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The voltage-gated Na\textsuperscript{+} channel Na\textsubscript{v}1.8 contains an ER-retention/retrieval signal antagonized by the β3 subunit

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Received 23 June 2008

Journal of Cell Science 121, 3243-3252 Published by The Company of Biologists 2008
doi:10.1242/jcs.026856

Summary

Voltage-gated Na\textsuperscript{+} channel (Na\textsubscript{v}) 1.8 contributes to the majority of the Na\textsuperscript{+} current that underlies the depolarizing phase of action potentials. Na\textsubscript{v}1.8 is mainly distributed intracellularly and its current amplitude can be enhanced by the β3 subunit. However, little is known about the mechanisms underlying its intracellular retention and the effects mediated by the β3 subunit. Here, we show that the β3 subunit promotes surface expression of Na\textsubscript{v}1.8 by masking its endoplasmic reticulum (ER)-retention/retrieval signal. The RRR motif in the first intracellular loop of Na\textsubscript{v}1.8 is responsible for retaining Na\textsubscript{v}1.8 in the ER and restricting its surface expression. The β3 subunit facilitates surface expression of Na\textsubscript{v}1.8. The intracellular C-terminus of the β3 subunit interacts with the first intracellular loop of Na\textsubscript{v}1.8 and masks the ER-retention/retrieval signal. Mutation of the RRR motif results in a significant increase in surface expression of Na\textsubscript{v}1.8 and abolishes the β3-subunit-mediated effects. Thus, the β3 subunit regulates surface expression of Na\textsubscript{v}1.8 by antagonizing its ER-retention/retrieval signal. These results reveal a novel mechanism for the effect of the Na\textsuperscript{+} channel β subunits on the α subunits.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/19/3243/DC1

Key words: Na\textsubscript{v}1.8, ER-retention/retrieval signal, Na\textsuperscript{+} channel β3 subunit

Introduction

Voltage-gated Na\textsuperscript{+} channels (Na\textsubscript{v}) comprise at least nine pore-forming α subunits, Na\textsubscript{v}1.1-1.9. Distribution of Na\textsubscript{v}1.8 is restricted to small and medium-sized dorsal root ganglion (DRG) neurons and their nociceptive afferent fibers (Benn et al., 2001). Na\textsubscript{v}1.8 current contributes to the depolarizing phase of action potentials (Renganathan et al., 2001). Importantly, Na\textsubscript{v}1.8 is mainly localized intracellularly and has to be recruited into the plasma membrane to exert its function (Okuse et al., 2002). However, the mechanisms that regulate the retention and trafficking of Na\textsuperscript{+} channels remain largely unknown.

Recent studies on the mechanisms for the assembly and trafficking of ion channels have mostly focused on their exit from the endoplasmic reticulum (ER), in which these proteins are synthesized and matured (Ma and Jan, 2002). One of the main strategies for quality control of ion channels is assembly-dependent export from the ER. Several transiently active ER-retention/retrieval signals have been identified in ion channel subunits (Ellgaard and Helenius, 2003). For ATP-sensitive potassium channels, an ER-retention/retrieval signal, RXR, has been found in both the α and β subunits (Zerangue et al., 1999). The hetero-octameric assembly of these subunits in the ER can reciprocally mask their ER-retention/retrieval signals, which leads to maturation of the channels and their transportation to the cell surface. This mechanism prevents incompletely assembled channels from being exported to the cell surface. Similarly, the intracellular C-terminus of the γ-aminobutyric acid receptor (GABA\textsubscript{B}1) subunit has been shown to contain an RSRR ER-retention/retrieval signal, and its assembly with the GABA\textsubscript{B2} subunit overcomes its ER localization (Margita-Mitrovic et al., 2000). Thus, masking of ER-retention/retrieval signals can be a crucial mechanism for regulating the surface expression of ion channels.

Most Na\textsuperscript{+} channels are associated with auxiliary β subunits (Catterall, 2000). Four different β-subunit genes, Scn1b (β1) (Isom et al., 1992), Scn2b (β2) (Isom et al., 1995), Scn3b (β3) (Morgan et al., 2000) and Scn4b (β4) (Yu et al., 2003), have been identified. An important role of these auxiliary subunits is to modulate the gating properties of Na\textsuperscript{+} channels. Recently, the β subunits have been shown to regulate surface expression of the α subunits. The β1 subunit increases the cell-surface density of Na\textsubscript{v}1.2 through β1-contactin and β1-ankyrin interactions (Kazarinova-Noyes et al., 2001; McEwen et al., 2004). Measurement of [\textsuperscript{3}H]saxitoxin binding shows that the expression level of Na\textsuperscript{+} channels on the cell surface is significantly reduced in DRG neurons of β2-knockout mice (Chen et al., 2002), suggesting that the β2 subunit plays an important role in ensuring surface expression of the α subunits. The β3 subunit has been reported to increase the amplitude of the Na\textsubscript{v}1.8 current (John et al., 2004; Shah et al., 2000). However, it remains unclear how surface expression of the α subunits is regulated by the β subunits.

In the present study, we demonstrate that the β3 subunit promotes the surface expression of Na\textsubscript{v}1.8 by masking the ER-retention/retrieval signal of this channel. We have identified an RRR motif in the first intracellular loop of Na\textsubscript{v}1.8 as an ER-retention/retrieval signal. When transiently expressed in COS-7 cells, the wild-type Na\textsubscript{v}1.8 was largely distributed within the ER.
However, mutation of the RRR motif resulted in a threefold increase in the surface expression of Na\textsubscript{1.8}, while the effect mediated by the C-terminus of the β3 subunit was abolished. Furthermore, we have demonstrated that the intracellular C-terminus of the β3 subunit interacts with the first intracellular loop of Na\textsubscript{1.8}. This interaction masks the ER-retention/retrieval signal of Na\textsubscript{1.8}, thus promoting its surface targeting. These results reveal a molecular mechanism for the regulation of cell-surface expression of Na\textsuperscript{+} channel α subunits by the β subunits.

