Identification of a 33-Kilodalton Cytoskeletal Protein with High Affinity for the Sodium Channel†

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ABSTRACT: The voltage-sensitive sodium channel is an intrinsic membrane protein that is nonrandomly distributed in neurons, suggesting a possible interaction with other cellular constituents. In this study, we have directly tested the hypothesis that components of the cytoskeleton interact with sodium channels. Utilizing the methods of sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blot overlay, we have identified a 33-kilodalton cytoskeletal protein (p33) that binds ^{32}P-labeled sodium channel purified from rat brain. This binding is a high-affinity (K_D < 1 nM) protein–protein interaction that is blocked by low concentrations of unlabeled sodium channels but is not blocked by monosaccharides, the complex glycoprotein fetuin, the transmembrane protein Na^+–K^+-ATPase, or bovine serum albumin. Levels of p33 are highest in lung and spleen while lower levels are found in brain, peripheral nerve, skeletal muscle, liver, and testes. This tissue distribution implies that the sodium channel may not be the only ligand for p33.

The segregation of membrane proteins into functional domains is a general feature of many cell types in adult tissue. For example, sodium channels are highly concentrated in the subsynaptic membrane of skeletal muscle cells (Beam et al., 1985; Angelides, 1986; Dreyfus et al., 1986) while calcium channels reside mainly in the transverse tubules (Almers et al., 1981). In myelinated neurons sodium channels are located in the axon initial segments (Catterall, 1981; Boudier et al., 1985; Wollner & Catterall, 1986) and nodes of Ranvier (Ellisman & Levinson, 1982; Waxman & Ritchie, 1985). Calcium channels, on the other hand, are found in the soma and nerve terminal but not along the axon (Hagiwara & Byerly, 1981).

Although distinct and functionally specialized membrane domains exist in many cell types, it is not known how these domains are maintained in adult tissue or how they are formed during the course of cellular differentiation. In erythrocytes it has been established that the lateral mobility of the anion transporter is restricted due to its interactions with components of the membrane skeleton [reviewed by Bennett (1985) and Marchesi (1985)]. Closely related analogues of the erythrocyte membrane skeleton proteins spectrin, ankyrin, actin, and protein 4.1 have been identified in nonerythroid tissues [reviewed by Moon and McMahon (1987)], and it seems likely that the cytoskeleton may perform a similar anchoring function in nonerythroid tissues. Koenig and Repasky (1985) have shown by immunofluorescence that spectrin is localized at the nodes of Ranvier and not at paranodal regions in goldfish neurons, suggesting that interaction with the spectrin-based membrane skeleton may play a role in maintaining the specific localization of neuronal proteins. Physiologically relevant interactions among some cytoskeletal components can be reformed in vitro by separation of proteins on SDS–polyacrylamide gels, followed by blot overlay methods to assay for protein–protein interactions (Baines & Bennett, 1985). In the present experiments, we have used the blot overlay approach to identify a novel 33-kilodalton (kDa)[1] cytoskeletal protein (p33) having a high affinity for the sodium channel. Future studies will determine whether p33 plays a role in the observed localization of sodium channels in neurons.

Experimental Procedures

Materials. [γ-^{32}P]ATP was obtained from Du Pont–New England Nuclear (Wilmington, DE). Saxitoxin (STX) was obtained from the National Institutes of Health and [^{3}H]STX was prepared according to the tritium exchange procedure of Ritchie et al. (1976) and purified and characterized as described previously (Waechter et al., 1983). Dulbecco's modified Eagle's medium (DMEM) used in cell culture was purchased from Gibco Laboratories (Santa Clara, CA), and fetal calf serum was purchased from HyClone Laboratories (Logan, UT). Trypsin was purchased from Sigma (St. Louis, MO). Male Sprague-Dawley rats were supplied by Tyler Labs (Bellevue, WA). Nitrocellulose (pore size BA85 0.45 μm) was obtained from Schleicher and Schuell (Keene, NH).

Cell Culture. N18 mouse neuroblastoma cells were grown as monolayers on 50-mL culture flasks in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. At confluence, each flask contained approximately 6×10^6 cells.

