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Identification of binding domains in the sodium channel $Na_V1.8$ intracellular N-terminal region and annexin II light chain p11

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Abstract The interaction of p11 (annexin II light chain) with the N-terminal domain of $Na_V1.8$, a tetrodotoxin-resistant sodium channel, is essential for the functional expression of the channel. Here we show that p11 binds to $Na_V1.8$ but not to sodium channel isoforms $Na_V1.2$, 1.5, 1.7 or $Na_V1.9$. The binding of amino acids 74–103 of $Na_V1.8$ to p11 residues 33–78 occurs in a random coiled region flanked by two EF hand motifs whose crystal structure has been established. As $Na_V1.8$ channel expression is associated with pain pathways, drugs that disrupt the $Na_V1.8$–p11 interaction and down-regulate channel expression may have analgesic activity.

Key words: Sensory neuron; Pain; Sodium channel; Annexin; S100 protein

1. Introduction

Voltage-gated sodium channels initiate and propagate action potentials in excitable cells. Ten distinct pore-forming α-subunits of voltage-gated sodium channels have been identified in rat [1]. The tetrodotoxin-resistant sodium channel $Na_V1.8$ (also known as SNS/PN3 or SCN10a) is expressed predominantly in nociceptive small diameter sensory neurones. $Na_V1.8$ is a functionally atypical sodium channel, and can give rise to slowly activating and inactivating currents that are insensitive to micromolar concentrations of tetrodotoxin [2,3]. Behavioural studies on $Na_V1.8$ null mutant mice have demonstrated a role for $Na_V1.8$ in the detection of noxious thermal, mechanical and inflammatory stimuli [4].

Although voltage-gated sodium channel activity resides within the α-subunits, the cellular localisation of channels, as well as aspects of their kinetics and levels of functional expression are regulated by accessory β-subunits [5]. Unlike other sodium channels, functional $Na_V1.8$ is poorly expressed in cell lines even in the presence of accessory β-subunits [6]. This suggests other regulatory proteins are involved in the functional expression of $Na_V1.8$.

Using a yeast two-hybrid screening, we previously identified annexin II light chain (p11) as a regulatory factor that facilitates the expression of $Na_V1.8$ on the plasma membrane [7,8]. p11, a member of the S100 family of small calcium binding proteins, binds directly to the N-terminus of $Na_V1.8$ and promotes the translocation of $Na_V1.8$ to the plasma membrane resulting in functional channels. We also showed that endogenous $Na_V1.8$ current in cultured small diameter dorsal root ganglion (DRG) neurones is inhibited by antisense down-regulation of p11 expression. p11 mRNA is expressed at high levels in DRG and at lower levels in heart, liver and brain, while none is expressed in kidney. p11 selectively interacts with the N-terminal region of $Na_V1.8$ and not the other four intracellular regions of $Na_V1.8$.

In the present study, we attempted to identify the binding domain in $Na_V1.8$ and p11. The binding domain for p11 was located at amino acid (aa) positions 74–103 in the N-terminus of $Na_V1.8$, while the binding domain for $Na_V1.8$ was identified between residues 33 and 78 in p11. This peptide lost binding ability to $Na_V1.8$ when cut between position 51 and 52. This suggests that the loop connecting the two EF hands and the first α-helix of the second EF hand plays a key role in binding to $Na_V1.8$. As direct association between $Na_V1.8$ and p11 is required for functional expression of $Na_V1.8$, disrupting this interaction may be a useful new approach to down-regulating $Na_V1.8$ and effecting analgesia.

2. Materials and methods

2.1. Molecular cloning of N-terminus of rat $Na_V1.8$ and p11

The segment encoding the N-terminus of rat $Na_V1.8$ (aa 1–127) was amplified by polymerase chain reaction (PCR) using full length $Na_V1.8$ cDNA [2,7,8] with the introduction of *Eco*RI and *Not*I sites in the 5′ and 3′ end respectively and cloned in-frame into pGEX-5X-1 (Amersham Pharmacia Biotech). The resultant glutathione *S*-transferase (GST) fusion protein was named GST-N. The N-terminal fragment was also divided into three parts, aa 1–25, 26–50 and 51–127, named N1, N2 and N3 respectively. The third segment (aa 51–127) was further cut into three smaller fragments. The three fragments, named N3-1 (aa 51–73), N3-2 (aa 74–103) and N3-3 (aa 104–127), and N1, N2 and N3 were also cloned into pGEX-5X-1 vector at *Eco*RI (5′) and *Not*I (3′) sites. The correct sequences and in-frame ligation into the expression vector of all PCR products were confirmed by sequencing.