**Results**

Na\textsubscript{1.8} is mainly retained in the ER

Na\textsubscript{1.8} is highly expressed in small and medium-sized DRG neurons in rat. Previous reports have shown that Na\textsubscript{1.8} is mainly localized intracellularly (Novakovic et al., 1998; Okuse et al., 2002). To further analyze the subcellular distribution of Na\textsubscript{1.8}, we constructed plasmids that expressed Na\textsubscript{1.8} with either a Myc (Na\textsubscript{1.8-Myc}) or GFP (Na\textsubscript{1.8-GFP}) tag at the C-terminus. The Na\textsubscript{1.8} constructs were verified by sequencing and evaluated by a functional assay. Na\textsubscript{1.8-GFP} was transfected into ND7-23 cells in order to evaluate the electrophysiological properties of Na\textsubscript{1.8} currents in transfected cells (John et al., 2004). This cell line is derived from rat DRG and does not express Na\textsubscript{1.8}, but it contains accessory Na\textsuperscript{+} channel β1 and β3 subunits. The tetrodotoxin-resistant Na\textsuperscript{+} current of Na\textsubscript{1.8} was detected and compared with that in DRG neurons. The Na\textsubscript{1.8} currents in Na\textsubscript{1.8-GFP}-expressing ND7-23 cells resembled the endogenously dissected Na\textsubscript{1.8} currents at the holding potential of ~70 mV (Rush et al., 2005) (supplementary material Fig. S1A), which indicates that transfected Na\textsubscript{1.8} is functionally identical to native Na\textsubscript{1.8}.

Because ND7-23 cells are round and do not show distinct subcellular structures, we used COS-7 cells to observe the subcellular localization of Na\textsubscript{1.8}. COS-7 cells are derived from African green monkey kidney fibroblast-like cells and lack Na\textsubscript{1.8} (supplementary material Fig. S1A). They are flat and are usually used to analyze the subcellular localization of proteins in organelles. In COS-7 cells transfected with Na\textsubscript{1.8-Myc} or Na\textsubscript{1.8-GFP}, the channel displayed a typical reticulum-like distribution, and was colocalized with the ER marker calnexin, but not with the Golgi marker GM130 (also known as GOLGA2) (Fig. 1A; supplementary material Fig. S1B). No cell-surface distribution of the channel was apparent. Although a Na\textsubscript{1.8} current was detected, the current amplitude was small in COS-7 cells transfected with Na\textsubscript{1.8-GFP} (supplementary material Fig. S1A). We thus conclude that the expressed Na\textsubscript{1.8} is mainly retained in the ER.

The RRR motif in the first intracellular loop of Na\textsubscript{1.8} is an ER-retention/retrieval signal

Membrane proteins with ER-retention/retrieval signals can be retained in the ER with limited surface expression (Margeta-Mitrovic et al., 2000; Nasu-Nishimura et al., 2006; Nilsson et al., 1989; Ren et al., 2003; Zerangue et al., 1999). Upon searching for the typical ER-retention/retrieval motifs, we found three RXR sites within the rat Na\textsubscript{1.8} sequence, two in the first and one in the second intracellular loop (Fig. 1B). The rat CD8α subunit, which shows distinct surface expression, was used as a control. We thus conclude that the expressed Na\textsubscript{1.8} is mainly retained in the ER.

**Fig. 1.** The RRR motif in the first intracellular loop of Na\textsubscript{1.8} serves as an ER-retention/retrieval signal.

(A) COS-7 cells transfected with Na\textsubscript{1.8-Myc} were immunofluorescently labeled with antibodies against Myc and the ER marker calnexin, or the Golgi marker GM130. (B) Schematic of the intracellular segments of Na\textsubscript{1.8} and the amino acid sequence of the first part of the first intracellular loop (1L). (C) Plasmids expressing Myc-CD8α, Myc-CD8α-KKTN, or a series of constructs with the C-terminus of CD8α linked to the second intracellular loop (2L) or the various segments of 1L of Na\textsubscript{1.8} were transfected into COS-7 cells. Transfected cells were immunofluorescently labeled with antibody against Myc or calnexin. The images are representative of at least three independent experiments. Scale bars: 10 μm.
model molecule to test whether these RXR sites could induce ER localization. In COS-7 cells, the transfected Myc-CD8α was exclusively present on the cell surface, as expected (Fig. 1C). We directly appended KKTN, the classical motif for ER localization (Cossins and Letourneau, 1994; Nilsson et al., 1989), to the C-terminus of Myc-CD8α (Myc-CD8α-KKTN). The transfected Myc-CD8α-KKTN was not distributed on the cell surface but rather was co-localized with calnexin (Fig. 1C). This represented a typical morphological pattern for ER localization. We further found a typical ER localization for the first intracellular loop of Na+,1.8 fused at the C-terminus of Myc-CD8α (Myc-CD8α-1L), but not for the second one (Myc-CD8α-2L) (Fig. 1C). To identify the functional motif for ER localization, we made a series of constructs by fusing various truncated segments of the first intracellular loop to the C-terminus of Myc-CD8α (Fig. 1B). Only those chimeric proteins that contained the 495RRR497 motif (Myc-CD8α-1L-1, Myc-CD8α-1L-1-1-3) co-localized with calnexin, whereas the chimeric proteins that contained the 458RPR460 motif (Myc-CD8α-1L-1-2) were mainly expressed on the cell surface (Fig. 1C). Furthermore, when 495RRR497 was substituted with alanine (Myc-CD8α-1L-1-3m), the chimeric proteins did not associate with ER structures, but appeared on the cell surface (Fig. 1C). These results indicate that the second RXR motif, RRR, in the first intracellular loop of Na+,1.8 functions as an ER-retention/retrieval signal.