Preparation of Cell Extracts. Typically, flasks of N18 cells were washed 2 times with phosphate-buffered saline (PBS) and treated with trypsin (25 mg mL^{-1}) in Hank's buffered balanced salts (without Ca^{2+} or Mg^{2+}) for 1 min at 37 °C to remove the cells from the flask. For whole cell extracts, cells were centrifuged, the pellet was resuspended in 10 volumes of SDS gel loading buffer [2.5% sodium dodecyl sulfate (SDS), 12.5% w/v glycerol, 125 mM Tris, pH 6.8, 5% β-mercaptoethanol (βME), 0.025% bromophenol blue], and the mixture was then boiled. To prepare Triton X-100 extracted cells, after treatment with trypsin the cells were centrifuged and the cell

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[1] Abbreviations: kDa, kilodalton(s); STX, saxitoxin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; βME, β-mercaptoethanol; SDS, sodium dodecyl sulfate; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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pellet was resuspended in Triton extraction buffer [100 mM KCl, 30 mM sucrose, 20 mM 1,4-piperazinediethanesulfonic acid (PIPES), 1 mM magnesium acetate, 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 0.5% Triton X-100], placed on ice for 1 min, and centrifuged (15000*g*) for 5 min at 4 °C. The insoluble cytoskeletal pellet was resuspended in 1 mL of SDS gel loading buffer, and 200 μL of 5× SDS gel loading buffer was added to the Triton X-100 soluble supernatant. Samples were boiled prior to separation of proteins on SDS–polyacrylamide gels (described below).

Protease Treatment of N18 Cells. To investigate the protease sensitivity and cellular location of p33, three identical culture flasks of N18 cells (6×10^6) were washed in PBS. A flask of control cells was treated with 2 mL of trypsin (25 mg mL^{-1}, as above) for 1 min at 37 °C to dislodge the cells from the flask. After 1 min, phenylmethanesulfonyl fluoride (PMSF) was added to 0.2 mM to inhibit trypsin activity, and the entire 2 mL was immediately boiled after addition of 2 mL of 2× SDS gel loading buffer. A second flask of cells was treated identically except that the cells were incubated with trypsin for 15 min at 37 °C. A third flask of cells was treated with trypsin for 1 min at 37 °C to dislodge the cells from the flask. Subsequently, Triton X-100 was added to 2%, and the cells were incubated at 37 °C for an additional 14 min. Examination by light microscopy revealed that after 30 s of incubation with Triton X-100 no intact cells remained, whereas the cells in the flask that did not contain Triton X-100 remained intact for the entire 15 min. An equal volume corresponding to 5% of each sample was assayed for the presence of p33 (see assay description below).

Tissue Preparation. To investigate the distribution of p33 in various tissues, approximately 0.5 g wet weight of several tissues was dissected from an adult male Sprague-Dawley rat and immediately frozen in liquid nitrogen. Samples were crushed into a fine powder with a mortar and pestle over dry ice and subsequently boiled in 10 volumes of SDS gel loading buffer, and protein content was determined as described (Peterson, 1977). To determine whether p33 was present in red blood cells, 500 μL of rat blood was added to 10 mL of 155 mM choline chloride, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and 100 μg mL^{-1} heparin, pH 7.5. The cells were spun in a clinical centrifuge (1000*g*) for 3 min at room temperature, and the red cell pellet was resuspended in 10 mL of choline chloride buffer (minus heparin), mixed gently, and spun as before. The final pellet was resuspended in 10 volumes of SDS gel loading buffer, vortexed, and boiled. Protein was determined as above.

Subcellular Fractionation. To study the subcellular distribution of p33 and to compare it with the distribution of sodium channel, a crude synaptosomal fraction was isolated from rat brain as described by Catterall et al. (1979). Briefly, rat brain tissue (2 g wet weight) was homogenized with 11 strokes of a teflon-to-glass homogenizer in 20 mL of homogenization buffer: 5 mM sodium phosphate, 0.32 M sucrose, pH 7.4, plus protease inhibitors (1 mM iodoacetamide, 1 mM PMSF, 1 μM pepstatin A). The suspension was centrifuged at 1000*g* for 10 min in a Sorvall SS34 rotor at 4 °C. The pellet was resuspended in 20 mL of ice-cold homogenization buffer and recentrifuged at 1000*g* as above. The supernatants from these two 1000*g* spins were combined and called S1. S1 was further centrifuged at 25000*g* for 30 min (SS34 rotor). The 25000*g* pellet (P2) was resuspended in 20 mL of lysis buffer (5 mM Hepes–Tris, pH 7.4, plus protease inhibitors as above), mixed by rotation for 30 min at 4 °C, and recentrifuged at 25000*g*. P1 and P2 were resuspended in 20 mL of homogenization buffer, and 1% of the volume was taken for separation on gels. Aliquots of 1% of the supernatant volumes were similarly taken. To assay the subcellular location of p33, all samples were electrophoresed, blotted to nitrocellulose, and probed with ^{32}P-labeled sodium channel as described below. To determine the location of sodium channel, the STX was used in a [^3H]STX binding assay as previously described (Catterall et al., 1979).

