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Three Distinct Motifs within the C-terminus of ASIC1a Regulate its Surface Trafficking

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Abstract

Various protein motifs play a key role in regulating protein biogenesis and trafficking. Here, we discovered that three distinct motifs regulate the trafficking of acid-sensing ion channel 1a (ASIC1a), the primary neuronal proton receptor which plays critical roles in neurological diseases including stroke, multiple sclerosis and seizures. Mutating the PDZ binding motif of ASIC1a increased its surface expression and current density. In contrast, mutating either a RRGK motif or a KEAKR motif reduced ASIC1a surface expression and acid-activated current density. Mutating or deleting the RRGK motif also reduced pH sensitivity and the rate of desensitization of ASIC1a. These changes were likely due to a change in ASIC1a biogenesis; mutating either the RRGK or KEAKR motif reduced *N*-glycosylation of ASIC1a while mutating the PDZ binding motif had the opposite effect. Our results demonstrate that these C-terminal motifs are important for ASIC1a trafficking and channel function. In addition, in contrast to multiple previous studies, which all show that K/R containing motifs lead to ER retention, our findings indicate that these motifs can also be required for efficient trafficking.

Keywords

ASIC1; trafficking; K/R rich motif; PDZ binding motif; glycosylation

INTRODUCTION

Distinct motifs within a protein play important roles in regulating the biogenesis and surface trafficking of membrane proteins. For example, the classical KDEL motif is critical for ER retention while the di-leucine (LL) motif controls protein trafficking through the secretory pathway (Pelham, 1988, Marsh et al., 1995, Johnson et al., 2001). Another common class of sorting signal is motifs containing lysine and arginine; the di-lysine (KK) and RXR motifs

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lead to ER retention (Letourneur et al., 1995, Teasdale and Jackson, 1996, Margeta-Mitrovic et al., 2000, Standley et al., 2000, Pagano et al., 2001, Scott et al., 2001). These studies indicate that identifying regulatory motifs is one important step toward a better understanding of protein biogenesis and trafficking. In addition, since the same motif may function differently in different proteins, revealing novel functions of a known motif will also provide new insight into the trafficking mechanism in general.

We study here trafficking of acid-sensing ion channel 1a (ASIC1a), the key proton receptor in brain neurons (Grunder and Chen, 2010, Noël et al., 2010, Zha, 2013). ASIC1a is responsible for most of acid-activated current and acid-induced Ca^{2+} increase in central neurons (Baron et al., 2002, Wemmie et al., 2002, Xiong et al., 2004, Zha et al., 2006, Jiang et al., 2009, Zha et al., 2009a). Consistent with an importance of ASIC1a in neuron physiology, *ASIC1a* null mice show changes in hippocampal LTP, learning, CO_2 sensing and fear-related behavior (Wemmie et al., 2002, Wemmie et al., 2003, Coryell et al., 2007, Coryell et al., 2009, Ziemann et al., 2009, Wu et al., 2013). More importantly, deleting *ASIC1a* protects neurons from damage induced by acidosis, brain ischemia and multiple sclerosis (Xiong et al., 2004, Yermolaieva et al., 2004, Gao et al., 2005, Sherwood and Askwith, 2008, Sherwood and Askwith, 2009, Duan et al., 2011). ASIC1a is also important in other diseases; for example, activation of ASIC1a is involved in seizure termination in mice (Ziemann et al., 2008). These studies demonstrate that ASIC1a contributes to multiple aspects of neuron physiology and, more importantly, plays a pivotal role in a variety of neurological diseases.

We and others have recently revealed the importance of ASIC1a trafficking in acidosis-induced changes in neurons (Chai et al., 2007, Chai et al., 2010, Jin et al., 2010, Jing et al., 2011, Duan et al., 2012, Jing et al., 2012, Zeng et al., 2013). However, we remain know little about the molecular mechanisms regulating ASIC trafficking. Here, to address this lack of knowledge on ASIC1a regulation, we performed systematic screening for trafficking motifs within the C-terminal intracellular tail of ASIC1a. With a combination of deletion and mutagenesis approaches, we found that the PDZ binding motif and two K/R containing motifs within ASIC1a C-terminus play important roles in ASIC1a trafficking and function.

