Weeks, J. R., Zehiring, W. A., and Catterall, W. A. (1992) Neuron 8, 59–70
25. Isom, L. L., De Jongh, K. S., Patton, D. E., Reber, B. F. X., Offord, J., Charbonneau, H., Walsh, K., Goldin, A. L., and Catterall, W. A. (1995) J. Biol. Chem. 270, 3306–3312
26. Ratcliffe, C. F., Qu, Y., McCormick, K. A., Tibbs, V. C., Dixon, J. E., Scheuer, T., and Catterall, W. A. (2000) Nat. Neurosci. 3, 437–444
27. Dib-Hajj, S. D., Hinson, A. W., Hargui, A., and Waxman, S. G. (1996) FEBS Lett. 384, 78–82
28. Oh, Y., Lee, Y. J., and Waxman, S. G. (1997) Neurosci. Lett. 234, 107–110
29. Oh, Y., and Waxman, S. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9985–9989
30. Fischer, E., Charbonneau, H., and Tenzer, N. K. (1991) Science 253, 401–406
31. Kaplan, M. R., Cho, M.-H., Ullian, E. M., Isom, L. L., Levinson, S. R., and Barres, B. A. (2001) Neuron 30, 105–119
32. Ackerman, M. J., and Clapham, D. E. (1997) N. Engl. J. Med. 336, 1575–1586
33. Escayg, A., MacDonald, B. T., Meisler, M. H., Baulac, S., Huberfeld, G., An-Gourfinkel, L., Brice, A., LeGuern, E., Mouland, B., Chaigne, D., Barsei, C., and Malafosse, A. (2000) Nat. Genet. 24, 343–345
34. Escayg, A., Heils, A., MacDonald, B. T., Haung, K., Sander, T., and Meisler, M. H. (2001) Am. J. Hum. Genet. 68, 866–873
35. Wallace, R. H., Scheffer, I. E., Hargui, A., Dibbens, R. M., Desai, R. R., Lerman-Sagie, T., Lev, D., Mazari, A., Brand, N., Ben-Zeit, B., Gokkman, L., Singh, R., Krenmidiotis, G., Gardner, A., Sutherland, G. R. Jr., George, A. L., Mulley, J. C., and Berkovic, S. F. (2001) Am. J. Hum. Genet. 68, 859–865
36. Wallace, R. H., Wang, D. W., Singh, R., Scheffer, I. E., George, A. L. Jr., Phillips, H. A., Saar, K., Reis, A., Johnson, A. W., Sutherland, G. R., Berkovic, S. F., and Mulley, J. C. (1998) Nat. Genet. 19, 366–370
37. Kordeli, E., Davis, J., Trapp, B., and Bennett, V. (1990) J. Cell Biol. 110, 1341–1352
38. Davis, J. Q., Lambert, S., and Bennett, V. (1996) J. Cell Biol. 135, 1355–1367
39. Lustig, M., Zanazzi, G., Sakurai, T., Blanco, C., Levinson, S. R., Lambert, S., Grumet, M., and Salzer, J. L. (2001) Curr. Biol. 11, 1864–1869
40. Michaely, P., and Bennett, V. (1993) J. Biol. Chem. 268, 22709–22709
Structural Requirements for Interaction of Sodium Channel β1 Subunits with Ankyrin

Jyoti D. Malhotra, Matthew C. Koopmann, Kristin A. Kazen-Gillespie, Nicholas Fettman, Michael Hortsch and Lori L. Isom

J. Biol. Chem. 2002, 277:26681-26688.
doi: 10.1074/jbc.M202354200 originally published online May 7, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202354200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 40 references, 23 of which can be accessed free at http://www.jbc.org/content/277/29/26681.full.html#ref-list-1