Sodium Channel Purification and Phosphorylation. Sodium channel was purified from rat brain by established procedures (Hartshorne & Catterall, 1984). Purified sodium channel was phophorylated with [γ-^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase as described previously (Costa & Catterall, 1984).

p33 Binding Assay. In the process referred to in the text as blot overlay, protein samples from all cell extracts and tissues were separated on SDS–polyacrylamide gels (10%) and transferred to nitrocellulose electrophoretically (Granger & Lazarides, 1984). The nitrocellulose "blots" were subsequently probed with ^{32}P-labeled sodium channel for at least 8 h in binding buffer (150 mM NaCl, 5 mM NaN_3, 1 mM EDTA, 15 mM Tris, pH 7.5, 0.1% gelatin, 0.1% Tween, 0.1% Triton X-100) to assay for the presence of p33, washed 5 times in binding buffer without sodium channel, and exposed to Kodak XAR-5 film for autoradiography.

Results and Discussion

The Sodium Channel Binds Specifically to p33. We initially investigated the interaction between purified ^{32}P-labeled sodium channel and proteins from N18 mouse neuroblastoma cells by separating the proteins of total N18 cell extracts on an SDS–polyacrylamide gel, blotting onto nitrocellulose, and probing the nitrocellulose with 0.125 pM ^{32}P-labeled sodium channel. A single band with a relative molecular mass of 33 000 was detected with the sodium channel probe (Figure 1A, lane 1). The binding was specifically blocked by addition of excess unlabeled sodium channel to the binding buffer (Figure 1A, lane 2). Increasing the concentration of sodium channel probe to 10 nM does not result in the specific labeling of any other protein bands (data not shown). The ability of unlabeled sodium channel to block the binding of ^{32}P-labeled sodium channel was the same regardless of the state of phosphorylation of the unlabeled sodium channel (data not shown). Purified rat brain sodium channels contain $\beta 1$ and $\beta 2$ subunits of 36 and 33 kDa, respectively (Hartshorne & Catterall, 1984). Therefore, we next investigated whether p33 was a previously described subunit of the sodium channel. When purified β subunits were electrophoresed, blotted onto nitrocellulose, and probed with ^{32}P-labeled sodium channel as in Figure 1, no binding to the β subunits was observed (data not shown). Therefore, we conclude that p33 is a new sodium channel ligand.

Disruption of the native conformation of the sodium channel blocks its binding to p33. When the disulfide linkages of the sodium channel are reduced and alkylated in the presence of SDS, the binding of channel to p33 is not detected (Figure 1B, lane 2). When the channel is reduced and alkylated in the absence of SDS (Figure 1B, lane 3), only a low level binding of the sodium channel to p33 is observed relative to a control blot (Figure 1B, lane 1). These data imply that the binding of the sodium channel to p33 requires the native conformation of the sodium channel.

The binding of sodium channel to p33 probably does not involve the carbohydrate domain of the sodium channel since the binding was sensitive to reduction and denaturation (above) and since the addition of several different sugars and glyco-