To confirm the ER-retention/retrieval signal, endoglycosidase H (Endo H) digestion was employed in transfected COS-7 cells. Endo H is able to digest high-mannose, N-linked carbohydrate moieties in the ER. Susceptibility of a glycoprotein to Endo H digestion suggests that it is retained in the ER (Nilsson et al., 1989). Because rat CD8α contains one N-linked and several O-linked carbohydrate units, it should be digested by Endo H if it resides in the ER. Immunoblotting of Myc-CD8α revealed bands with a molecular weight of 25-37 kDa. The majority of Myc-CD8α was concentrated in the higher molecular weight bands and was resistant to Endo H digestion, representing the mature form of the protein, whereas a small amount of Myc-CD8α in the lower molecular weight band was sensitive to Endo H digestion, representing the immature form of the protein in the ER (Fig. 2). In contrast to the immunoblotting pattern of Myc-CD8α, Myc-CD8α-KKTN was concentrated only in the band sensitive to Endo H digestion (Fig. 2), which was consistent with its distribution in the ER (Fig. 1C). Importantly, we found that a large proportion of Myc-CD8α-1L and Myc-CD8α-1L-1 was sensitive to Endo H digestion, whereas only a fraction of Myc-CD8α-2L or Myc-CD8α-1L-2 displayed sensitivity to Endo H digestion (Fig. 2). A large proportion of Myc-CD8α-1L-1-3 was found to be sensitive to Endo H digestion, whereas Myc-CD8α-1L-1-1, Myc-CD8α-1L-1-2 and Myc-CD8α-1L-1-3m were not (Fig. 2). Thus, the RRR motif, but not the RPR motif, in the first intracellular loop of Na+,1.8 mediates residence of the protein in the ER.

The RRR ER-retention/retrieval signal restricts the surface expression of Na+,1.8

To verify the effect of the RRR ER-retention/retrieval signal on the surface expression of the protein, we carried out permeabilized and non-permeabilized immunostaining, conditions under which either the total protein or just the protein on the plasma membrane of transfected COS-7 cells, respectively, is labeled (Fig. 3). A Myc tag was inserted between the signal peptide and mature protein of CD8α, and non-permeabilized immunostaining was performed with Myc antibody before paraformaldehyde fixation. As demonstrated above, transfected Myc-CD8α exhibits the typical cell-surface expression, whereas Myc-CD8α-KKTN exhibits prominent localization in the ER (Fig. 3). We found that cells transfected with Myc-CD8α-1L, Myc-CD8α-1L-1 and Myc-CD8α-1L-1-3 all showed weak fluorescent labeling on the cell surface (Fig. 3). However, cells transfected with Myc-CD8α-2L, Myc-CD8α-1L-1-2 or Myc-CD8α-1L-1-3m displayed prominent surface labeling (Fig. 3). The difference in surface labeling was not due to different levels of expression because all constructs had a comparable expression level as judged by whole-cell permeabilized staining. The above morphological results were confirmed by quantitative analysis of protein levels by cell-surface biotinylation and immunoblotting (Fig. 4A,B). In COS-7 cells transfected with Myc-CD8α fused with various segments of the first intracellular loop that contained an RRR motif, a lower level of surface expression of the chimeric proteins was detected as compared with expression of proteins lacking this motif. However, cells transfected with Myc-CD8α-1L-1-3m resumed prominent surface expression. Thus, the RRR ER-retention/retrieval signal in the first intracellular loop of Na+,1.8 restricts its surface expression.

The surface expression of the full-length channel was further examined when the RRR ER-retention/retrieval motif was mutated or removed. We mutated the RRR motif to alanine or replaced the first intracellular loop with the second one in full-length Na+,1.8. Since the transfection efficiency of full-length Na+,1.8 was low in COS-7 cells and the quantity of channel protein produced was not sufficient for biochemical studies, we chose HEK293 cells with their higher transfection efficiency for these experiments. HEK293 cells do not express Na+,1.8 and the transfected channel was functional (supplementary material Fig. S1A). Two days after transfection with Na+,1.8-GFP or its mutants, cell-surface biotinylation and immunoblotting showed that mutation of the RRR motif, or replacement of the first intracellular loop with the second one, in full-length Na+,1.8 caused a threefold increase in surface expression (Fig. 4C). These results suggest that the RRR ER-retention/retrieval signal in the first intracellular loop of Na+,1.8 contributes to a lower level of surface expression of the channel.
Fig. 3. The RRR ER-retention/retrieval signal reduces surface expression of the CD8α chimeras. COS-7 cells were transfected with plasmids expressing Myc-CD8α, Myc-CD8α-KKTN, or with a series of constructs with the C-terminus of CD8α linked to 2L or to the various segments of 1L of Nav1.8. Non-permeabilized and permeabilized immunofluorescence labeling were carried out with antibodies against Myc. The images are representative of at least three independent experiments. Scale bar: 10 μm.