Experimental Procedures

Constructs and Reagents

Untagged and N-terminal HA-tagged wild-type mouse ASIC1a has been described earlier (Zha et al., 2006, Zha et al., 2009b, Jing et al., 2011). Various ASIC1a truncation and mutagenesis mutants were generated by PCR or with a Quickchange mutagenesis kit (Stratagene, La Jolla, CA), following manufacturer's instructions. All constructs were verified by sequencing. Rabbit anti-ASIC1 was kindly provided by Dr. John Wemmie (Wemmie et al., 2003). Other antibodies used were: mouse anti-tubulin (University of Iowa Developmental Hybridoma Bank), mouse monoclonal anti-HA (Santa Cruz, Santa Cruz, CA and Syd Labs, Malden, MA), goat anti-ASIC1 (sc-13905, Santa Cruz), HRP- and Dylight 680- and 800-conjugated secondary antibodies (Pierce, Rockford, IL), Alexa 680- and 800-conjugated secondary antibodies (Invitrogen, CA & Li-cor, Lincoln, NE). Other reagents used: Endo H (New England Biolabs, Ipswich, MA); NHS-sulfo-LC-biotin and NeutrAvidin Beads (Pierce); culture media and serum (HyClone or Invitrogen); lipofectamine 2000 (Invitrogen).

CHO cell culture and transfection

CHO-K1 cells were purchase from ATCC. CHO cell culture and lipofectamine 2000 mediated transfection were performed as described earlier (Jing et al., 2011).

Surface Biotinylation, De-glycosylation, NeutrAvidin Pull-down and Western Blot

Surface biotinylation, de-glycosylation, and NeutrAvidin pulldown were performed similar to what was described earlier (Jing et al., 2011, Jing et al., 2012). The samples were separated by 10% SDS-PAGE and transferred to PVDF or nitrocellulose membranes. Blotting was performed using standard chemiluminescence or fluorescence detection according to instructions of the Odyssey Imaging System (Li-cor), similar to what was described earlier (Zha et al., 2006, Jing et al., 2011). Antibody dilutions were: rabbit anti-ASIC1a 1:5K-30K; goat anti-ASIC1 1:500; monoclonal anti-HA 1:1K-2K; monoclonal anti-tubulin 1:30K; HRP conjugated goat anti-rabbit or anti-mouse 1:12000, donkey or goat anti-rabbit Alexa 680 1:16,000, and donkey or goat anti-mouse Dylight 800 1:16,000. Blots were developed using a Kodak automated film processing system, imaged using a digital Chemiluminescence imager (Fuji), or visualized using an Odyssey Infrared Imaging System according to manufacturer's instructions. Densitometry of imaged bands was performed as described earlier (Zha et al., 2006, Jing et al., 2011).

Electrophysiology

Whole-cell patch-clamp recordings were performed as described previously (Chu et al., 2006, Jiang et al., 2010, Jiang et al., 2011, Jing et al., 2011). During each experiment, a voltage step of -10 mV from the holding potential was applied periodically to monitor the cell capacitance and the access resistance. Recordings in which either the access resistance or the capacitance changed by more than 10% during the experiment were excluded from data analysis. To determine the time constant of the desensitizing portion of the ASIC currents, pH 6.0-activated currents were fitted by a single, standard exponential equation using Clampfit 10.2.

Statistical analysis

For biochemical data, we used paired t-test, or for multiple comparisons, one way ANOVA followed by Mann-Whitney test. For electrophysiological data, we used unpaired t-test when comparing two samples, or one-way ANOVA followed by a Bonferroni comparison of groups using either the least squares-adjusted means or the two-tailed unpaired Student's t-test. Data were reported as mean $\pm$ s.e.m. for the number of samples indicated. Differences were considered significant if $p < 0.05$.