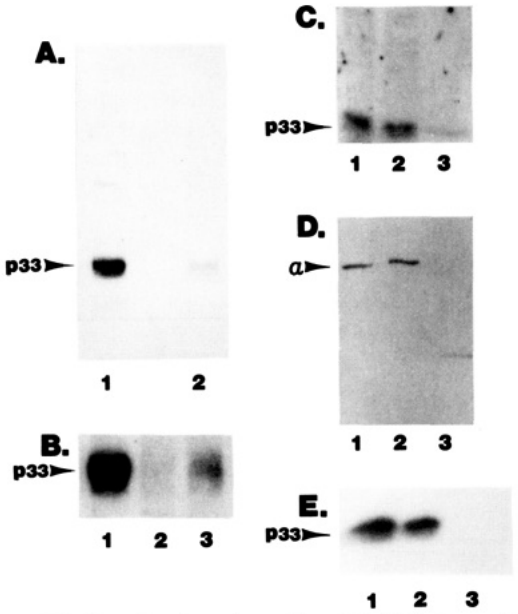

FIGURE 1: Binding of sodium channel to p33. (A) Autoradiogram of a blot overlay of solubilized N18 cells probed with 0.125 pM ^{32}P-labeled sodium channel (lane 1) or ^{32}P-labeled sodium channel plus a 1000-fold excess of unlabeled sodium channel (lane 2). In addition to the band observed at 33 kDa, another band of variable intensity is observed at the dye front (data not shown). As this band is often absent, and remains after protease treatment of N18 cells, this interaction may be due to carbohydrate or lipid domains of the sodium channel and is therefore not analyzed further in this study. (B) Sensitivity of sodium channel to denaturation. N18 cell extracts transferred to nitrocellulose were probed with ^{32}P-labeled sodium channel that had been either untreated (lane 1), reduced in the presence of SDS with 10 mM βME for 10 min at 20 °C, followed by alkylation with 30 mM iodoacetamide for 30 min at 20 °C (lane 2), or reduced and alkylated in the absence of SDS (lane 3). (C) Sensitivity of p33 to protease treatment. N18 cells were trypsinized for 1 min (lane 1), 15 min in the absence of Triton X-100 (lane 2), or 15 min in the presence of 2% Triton X-100 (lane 3). (D) Sensitivity of α-fodrin to protease treatment. Lanes are identical with those in (C). The nitrocellulose blot was probed with polyclonal antibodies specific for α-fodrin (Giebelhaus et al., 1987). (E) Autoradiogram of blot overlay of whole N18 cells (lane 1), Triton X-100 insoluble fraction of N18 cells (lane 2), and Triton X-100 soluble fraction of N18 cells (lane 3). Volumes of the Triton X-100 insoluble and soluble fractions were adjusted to equivalent proportions.

proteins to the binding buffer did not decrease the binding activity. Glucose, sucrose, galactose, mannose, *N*-acetylglucosamine, and the complex glycoprotein fetuin at 1 mg mL^{-1} were unable to block the binding of sodium channel to p33 (data not shown). Addition of calcium (5 mM), bovine serum albumin (50 mg mL^{-1}), or the transmembrane protein Na^+–K^+-ATPase also had no effect (data not shown).

Association of p33 with the Cytoskeleton. We next used protease treatment to investigate whether p33 was an intracellular protein. Treatment of intact N18 cells with trypsin for 1 (Figure 1C, lane 1) or 15 min (Figure 1C, lane 2) did not diminish the observed binding. When the integrity of the cell membranes was disrupted with Triton X-100 and the solubilized cells were treated with trypsin for 15 min, sodium channel binding was virtually undetectable (Figure 1C, lane 3). To show that the trypsin was excluded from cells in the absence of Triton X-100, and that trypsin had access to intracellular proteins upon addition of Triton X-100, antibodies against the membrane skeleton protein α-fodrin (nonerythroid α-spectrin) (Giebelhaus et al., 1987) were used to probe a Western blot identical with that shown in Figure 1C. Exposure of intact N18 cells to trypsin for 1 (Figure 1D, lane 1) or 15 min (Figure 1D, lane 2) did not result in the cleavage of α-fodrin. If, however, the cell membranes were disrupted, α-fodrin was cleaved by trypsin (Figure 1D, lane 3). The susceptibility of p33 to proteolysis in permeabilized cells, but not in intact cells, supports the hypothesis that p33 is an intracellular protein. The sensitivity of p33 to proteolysis probably accounts for the occasional appearance of a doublet at 33 kDa (Figure 1C, lanes 1 and 2, and Figure 3B,C, all lanes).