Fig. 4. The RRR ER-retention/retrieval signal reduces surface expression of Nav1.8. (A,B) COS-7 cells transfected with a plasmid expressing Myc-CD8α, Myc-CD8α-KKTN, or with a series of constructs with the C-terminus of CD8α linked to 2L or to the various segments of 1L of Nav1.8, were subjected to cell-surface biotinylation/immunoblotting. Representative immunoblot and quantitative analyses are shown. Actin served as an internal control for protein loading. The ratio of immunoblot intensity of surface versus total protein was calculated and data were plotted as a percentage of the controls (n>3). **P<0.01 versus cells transfected with the plasmids indicated; ***P<0.01 versus Myc-CD8α-1L-1-expressing cells. (C) HEK293 cells were transfected with plasmids expressing Na1.8 or Na1.8 mutants in which 495RRR497 was mutated to alanine (Na1.8m-Myc), or the first intracellular loop was replaced by the second one (Na1.8r-Myc), and subjected to cell-surface biotinylation/immunoblotting. Data were plotted as a percentage of Na1.8-Myc. *P<0.05 versus cells transfected with Na1.8-Myc (n>3).
The C-terminus of the β3 subunit masks the RRR ER-retention/retrieval signal

The Na⁺ channel β subunits regulate surface expression of the α subunits (Kazarinova-Noyes et al., 2001; McEwen et al., 2004), and the β3 subunit increases the amplitude of the Na⁺,1.8 current (John et al., 2004; Shah et al., 2000). Therefore, we explored whether the β3 subunit could regulate the effect of the RRR ER-retention/retrieval signal of Na⁺,1.8. The effect of the β1 subunit, which exhibits 50% homology with the β3 subunit, was also studied. In COS-7 cells that co-expressed Myc-CD8α-1L-1-3 and the β3 subunit, cell-surface biotinylation and immunoblotting showed that the surface expression of Myc-CD8α-1L-1-3 was significantly promoted by the β3 subunit (Fig. 5A). However, co-expression of the β1 subunit did not enhance the surface expression of Myc-CD8α-1L-1-3 (Fig. 5A).

Having found the RRR ER-retention/retrieval signal in the intracellular loop of Na⁺,1.8, we investigated the role of the intracellular C-terminus of the β3 subunit in regulating cell-surface targeting of the α subunits. We modified a system that has been used to examine the reciprocal effect of juxtaposition of various cytoplasmic domains on their ability to mask ER-retention/retrieval signals (Letourneur et al., 1995). We constructed a GFP-tagged CD8β subunit (CD8β-GFP) and inserted the C-terminus of the β3 subunit between CD8β and the GFP tag (CD8β-β3C-GFP). In our system, Myc-CD8α displayed distinct surface expression, whereas CD8β-GFP was localized in the ER in single-plasmid-transfected COS-7 cells (Fig. 5B). However, CD8β-GFP exhibited exactly the same pattern as Myc-CD8α in double-plasmid-transfected cells (Fig. 5B). Thus, efficient assembly between CD8β and CD8α enables the surface expression of CD8β. Then, CD8β-β3C-GFP or CD8β-GFP was co-transfected with Myc-CD8α-1L-1. Seventy percent of CD8β-β3C-GFP-co-transfected cells showed distinct surface expression of Myc-CD8α-1L-1, whereas only 5% of CD8β-GFP-co-transfected cells displayed this pattern. As a negative control, CD8β-β3C-GFP did not have any effect on the ER localization of Myc-CD8α-KKTN. The cell-surface biotinylation and immunoblotting results showed that CD8β-β3C-GFP, but not CD8β-GFP, significantly enhanced the surface expression of Myc-CD8α-1L-1 (Fig. 5C) and Myc-CD8α-1L-1-3 (Fig. 5D). However, CD8β-β3C-GFP did not promote surface targeting of either Myc-CD8α-1L-1 or Myc-CD8α-1L-1-2 (Fig. 5D). These results suggest that the C-terminus of the β3 subunit masks the ER-retention/retrieval signal of the RRR motif through juxtaposition upon assembly of CD8α and CD8β.

**Fig. 5.** The C-terminus of the β3 subunit masks the RRR ER-retention/retrieval signal. (A) COS-7 cells were co-transfected with plasmids expressing Myc-CD8α-1L-1-3 and β1-GFP or β3-GFP, and subjected to cell-surface biotinylation/immunoblotting. Representative immunoblot and quantitative analyses are shown. Actin served as an internal control for protein loading. Data were plotted as a percentage of controls. *P<0.05 versus cells transfected with Myc-CD8α-1L-1-3 only (n=3). (B) COS-7 cells were co-transfected with plasmids expressing CD8β-GFP and Myc-CD8α, CD8β-β3C-GFP and Myc-CD8α-1L-1, CD8β-GFP and Myc-CD8α-1L-1, or CD8β-β3C-GFP and Myc-CD8α-KKTN. Cells were immunofluorescently labeled with antibody against Myc. The images are representative of at least three independent experiments. Scale bar: 10 μm. (C,D) COS-7 cells were co-transfected with CD8β-GFP or CD8β-β3C-GFP plus Myc-CD8α-1L-1, Myc-CD8α-1L-1-2, Myc-CD8α-1L-1-3, and subjected to cell-surface biotinylation/immunoblotting. *P<0.05 versus cells co-transfected with CD8β-GFP (n=3).
The C-terminus of the β3 subunit interacts with RRR-motif-containing segments and promotes their surface expression
In the above system, the C-terminus of the β3 subunit was juxtaposed to the segments of the first intracellular loop of Na_1.8 through interaction between CD8α and CD8β. To explore the underlying mechanism that keeps the RRR-motif-containing segments in the first intracellular loop of Na_1.8 close to the C-terminus of the β3 subunit in native molecules, we investigated their interaction. To ensure the correct membrane insertion of the C-terminus of the β3 subunit, we constructed a truncated mutant that contained the transmembrane domain, the C-terminus of the β3 subunit (amino acids 160-215) and the GFP tag at the C-terminus (TM+β3C-GFP). Co-immunoprecipitation showed that TM+β3C-GFP interacted with Myc-CD8α-1L-1 (Fig. 6A) and Myc-CD8α-1L-1-3 (Fig. 6C), but not with Myc-CD8α-1L-1-2 or Myc-CD8α-1L-1-3m (Fig. 6C). In parallel, surface expression of Myc-CD8α-1L-1 (Fig. 6B) or Myc-CD8α-1L-1-3 (Fig. 6D,F), but not Myc-CD8α-1L-1-2 or Myc-CD8α-1L-1-3m (Fig. 6E,F), was increased in COS-7 cells when co-transfected with TM+β3C-GFP. Thus, the C-terminus of the β3 subunit interacts with the first intracellular loop of Na_1.8, which leads to the steric juxtaposition and masking of the ER-retention/retrieval signal.