Full length rat cDNA for p11 was previously subcloned into the pBS500 expression vector to generate green fluorescent protein (GFP)-p11 fusion protein as described [7]. The resultant plasmid was designated pBS-GFP/p11. Full length p11 was also divided into three fragments (aa 1–32, 33–77 and 78–95) and cloned in-frame into the *Nco*I/*Xba*I sites in pBS500 to express the fragments as GFP fusion proteins. The three fragments, named p11-1, p11-2 and p11-3, and full length p11 were transfected individually into COS-7 cells grown in 100 mm dishes in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum and 5% streptomycin–penicillin using lipofection.

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Abbreviations: DRG, dorsal root ganglion; GST, glutathione *S*-transferase; GFP, green fluorescent protein

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The subsequent p11 proteins were extracted from transfected COS-7 cells by lysis buffer (4% sodium dodecyl sulphate (SDS), 10 mM sodium phosphate, pH 7.4) 3 days after transfection as described [7]. Fragment p11-2 was further cut into two parts (aa 33–51 and 52–77) after confirming a direct interaction between the N-terminus of rNa$_V$1.8 and the p11-2 fragment. The two smaller fragments, named p11-2-1 (aa 33–51) and p11-2-2 (aa 52–78) along with another fragment named p11-1A (aa 1–51), were cloned in-frame into pBS500 as above. The electrophoretic mobility of the p11 fragments on the gel appears approximately 30 kDa higher due to fused GFP.

2.2. Molecular cloning of partial rNa$_V$1.2, rNa$_V$1.5, rNa$_V$1.7 and rNa$_V$1.9

mRNA was extracted from 3 week old rat heart, brain, and DRG tissue as described [9]. Using reverse transcription PCR, the 84 base pairs that correspond to the N3-2 region of Na$_V$1.8 in Na$_V$1.2, Na$_V$1.5, Na$_V$1.7, and Na$_V$1.9 were amplified and cloned in-frame into the *Eco*RI/*Not*I sites of pGEX-5X-1.

2.3. GST pull-down assay

The GST/Na$_V$1.8 N-terminal fusion proteins, designated GST-N, -N1, -N2, -N3, -N3-1, -N3-2 and -N3-3, were produced in *Escherichia coli* BL-21 and affinity purified on glutathione-Sepharose beads [7]. To examine the binding region of the N-terminus of Na$_V$1.8 to p11 in vitro, glutathione-Sepharose beads preincubated with GST-N1, -N2 and -N3 were incubated at 4°C overnight with full length p11 fusion protein extracted from transfected COS-7 cells in phosphate buffered saline (PBS). After intensive washing with ice-cold PBS, the bound proteins were denatured in sample buffer (100 mM Tris–HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol), separated by 10–12% SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech). Primary anti-GFP antibody (Santa Cruz Biotechnology) in 1:800 dilutions was applied for 1 h at room temperature. Secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse IgG, Amersham Pharmacia Biotech) in 1:2000 dilutions was applied for 1 h at room temperature. ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech) was applied according to the manufacturer's instructions and the blot was exposed to BioMax film (Kodak). The same protocol was used to see whether p11 binds to the same 28 aa region in Na$_V$1.2, Na$_V$1.5, Na$_V$1.7 or Na$_V$1.9 as in the N3-2 region on Na$_V$1.8 using anti-His$_6$ tag antibody as a primary antibody.

To examine the exact binding region of p11 to the N-terminus of Na$_V$1.8 in vitro, glutathione-Sepharose beads were preincubated with purified GST-N, the entire 127 aa N-terminus of Na$_V$1.8 fused to GST, and incubated at 4°C overnight with one of the three fragmented p11 fusion protein extracted from transfected COS-7 cells in PBS.