Extraction of cells with Triton X-100 leaves an insoluble residue containing the cytoskeleton and associated proteins [reviewed by Bennett (1985) and Marchesi (1985)]. Figure 1E shows an experiment in which we extracted N18 cells with Triton X-100 and assayed the insoluble (Figure 1E, lane 2) and soluble (Figure 1E, lane 3) fractions for the presence of p33. All of p33 is retained in the Triton-insoluble fraction (Figure 1E, lane 2), consistent with the association of p33 with the cytoskeleton.

p33 Has High Affinity for the Sodium Channel. As the above data suggest that the sodium channel binds to an intracellular protein of 33 kDa, we next examined whether this binding was saturable and was of high affinity. The binding of ^{32}P-labeled sodium channel to p33 can be completely inhibited by increasing concentrations of unlabeled sodium channel, with half-maximal inhibition at less than 0.1 nM (Figure 2A). The binding is saturable and exhibits an apparent K_D of less than 1 nM (Figure 2B). Although more accurate estimates of the affinity will require purification of p33 and development of solution binding assays, the present data show that the binding of sodium channel to p33 is specific and is of high affinity.

Distribution of p33. Specific binding of ^{32}P-labeled sodium channel was used as an assay to identify p33 in several tissues from adult rat. Proteins from different tissues were separated on SDS–polyacrylamide gels (Figure 3A), and p33 was assayed by blot overlay with ^{32}P-labeled sodium channel (Figure 3B). The greatest levels of p33 were detected in N18 cells, lung, and spleen (Figure 3B, lanes 1, 5, and 10, respectively). Although we were unable to detect the protein in 50-μg protein samples of skeletal muscle, brain, red blood cells, and peripheral nerve (Figure 3B, lanes 6–9), analysis of larger protein samples (250 μg) revealed p33 in brain, peripheral nerve, and skeletal muscle (Figure 3C, lanes 1–3) but not in red blood cells.

The distributions of p33 and sodium channels in subcellular fractions of rat brain were determined by using established procedures for isolating synaptosomes (Catterall et al., 1979). Greater than 80% of the sodium channel remains soluble in the 1000*g* supernatant (S1) (Figure 4A). After the 25000*g* spin of S1, essentially all of the recoverable sodium channel is detected in the 25000*g* pellet (P2), similar to previous reports (Catterall et al., 1979). The distribution of p33 through this synaptosomal preparation is different. All of p33 is brought down in the 1000*g* pellet (P1, Figure 4B), which contains intact cells, nuclei, and some cytoskeletal proteins. The membrane skeleton protein α-fodrin (nonerythroid α-spectrin) is also found in P1 to a significant extent (Figure 4C). These experiments demonstrate that while p33 does not remain associated with the sodium channel through a synaptosomal preparation, it does cofractionate with a major proportion of the spectrin-based membrane skeleton. We suggest that these observations reflect the preferential binding of p33 to cytoskeletal elements that fractionate into P1. This observation is analogous to the behavior of the membrane skeleton protein ankyrin. Erythroid ankyrin forms a linkage between the anion transporter and spectrin but is found preferentially in the