The RRR motif conveys the effect of the C-terminus of the β3 subunit on Na_1.8
To investigate whether the RRR motif in full-length Na_1.8 interacts with the C-terminus of the β3 subunit and to address whether such an interaction plays a role in regulating the targeting of Na_1.8, we co-transfected the latter with either full-length Na_1.8 or its mutant in which the RRR motif was changed to alanine (Na_1.8m-Myc). The results showed that the C-terminus of the β3 subunit interacted with Na_1.8 (Fig. 7A) and promoted surface expression of Na_1.8 (Fig. 7B). However, when co-transfected with the latter, the C-terminus of the β3 subunit continued to interact with the mutant (Fig. 7A) but failed to promote its surface targeting (Fig. 7C). This suggests that another domain(s) of Na_1.8 is also involved in its interaction with the C-terminus of the β3 subunit, and that the C-terminus of the β3 subunit promotes surface expression of Na_1.8 by masking its RRR motif.

Finally, we investigated whether the C-terminus of the β3 subunit could promote surface expression of Na_1.8 in DRG neurons. The transactivator of transcription (Tat) peptide, an 11-amino-acid protein-transduction domain of human immunodeficiency virus, has been used as a cell-penetrating vector to deliver small cargoes or large molecules (Lindgren et al., 2000). To deliver the C-terminus of the β3 subunit into DRG neurons, we generated a Tat fusion protein of the C-terminus of the β3 subunit with a glutathione S-transferase (GST) tag at the N-terminus (GST-Tat-β3C) (Fig. 7D). When cultured DRG neurons were incubated with this fusion protein for 12 hours, GST-Tat-β3C penetrated into the cells and significantly increased the surface expression of endogenous Na_1.8 (Fig. 7D). Thus, the C-terminus of the β3 subunit is sufficient for promoting the surface expression of Na_1.8 in DRG neurons.

Discussion
In the present study, we have uncovered a molecular mechanism for intracellular targeting of Na_1.8. We have also demonstrated that the β3 subunit interacts with Na_1.8 to promote its surface expression. Our results reveal that an RRR ER-retention/retrieval signal in the first intracellular loop of Na_1.8 plays an important role in regulating the intracellular distribution and surface expression of this channel. Interaction of the β3 subunit with the RRR motif masked this ER-retention/retrieval signal and thus increased the surface expression of Na_1.8. These findings delineate a novel mechanism for a functional relationship between the Na^+ channel α and β subunits.

The ER-retention/retrieval signal in the first intracellular loop of Na_1.8
ER retention/retrieval has been proposed as an indispensable quality-control step for multimeric protein complexes, including many ion channels. Thus far, the cytoplasmic and C-terminus-localized di-lysine KXXX motif, and the internally positioned RXR motif, have been well characterized as ER-retention/retrieval signals

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**Fig. 6.** The C-terminus of the β3 subunit interacts with segments that contain the RRR motif and promotes their surface expression. The C-terminus of the β3 subunit with the transmembrane domain (TM+β3C-GFP) was co-transfected with Myc-CD8α-1L-1, Myc-CD8α-1L-1-2, Myc-CD8α-1L-1-3 or Myc-CD8α-1L-1-3m in COS-7 cells. (A,C) Proteins were immunoprecipitated with GFP-specific antibody and cell lysates subjected to immunoblotting with antibodies against Myc or GFP as indicated. All experiments were repeated three times. (B,D,F) Transfected cells were subjected to cell-surface biotinylation/immunoblotting. Representative immunoblots are shown. Actin served as an internal control for protein loading. Quantitative data were plotted as a percentage of controls. *P<0.05 versus the control cells or the cells indicated (n=3).
Mechanisms for the effect of the Na\(^+\) channel β subunits on the α subunits

Modulation of the electrophysiological properties and trafficking of the Na\(^+\) channel α subunits is considered a major function of the β subunits. The functional domains of the β1 subunit that are involved in regulating Na\(^+\) channels have been studied. Both intracellular and extracellular interacting domains of the β1 subunit are required for high-affinity association with Na\(^+\) channels. The ER-retention/retrieval motif in Na\(^+\) channels is a major determinant of the localization in the ER. The surface expression of several other transmembrane proteins (e.g., GABA receptors, kainate receptors) is also regulated by this RXR motif (Margeta-Mitrovic et al., 2000; Nasu-Nishimura et al., 2006; Ren et al., 2003).

Here, we have demonstrated that the RRR motif in the first intracellular loop of Na\(^+\) channels in rat, found that the RRR motif in Na\(^+\) channels was not conserved in other Na\(^+\) channels. Since there are other potential ER-retention/retrieval motifs, such as the basic-amino-acid-rich region RRRKKRRKRK in Na\(^+\) channels, and RKR in Na\(^+\), these Na\(^+\) channels might possess ER-retention/retrieval properties similar to those of Na\(^+\) channels. When we compared the Na\(^+\) sequence between species, we found that the RRR ER-retention/retrieval signal in rat was replaced by KRR in human, and the RPR sequence was switched to RHR. The RHR sequence, rather than KRR, serves as an ER-retention/retrieval signal in the CD8α system (our unpublished data). Therefore, the ER-retention/retrieval mechanism might exist in other Na\(^+\) channel isoforms and in Na\(^+\), 8 of other species.