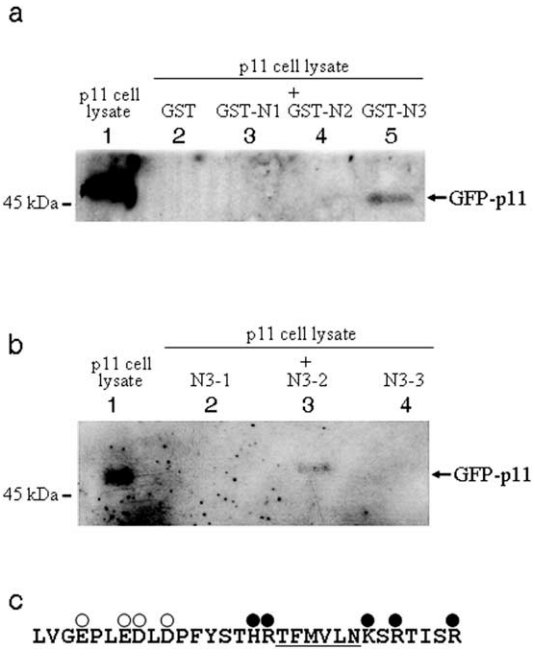

Fig. 1. Mapping of the Na$_V$1.8 binding domain to p11 by GST pull-down assay. a: GFP-p11 binds directly to N3 region (aa 51–127). b: GFP-p11 binds to N3-2 (aa 74–103). c: Protein sequence of N3-2 region of Na$_V$1.8. Acidic amino acids (open circles) and basic amino acids (closed circles) are shown. Putative β-strand is underlined.

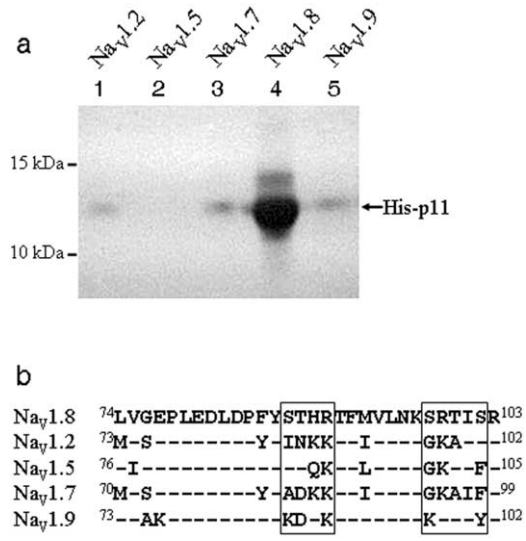

Fig. 2. Specificity of p11 to the voltage-gated sodium channels. a: His$_6$-tagged p11 binds strongly to Na$_V$1.8 in GST pull-down assay, while Na$_V$1.2, Na$_V$1.7 and Na$_V$1.9 show weak affinity to p11. b: Sequence alignment of N3-2 domain in the rat voltage-gated sodium channels. Two domains of Na$_V$1.8 with low homology to other sodium channels are shown in rectangle.

2.4. 3D structure analysis of possible Na$_V$1.8 binding site in p11

Computational 3D data of human p11 chain A and B [10], PDBid 1BT6, were analysed by Protein Explorer (http://www.rcsb.org/pdb/).

3. Results

3.1. p11 binds to aa 74–103 of the N-terminus of Na$_V$1.8

To identify the binding region of p11 to the N-terminus of Na$_V$1.8 in vitro, we expressed the GFP-p11 fusion protein in COS-7 cells, and divided the N-terminal domain of Na$_V$1.8 into three fragments (aa 1–25, 26–50 and 51–127) and expressed them as GST fusion proteins, N1, N2 and N3, in BL21 bacterial cells. Fig. 1a shows the N3 region of Na$_V$1.8 (lane 5) pulled down with GFP-p11 fusion proteins efficiently in an GST pull-down assay. Neither purified GST, GST-N1 nor GST-N2 pulled down GFP-p11 protein (lanes 2–4), demonstrating that GFP-p11 lysate binds specifically to aa 51–127 of Na$_V$1.8. This region was further divided into three smaller fragments and expressed as GST fusion protein, named N3-1 (aa 51–73), N3-2 (aa 74–103) and N3-3 (aa 104–127). Fig. 1b shows that N3-2 pulled down GFP-p11 fusion proteins specifically (lane 3), demonstrating that GFP-p11 binds to aa 74–108 of Na$_V$1.8. The amino acid sequence of this region (Fig. 1c) shows a characteristic cluster of acidic (open circles) and basic (closed circles) amino acids divided by putative β-strand (underlined). These residues are well conserved in other species (mouse, human and dog).