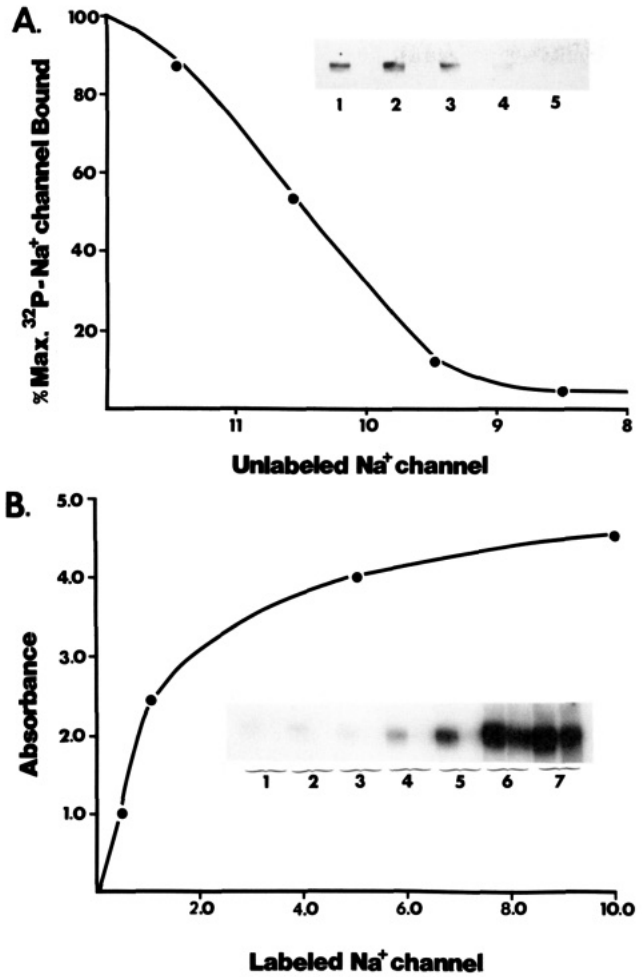

FIGURE 2: Inhibition curve and saturation isotherm for sodium channel binding to p33. (A) Inhibition of ^{32}P-labeled sodium channel binding to p33 by increasing concentrations of unlabeled sodium channel. Inset: Autoradiogram of p33 bands probed with ^{32}P-labeled sodium channel and increasing concentrations of unlabeled channel. Nitrocellulose strips (0.5 cm) containing 50 μg of protein were incubated with 12.5 pM ^{32}P-labeled sodium channel plus 0 pM, 5 pM, 50 pM, 500 pM, and 5 nM unlabeled sodium channels in lanes 1–5, respectively. After exposure of the nitrocellulose strips to preflashed Kodak XAR-5 film, binding was estimated by measuring the density of each band with an LKKB 2222-010 Ultroscan XL laser densitometer. Absorbance in arbitrary units is plotted on the ordinate, and the negative log of the sodium channel concentration (M) is plotted on the abscissa. (B) Concentration dependence of specific binding of sodium channel to p33. Inset: Autoradiogram of saturation assay performed on nitrocellulose strips. Total binding (first lane of each pair) and nonspecific binding (second lane of each pair) were determined for the following concentrations of ^{32}P-labeled sodium channel: 0.05 (lanes in 1), 0.10 (lanes in 2), 0.25 (lanes in 3), 0.50 (lanes in 4), 1.0 (lanes in 5), 5.0 (lanes in 6), and 10.0 nM (lanes in 7). Addition of 20 nM unlabeled sodium channel defined the nonspecific binding. Absorbance in arbitrary units is plotted on the ordinate, and sodium channel concentration (nM) is plotted on the abscissa (only lanes 4–7 are plotted).

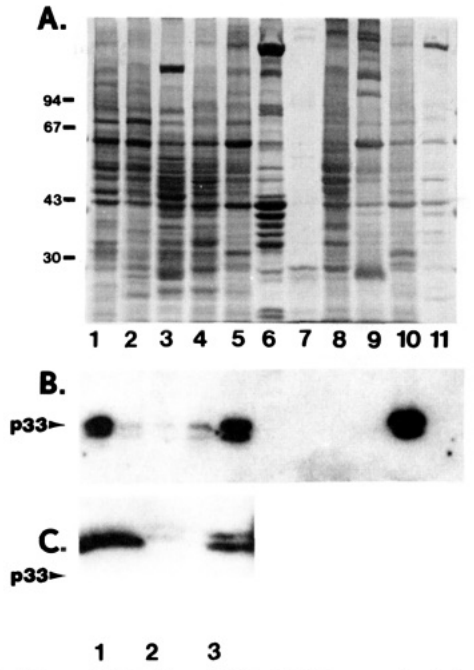

FIGURE 3: Tissue distribution of p33. (A) Coomassie stained gel of total protein from N18 cells (lane 1) and the following rat tissues (50 μg of protein per lane): testes (lane 2), liver (lane 3), kidney (lane 4), lung (lane 5), skeletal muscle (lane 6), blood (lane 7), brain (lane 8), peripheral nerve (lane 9), spleen (lane 10), and heart (lane 11). (B) Autoradiogram of blot overlay of tissue samples (lane designation as in panel A) probed with ^{32}P-labeled sodium channel. (C) Overloaded samples of approximately 250 μg of protein per lane of extracts of brain (lane 1), peripheral nerve (lane 2), and skeletal muscle (lane 3).