The ER-retention/retrieval signals are effective only when they are positioned within their active zones. The active zone is determined by the length of spacer that separates the cytoplasmic side of the transmembrane domain from the ER-retention/retrieval signal. RXR is functional only when it is positioned distal to the membrane leaflet (Shikano and Li, 2003). Our CD8α-fused chimeras, we retained the entire 26-amino-acid C-terminus of CD8α. Various truncated segments of the intracellular loop of Na\(^+\) channels were directly appended to the C-terminus of CD8α. CD8α-1L-1-3 contained 57 amino acids between the cytoplasmic side of the transmembrane domain and the RXR ER-retention/retrieval signal, thus, the signal was located within its active zone (Shikano and Li, 2003). In the case of CD8α-1L-1-2 or CD8α-2L, the length of the spacing peptide that separated the RPR or RFR sequence from the cytoplasmic side of the transmembrane domain was 55 or 70 amino acids, which were also located within the active zone of the RXR ER-retention/retrieval signal. Thus, the lack of ER localization might be explained by the non-function of the RPR and RFR sequences, rather than inappropriate spacing. In native Na\(^+\), the length of the spacing peptide that separates the RRR motif from the membrane leaflet is 96 amino acids, which are located within the active zone of the RXR ER-retention/retrieval signal. Thus, this RRR ER-retention/retrieval signal contributes to the ER localization of Na\(^+\).

**Fig. 7.** Mutation of the RRR motif in Na\(^+\) disrupts β1-promoted surface expression of the channel. (A) TM+β3C-GFP was co-expressed with either Na\(^+\) or Na\(^+\)Myc in HEK293 cells. Proteins were immunoprecipitated with Myc antibody and cell lysates subjected to quantitative analyses. IB: biotinylation/immunoblotting. Representative immunoblot and (B) Nav1.8β3C-GFP (C) in HEK293 cells. Cultured DRG neurons were transfected with TM+β3C-GFP (B), and Nav1.8β3C-GFP (C) in HEK293 cells. Cultured DRG neurons were transfected with TM+β3C-GFP (B), and Nav1.8β3C-GFP (C) in HEK293 cells. Cultured DRG neurons were transfected with TM+β3C-GFP (B), and Nav1.8β3C-GFP (C) in HEK293 cells. Cultured DRG neurons were transfected with TM+β3C-GFP (B), and Nav1.8β3C-GFP (C) in HEK293 cells. Cultured DRG neurons were transfected with TM+β3C-GFP (B), and Nav1.8β3C-GFP (C) in HEK293 cells. Cultured DRG neurons were transfected with TM+β3C-GFP (B), and Nav1.8β3C-GFP (C) in HEK293 cells.
channels via interaction with components of the cytoskeleton, such as ankyrin G (Malhotra et al., 2000). In the present study, we demonstrate a novel function and mechanism for the intracellular domain of the β subunit, namely, promoting surface expression through masking the ER-retention/retrieval signal of the α subunit.

Protein complexes destined for the plasma membrane seem to follow a common pathway in which assembly is required to preclude ER localization. One potential mechanism is that the ER-retention/retrieval signal of the protein is masked and sterically antagonized by its partner (Zerangue et al., 1999; Margeta-Mitrövic et al., 2000). In our study, the RRR motif in the first intracellular loop of Na\(_\text{v}1.8\) was found to serve as an ER-retention/retrieval signal. The C-terminus of the β subunit interacted with the RRR-motif-containing segments of Na\(_\text{v}1.8\). Thus, assembly of the β subunit with Na\(_\text{v}1.8\) masks the RRR ER-retention/retrieval signal, and this enables the channel to be exported to the cell surface. However, when the RRR ER-retention/retrieval signal was mutated, the interaction between Na\(_\text{v}1.8\) and the C-terminus of the β subunit was not disrupted.

Other parts of Na\(_\text{v}1.8\) also interact with the C-terminus of the β subunit (our unpublished data). Although these interactions might also have led to the steric juxtaposition of two molecules, the RRR motif conveyed the full effect of the C-terminus of the β subunit, and this enables the channel to be exported to the cell surface. Changes in the expression of the β subunit (Shah et al., 2000; Takahashi et al., 2003). We suspect that upregulation of the β3 subunit might contribute to Na\(_\text{v}1.8\) redistribution in injured DRG neurons. It has been proposed that small chemicals that inhibit the function of the β3 subunit may alleviate pain sensation (Isom, 2002). Therefore, specific disruption of the interaction between Na\(_\text{v}1.8\) and the β3 subunit might be a method to reduce the surface expression of Na\(_\text{v}1.8\) and relieve chronic pain.