3.2. p11 specifically binds to $Na_V1.8$ among voltage-gated sodium channels

As p11 is expressed in various tissues including heart and brain [7], it is important to study the specificity of affinity between p11 and voltage-gated sodium channels. We tested the binding ability of p11 to $Na_V1.2$, $Na_V1.5$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ using His-tagged p11 in a GST pull-down assay. Fig. 2a shows $Na_V1.8$ pulled down p11 efficiently (lane 4). $Na_V1.2$, $Na_V1.7$ and $Na_V1.9$ show weak binding to p11, while $Na_V1.5$ does not show affinity to p11. By comparing protein sequences, two domains of $Na_V1.8$ showed particularly low homology to other sodium channels (Fig. 2b, rectangle). The two domains are well conserved in other species (mouse, human, and dog). These domains may play a key role in binding to p11.

3.3. $Na_V1.8$ binds to aa 33–78 of p11

To determine the region of p11 that binds to $Na_V1.8$, p11 was divided into three fragments, p11-1 (aa 1–32), p11-2 (33–77) and p11-3 (78–95). These fragments were subjected to GST pull-down assays against the N-terminus of $Na_V1.8$, GST-N. Fig. 3a shows that fragment p11-2 binds specifically to the N-terminus of $Na_V1.8$ (lane 5). GST-N did not pull down cell lysates p11-1 and p11-3 (lanes 4 and 6). Aa 33–77 (p11-2) of p11 was further divided into two smaller fragments, p11-2-1 (aa 33–51) and p11-2-2 (aa 52–77). Fig. 3b shows that none of the smaller fragments including another segment, p11-1A (aa 1–51), binds to the N-terminus of $Na_V1.8$ (lanes 6–8). There are two EF hand motifs in p11 (Fig. 3c), although they do not have the ability to bind calcium owing to mutations [10]. Fragment p11-2-2 (Fig. 3c, underlined in magenta) holds the second EF hand motif almost entirely, while p11-2-1 (underlined in cyan) consists of the linker region of the two EF hand motifs. This suggests the loop connecting the two EF hands, designated L_{II} in [7], and the first α-helix of the second EF hand (H_{III}) play key roles in binding to $Na_V1.8$.

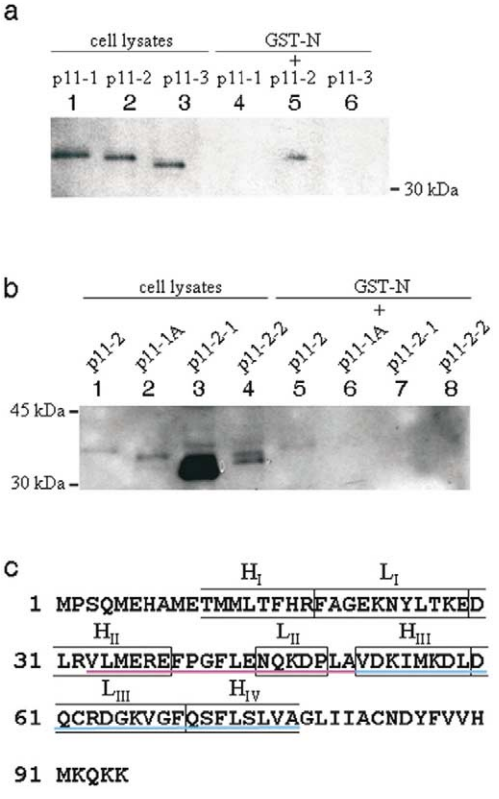

Fig. 3. Mapping of p11 binding domain to $Na_V1.8$ by GST pull-down assay. a: N-terminal domain (127 residues) of $Na_V1.8$, GST-N, binds p11-2 region (aa 33–77) of p11. b: GST-N does not bind to either p11-2-1 (aa 33–51) or p11-2-2 (aa 52–77). c: Full length rat p11 sequence is shown. The first EF hand consists of the H_I-L_I-H_{II} region, while H_{III}-L_{III}-H_{IV} forms the second EF hand motif. p11-2-1 and p11-2-2 regions are shown with magenta and cyan underline respectively.