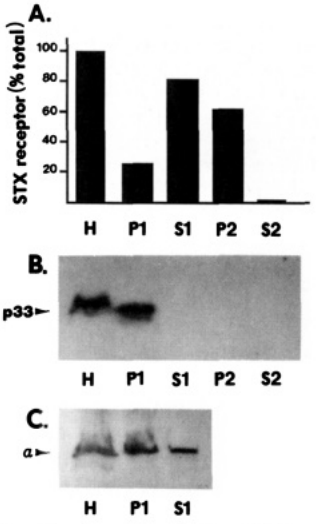

FIGURE 4: Subcellular fractionation of p33. (A) Bar graph of relative levels of sodium channel (assayed by amount of bound STX) (Catterall et al., 1979) from rat brain homogenate (H), 1000*g* pellet (P1), 1000*g* supernatant (S1), 25000*g* pellet (P2), and 25000*g* supernatant (S2). (B) Autoradiogram of blot overlay of fractionation steps described in (A). ^{32}P-Labeled sodium channel was used as a probe. (C) Autoradiogram of Western blot of H, P1, and S1 probed with polyclonal antibodies against α-fodrin (Giebelhaus et al., 1987).

cytoskeletal fraction of red blood cells [reviewed by Bennett (1985) and Marchesi (1985)]. Whether alternative procedures for subcellular fractionation maintain the hypothesized binding of p33 to sodium channels is presently under investigation.

Possible Function of p33. We speculate that p33 may be involved in the linkage between the cytoskeleton and selected membrane proteins, serving to localize them into discrete functional domains. That p33 may serve specialized functions in differentiated cells is consistent with our observation that levels of p33 vary greatly among different tissue types. The observation that lung and spleen exhibit the highest levels of p33 by the sodium channel binding assay implies that there are ligands for p33 other than sodium channel, which is absent from these tissues. The ability of p33 from lung and spleen to bind brain sodium channel, however, implies that some p33 ligands may have a conserved p33 binding domain. Recent work provides an example of a single membrane skeleton protein that binds different ligands in a tissue-specific manner. Ankyrin binds the anion transporter in erythrocytes [reviewed by Bennett (1985) and Marchesi (1985)] and the Na^+–K^+-ATPase in epithelial cells (Nelson & Veshnock, 1987).

Moreover, erythrocyte ankyrin binds the Na^+-K^+-ATPase from epithelial cells in vitro, suggesting the presence in ankyrin of conserved binding domains for two distinct integral membrane proteins.

These data present the first evidence for the interaction of the sodium channel with another cellular protein. It is likely that this finding will have implications for other cell types due to the presence of p33 in tissues not containing sodium channels. Further studies of the interaction of the sodium channel with p33 may enable us to obtain a better understanding of the mechanisms by which plasma membranes become functionally specialized during differentiation.

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Sequence Specificity of DNA Cleavage by Bis(1,10-phenanthroline)copper(I)†

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ABSTRACT: The bis(1,10-phenanthroline)copper(I) complex is a relatively simple molecule previously shown to cause DNA cleavage with a strong preference for gene control regions such as the Pribnow box. Sequence level mapping of sites of $[(Phen)_2Cu^I]^+$ cleavage in >2000 bases in histone genes and the plasmid pUC9 showed that the specificity for control regions is related to a predominant preference for minor groove binding at TAT triplets, which were cleaved most strongly at the adenosine sugar ring. The related sequences TGT, TAAT, TAGPy, and CAGT (Py = pyrimidine) were moderately preferred, while CAT and TAC triplets, PyPuPuPu quartets, PuPuPuPy quartets, and CG-rich PyPuPuPy quartets were cleaved with low to average frequency. Polypurine and polypyrimidine sequences were cleaved with low frequency. The sequence preferences of $[(Phen)_2Cu^I]^+$ can be ascribed predominantly to (i) a requirement for binding in the minor groove at a pyrimidine 3′ → 5′ step and (ii) stereoelectronic effects of the 2-amino group of guanine in the minor groove, which inhibit binding. Although the reagent appears primarily to recognize sequence features at the triplet or quartet level, lower than expected cleavage was observed for two TAT sequences adjacent to several other preferred sequences and higher than expected cleavage was observed at CAAGC sequences, suggesting that longer range sequence-dependent DNA conformational effects influence specificity in certain cases.

The bis(1,10-phenanthroline)copper(I) complex $[[(Phen)_2Cu^I]^+]$ and other phenanthroline complexes have attracted considerable interest as probes of local DNA conformation and as reagents for "footprinting" binding sites of proteins and other ligands to DNA (Barton, 1986; Sigman, 1986). $[(Phen)_2Cu^I]^+$ appears to recognize DNA with con-

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