### Materials and Methods

#### Plasmid construction

To obtain the full-length Na\(_\text{v}1.8\) (GenBank accession number US3833), four segments of Na\(_\text{v}1.8\) (Scn1a) were cloned into pcDNA3.1 from a rat DRG cDNA library and sequenced. The pEGFP-N3 vector for Myc-CD8 \(\beta\) subunit was constructed by PCR and subcloned into the pEGFP-N3 vector. The plasmid of Na\(_\text{v}1.8\) with RRR mutated to alanine was constructed according to the following procedure. It was cloned into the pEGFP-N3 vector using the pEGFP-N-3' primer and the following primer with RRR mutated to alanine: 5'-CACGGATCTAGACAGCACCTCCTGTAAGAGGCGAT-3'. The first intracellular loop of Na\(_\text{v}1.8\) was replaced by the above, amplified segment to obtain the desired construct. The β3 subunit from the rat DRG cDNA library was amplified with primers 5'-TAAAGCTTATGGGAAGCT-3' and 5'-TACGGATCTTACGCCGCACT-3'. The second intracellular loop of Na\(_\text{v}1.8\) was amplified with primers 5'-TATACGATGTTTCAGAGGTCTCCATGCAAAGCCTC-3' and 5'-TTATACGATGTTTCAGAGGTCTCCATGCAAAGCCTC-3'. Then, both the β1 and β3 subunits were cloned into the pEGFP-N3 vector. The C-terminus of the β3 subunit with the signal peptide and transmembrane domain (TM1-3βC, β3 [1-24, 160-215]) was constructed by the two-round PCR method. First-round PCR amplifies TM-5βC with primers 5'-TACAGGATCTAGACAGCACCTCCTGTAAGAGGCGAT-3' and 5'-GAGGATCTTACGCCGCACT-3'. Then, the β1 and β3 subunits were cloned into the pEGFP-N3 vector. The plasmid of Nav1.8 with RRR mutated to alanine was constructed according to the following procedure. It was cloned into the pEGFP-N3 vector using the pEGFP-N-3' primer and the following primer with RRR mutated to alanine: 5'-CACGGATCTAGACAGCACCTCCTGTAAGAGGCGAT-3'. The first intracellular loop of Na\(_\text{v}1.8\) was replaced by the above, amplified segment to obtain the desired construct.

#### For Myc-CD8 β subunit

For Myc-CD8 β subunit from a rat thymus library were amplified using the following primers: 5'-CATGGTGATTTCACTAGACAGCACCTCCTGTAAGAGGCGAT-3' and 5'-TACGCGGAGGAGGCTGTCAGTGTAAGAGGCGAT-3'. The PCR products were purified from 1% agarose gels and then used as templates for the second-round PCR with primers 5'-CTACGCCGCGCGCTGTCAGTGTAAGAGGCGAT-3' and 5'-GAGGATCTTACGCCGCACT-3'. Then, the β1 and β3 subunits were cloned into the pEGFP-N3 vector. The C-terminus of the β3 subunit with the signal peptide and transmembrane domain (TM1-3βC, β3 [1-24, 160-215]) was cloned into the pEGFP-N3 vector.

CD8β and CD8β from a rat thymus library were amplified using the following primers: 5'-CATGGTGATTTCACTAGACAGCACCTCCTGTAAGAGGCGAT-3' and 5'-TACGCGGAGGAGGCTGTCAGTGTAAGAGGCGAT-3'. The PCR products were purified from 1% agarose gels and then used as templates for the second-round PCR with primers 5'-CTACGCCGCGCGCTGTCAGTGTAAGAGGCGAT-3' and 5'-GAGGATCTTACGCCGCACT-3'. Then, the β1 and β3 subunits were cloned into the pEGFP-N3 vector. The C-terminus of the β3 subunit with the signal peptide and transmembrane domain (TM1-3βC, β3 [1-24, 160-215]) was cloned into the pEGFP-N3 vector.
CD8β and the EGFP tag using primers 5'-CATGGGATCTCAGAAAAGGTCTC-TAAAGGCACGGA-3' and 5'-ATGGCATCCCTCCTCAACAGCTACAGA-GTT-3'.

GST-Tat was constructed for sense and antisense oligonucleotides 5'-GGC-GGATCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGGCCTCGAG-CGG-3' and 5'-GGCTGAAAGCCTCGTCTGCTCCTGCTGCCATTAGGCTCGG-3'. After denaturing and annealing, the oligonucleotides were inserted into the pGEX-KG vector. GST-Tat-β3C was constructed based on GST-Tat. The C-terminus of the β3 subunit was inserted into the C-terminus of GST-Tat using primers 5'-GGCCTGAGAGGAGTTCTCT-3' and 5'-CCCAAAGCTT-TATTTGCTCAGGG-3'.

Cell culture and transfection
HEK293 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). HEK293 cells were transiently transfected with plasmids using the calcium phosphate method. Two days after transfection, the cells were collected for the different assays. ND7-23 cells, derived from mouse neuroblastoma and rat DRG neurons, were obtained from the European Collection of Cell Cultures (ECACC; Porton Down, UK) and maintained in DMEM (Invitrogen) supplemented with 1% FBS. African green monkey kidney COS-7 cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS. African green monkey kidney COS-7 cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS. ND7-23 or COS-7 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen). Two days after transfection, the cells were used for the different assays.

Cell-surface biotinylation and western blotting
Experiments were carried out according to a modification of our published protocol (He et al., 2003). Briefly, transfected HEK293 and COS-7 cells, or cultured DRG neurons, were incubated with Sulfos- NHS-Biotin (Pierce, Rockford, IL) for 45 minutes at 4°C, and then lysed in immunoprecipitation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.5 mg/ml BSA) for 1 hour. Freshly prepared lysates were incubated overnight with Immunopure Immobilized Streptavidin (Pierce). After washing, the beads were incubated for 20 minutes at 50°C in SDS-PAGE loading buffer. The samples were separated on SDS-PAGE gels, transferred, probed with antibodies, and visualized using ECL reagents (Amersham Pharmacia, Little Chalfont, UK). Mouse antibodies against GFP (1:1000; Roche, Indianapolis, IN), Myc (1:5000; provided by Jin-Qiu Zhou, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) and actin (1:10,000; Chemicon, Temecula, CA) and rabbit antibody against Na1.8 (1:8 (Alomone Labs, Jerusalem, Israel) were used. The immunoreactive bands were quantified with Image-Pro Plus software (Media Cybernetics, Bethesda, MD), and quantification using Sigma Plot 8.0 (Systat Software, Chicago, IL) was based on at least three independent experiments. The data were processed for mean ± s.e.m. and analyzed by paired Student’s t-test.