A 3D model of the human p11 chain A [10], which shares 95% homology with rat p11, is shown in Fig. 4. Fig. 4a,d shows p11-2-1 and p11-2-2 domains in magenta and cyan respectively. Interestingly, p11-2-1 and p11-2-2 domains are connected by a hydrogen bond between Asp^{48} and Lys^{54} (Fig. 4c) with distance of O(Asp^{48})–N(Lys^{54}) 2.421 Å. As Asp^{48} locates in p11-2-1 and Lys^{54} is in p11-2-2, this may be one of the reasons why cutting p11-2 into p11-2-1 and p11-2-2 resulted in loss of binding ability to $Na_V1.8$. It is difficult to identify the actual binding site for $Na_V1.8$ within the p11-2 domain due to lack of knowledge of the 3D structure of the N3-2 domain of $Na_V1.8$. However, we came up with two possible areas as binding pockets for $Na_V1.8$ by analyzing the 3D structure of p11. Fig. 4b shows amino acids forming one of the possible binding sites for $Na_V1.8$. This pocket consists of different types of amino acids, two acidic amino acids (Asp^{53} and Asp^{58}), two basic amino acids (Lys^{57}, Arg^{63}) and Leu^{50}. From an alternative angle of view (Fig. 4e), a unique pocket consists of three acidic amino acids (Glu^{36}, Glu^{44} and Asp^{53}) and Pro^{49} can be seen. One of these pockets may be responsible for binding to $Na_V1.8$. Although the 3D structure of the L_{II} region of p11 is slightly different between chains A and B in the p11 dimer [10], the 3D conformation of the putative binding pockets shown in Fig. 4b,e is well preserved in chain B (data not shown). These listed amino acids are all well conserved in other species (mouse, human, cow, pig, and chicken).

4. Discussion

In the present study, we identified the putative interactive domains in the $Na_V1.8$–p11 complex. Because p11 is known to exist as a dimer in a heteromeric annexin II complex, it is possible that two molecules of $Na_V1.8$ bind to a p11 homodimer. Recently, the 3D structure of the complex of p11 with annexin II heavy chain has been proposed in two possible models, a heterotetrameric and a hetero-octameric model [11]. In both models, the putative binding domains for $Na_V1.8$ are the most peripheral and exposed. This suggests that there is good accessibility for p11 binding to $Na_V1.8$.

Since we reported that p11 binds directly to $Na_V1.8$ and facilitates its functional expression on the plasma membrane [7], other groups have also reported p11 as a co-factor for expression of other membrane proteins. TASK-1, the 2P domain potassium channel, interacts with p11 and the association appeared to be essential for trafficking of TASK-1 to the plasma membrane [12]. TRPV5 and TRPV6, the epithelial calcium channels which belong to the superfamily of transient receptor potential (TRP) channels, also associate with p11 and subsequently move into the plasma membrane [13]. Both TASK-1 and TRPV5/6 have an internal type I PDZ consensus binding sequence Ser/Thr-X-Val [14] in their C-termini, Ser-Ser-Val in TASK-1 and Thr-Thr-Val in TRPV5/6. This se-