Immunoprecipitation
Cells were lysed in immunoprecipitation buffer and the supernatants incubated with 1 g/l mouse antibody against GFP or rabbit antibody against Myc (Sigma, St Louis, MO) overnight at 4°C, followed by incubation with protein G-Sepharose beads (Amersham Biosciences). The immunoprecipitated proteins and 5–10% of total lysates were analyzed by western blotting as described above.

Immunocytochemistry
The transfected COS-7 cells were fixed with 4% paraformaldehyde at 4°C for 15 minutes. For co-localization staining of Na1.8-Myc, Na1.8-GFP or Myc-CD8α, donkey anti-rabbit secondary antibody conjugated with rhodamine was for 45 minutes. For co-localization staining of Nav1.8-Myc, Nav1.8-GFP or Myc-CD8α, donkey anti-rabbit secondary antibody conjugated with rhodamine was for 45 minutes. For co-localization staining of Nav1.8-Myc with the Golgi marker GM130, the samples were examined using a Leica DMRE microscope with a 63× oil-immersion lens (numerical aperture 1.32), and images were taken with the SP2 laser-scanning confocal system at ~20°C (Leica, Germany).

Endo H digestion
Transfected COS-7 cells were harvested and lysed in 300 μl RIPA lysis buffer (150 mM NaCl, 30 mM HEPES pH 7.5, 10 mM NaF, 1% Triton X-100, 0.01% SDS, 0.1 mM PMSF, 1 mM mg/ml pentatin A, 1 mg/ml leupeptin) for 1 hour at 4°C. The cell lysates were centrifuged at 12,000 × g for 10 minutes and the supernatants collected. Proteins were denatured and treated with 0.01 U Endo H (Sigma) to remove high-mannose N-glycans, according to the manufacturer’s instruction. The samples were analyzed by SDS-PAGE and western blotting as described above.

Preparation of dissociated DRG neurons
Male Sprague-Dawley rats (body weight 100-120 g; Shanghai Center of Experimental Animals, Chinese Academy of Sciences) were used according to the policy of the Society for Neuroscience (USA) on the use of animals. The experiment was approved by the Committee of Use of Laboratory Animals and Common Facility, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and sacrificed. The DRGs were dissected and digested with 1 mg/ml collagenase type 1A, 0.4 mg/ml trypsin type 1 and 0.1 mg/ml DNase I (all from Sigma) in DMEM at 37°C for 30 minutes, and then triturated and cultured in DMEM supplemented with 10% FBS for drug treatment. Other dissociated DRGs were plated on coverslips for electrophysiological measurements in an extracellular solution: 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES and 10 mM glucose (pH 7.4).

GST-fused Tat protein expression and purification
Escherichia coli BL21 (DE3) was transformed with constructs encoding GST fusion proteins. A minicircle for each construct was inoculated and grown overnight at 37°C. Then, a large amount of culture was inoculated from the minicircle and allowed to grow to OD600 0.5-0.9. The culture was induced to produce recombinant protein with 1 mM isopropyl-β-D-thiogalactopyranoside and allowed to grow for 4 hours at 37°C. The bacteria were harvested by centrifugation, resuspended and sonicated in ice-cold PBS buffer (157 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) containing protease inhibitor (100 μM PMSF, Roche). After centrifugation (12,000 g for 10 minutes), the supernant was added to a column packed with a bed resin of glutathione-Sepharose beads and allowed to flow through by gravity. After washing with PBS, the fusion protein was eluted with GST elution buffer (50 mM Tris, pH 8.0) that contained 10 mM glutathione. Protein was analyzed quantitatively by the Bradford assay using bovine serum albumin as a standard. Fractions containing the GST fusion protein were concentrated using Amicon Ultra 4 concentrators (5000 molecular weight cut-off; Millipore, Bedford, MA).

Drug treatment
For the dissociated DRG neurons, proteins containing Tat peptide were added to the culture medium 0, 2 and 14 hours after dissociation, and then cell-surface biotinylation labeling was performed 4 hours after the final treatment.

Electrophysiology
Whole-cell patch-clamp recording was performed as previously described with some modification (Russ et al., 2005), using an EPC9 amplifier (HEKA Elektronik, Germany). Briefly, the dissociated DRG neurons and Na1.8-GFP-expressing ND7-23, COS-7 and HEK293 cells on coverslips were placed in the recording chamber, which contained the following solution: 120 mM NaCl, 20 mM tetrathylammonium chloride, 5 mM CsCl, 1 mM MgCl2, 1 mM CaCl2, 0.1 mM CdCl2, 10 mM HEPES and 10 mM t-glucose (pH 7.4). Electrode resistance was maintained at around 6-8 MΩ when filled with the following solution: 140 mM CsF, 1 mM EGTA, 10 mM NaCl, 10 mM HEPES (pH 7.3). Neurons with a diameter of 20-25 μm were recorded with a solution that contained 1 μM tetrodotoxin. The protocol used to isolate the tetrodotoxin-resistant Na1.8 current was as follows: a holding potential of ~70 mV and then a series of 100-millisecond pulses from ~20 to ~50 mV. The current density was calculated by dividing the peak current by the cell capacitance as recorded by whole-cell capacitance compensation with amplifier circuitry. The data acquired with Pulse software (HEKA Elektronik) were analyzed using Igor Pro 4.01 (WaveMetrics, Lake Oswego, OR).

We acknowledge Xu Zhang and Cheng He for critical comments on the experiments and manuscript and Cheng-Biao Wu for comments on the revised manuscript. This work was supported by grants from the National Natural Science Foundation of China (30570574, 30325024, 30621091, 30623003), National Basic
Research Program of China 2007CB914501 and STCSM 06DZ22032.

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