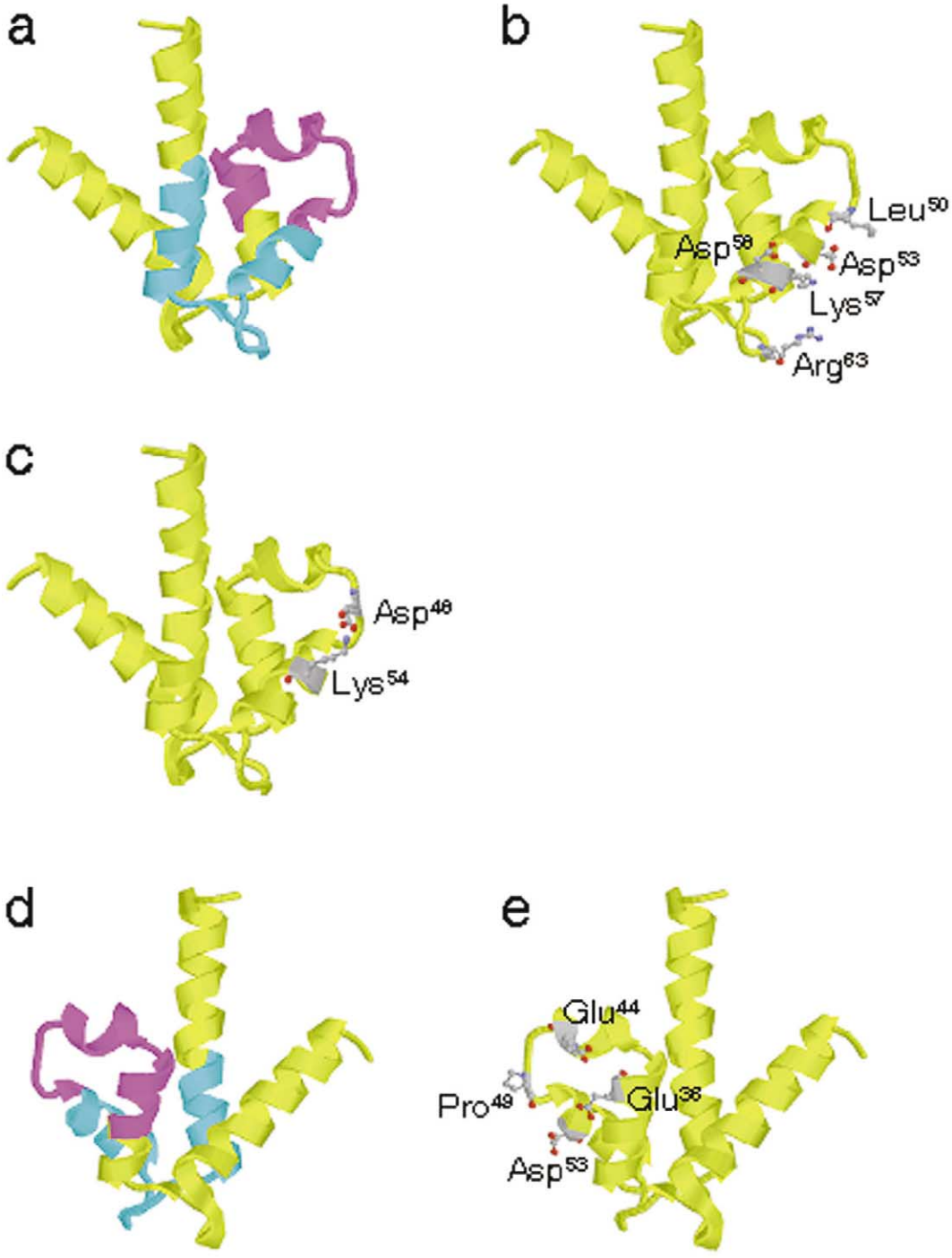

Fig. 4. Computational 3D structure of p11 chain A. a,d: p11-2-1 and p11-2-2 regions are shown in magenta and cyan respectively. c: Two amino acids forming a hydrogen bond (Asp^{48} and Lys^{54}) are shown. Negatively charged atoms are shown in red and positive ones are shown in blue. b,e: Putative $Na_V1.8$ binding pockets are shown with amino acids exposed. d,e: Alternative view of angle to a–c.

quence does not exist in the N3-2 domain of $Na_V1.8$. Therefore $Na_V1.8$ is likely to use a novel binding motif to associate with p11. Neither study [13,14] included an examination of binding domains of the channels to p11. It is important to know if these channels share the same binding site in p11 in terms of developing drugs to disrupt specifically the interaction between p11 and $Na_V1.8$.

In this study, we found that p11 specifically binds to $Na_V1.8$ and not other voltage-gated sodium channels. It is also important to know if other S100 proteins bind to $Na_V1.8$. Twenty members of the S100 protein family have been identified so far [15]. In DRG, S100 proteins are mainly expressed in large diameter neurones, while only 8% of small diameter neurones (cell body size $< 600\ \mu m^2$, corresponds to a cell diameter $< 27.6\ \mu m$) expressed S100 protein detected using pan-S100 protein antibody [16]. In contrast, we showed that p11 is highly expressed in small diameter nociceptive neurones in DRG [7]. The putative $Na_V1.8$ binding sites in

p11 proposed in this study (Fig. 4b,e) are conserved in S100A11 (also known as S100C, calgizzarin, or annexin I light chain); however, there is no apparent homology with other S100 proteins. S100A11 is expressed in some populations of small DRG neurones at a modest level, and up-regulated by axotomy in most DRG neurones [17]. This raises the possibility that $Na_V1.8$ interacts not only with p11, but also with S100A11 in DRG neurones.

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