RESULTS

Identification of motifs which are important for ASIC1a surface expression

To narrow down the C-terminal region which is important for ASIC1a trafficking, we performed serial deletion of the C-terminus of ASIC1a, and analyzed their effect on surface expression in Chinese hamster ovary (CHO) cells. Deleting amino acids 464–526 significantly ($p = 0.0009$, Mann-Whitney test) reduced ASIC1a surface:total ratio, while deleting amino acids 495–526 had no significant effect (Fig 1A, B). These data suggest that the region between amino acids 464–494 is important for ASIC1a surface expression. One distinct feature of this region is that its N-terminal portion (amino acids 464–475) contains multiple basic residues (Fig 1A). Previous studies have shown that motifs containing clusters of K/R are important for different aspects of protein-protein interaction, including trafficking (Letourneur et al., 1995, Margeta-Mitrovic et al., 2000, Standley et al., 2000, Pagano et al., 2001, Scott et al., 2001, Ren et al., 2003). Therefore, we hypothesized that this K/R rich region contains motifs that regulate ASIC1a trafficking. To test this hypothesis, we generated a $\Delta 6$ mutant, which has amino acids 464–469 ($_{464}\text{CRRGKC}$) deleted, an AAGA mutant, which has amino acids $_{465}\text{RRGK}$ mutated to AAGA, and an AEAAG mutant, which has amino acids $_{471}\text{KEAKR}$, mutated to AEAAG. Since mutating similar K/R type motifs

increases surface presence of multiple channels (Letourneur et al., 1995, Margeta-Mitrovic et al., 2000, Standley et al., 2000, Pagano et al., 2001, Scott et al., 2001), we predicted to observe an increase in ASIC1a surface expression in these mutants. Surprisingly, all three mutants reduced ASIC1a surface expression (Fig. 1C–E). These data suggest that the ₄₆₅RRGK and ₄₇₁KEAKR motifs are required for efficient surface trafficking of ASIC1a.

In our truncation study, deleting the last 25 amino acids increased ASIC1a surface:total ratio (Fig 1A, B), suggesting that a motif within this region inhibits ASIC1a surface expression. Since ASIC1a contains a PDZ binding motif (Duggan et al., 2002, Hruska-Hageman et al., 2002), we asked whether the PDZ binding motif of ASIC1a is responsible for the observed effect. Mutating the last three amino acids from FTC to GTG led to a significant increase in ASIC1a surface levels (Fig 1F). This result indicates that PDZ-dependent interactions have an inhibitory effect on ASIC1a surface expression.

Next, to address the functional importance of the identified motifs, we studied acid-activated currents in CHO cells expressing wild-type or mutant ASIC1a; all responded to acidic stimulation in a dose-dependent manner (Fig. 2A, B). Consistent with our biochemical analysis, the $\Delta 6$, AAGA and AEAAG mutants significantly reduced pH 6-activated current while the GTG mutant had the opposite effect (Fig. 2C, D). We further analyzed current properties and found that mutating either the RRGK or the KEAKR motif reduced the rate of desensitization while the mutating the PDZ binding motif had no effect (Fig. 2E). In addition, both $\Delta 6$ and AAGA reduced pH sensitivity (Fig. 2F). The pH₅₀ of $\Delta 6$ and AAGA was reduced by 0.25 and 0.21 pH units, respectively; both significantly lower than that of WT ($p < 0.05$). These data provide additional support to our surface expression data, and further show that the RRGK motif plays an important role in regulating ASIC1a channel properties.

Effects of the identified motifs on N-glycosylation and channel function of ASIC1a

One major way for intracellular motifs to control channel trafficking is through regulating the biogenesis process, which is marked by the maturation of *N*-linked glycans. Our recent data also show that *N*-glycosylation of ASIC1a is important for its trafficking and channel function (Jing et al., 2012). We therefore hypothesized that the identified C-terminal motifs regulate *N*-glycosylation of ASIC1a. To test this hypothesis, we transfected CHO cells with wild-type ASIC1a or different ASIC1a mutants, performed surface biotinylation, and treated cell lysates with Endo H, which removes "immature" *N*-linked glycans that have not been further processed within mid-to late-Golgi. All three mutants disrupting the K/R rich motifs, $\Delta 6$, AAGA and AEAAG, significantly ($p < 0.05$, paired t-test) reduced *N*-glycosylation of ASIC1a (Fig. 3A). In contrast, the GTG mutant increased *N*-glycosylation of ASIC1a at the surface (Fig. 3B). These data parallel with our surface expression and electrophysiology data, and suggest that the effect of these motif mutants may, at least in part, due to changes in their biogenesis.

DISCUSSION

Trafficking of ion channels plays important roles in regulating neuronal function (Collingridge et al., 2004). Despite the importance of ASIC1a in multiple neurological diseases (Xiong et al., 2008, Sluka et al., 2009, Noël et al., 2010), there are few reports on identifying motifs that regulate ASIC1a trafficking. Here, we performed a thorough examination of ASIC1a C-terminal tail and revealed several distinct motifs regulating ASIC1a surface expression and channel properties. Of note, the RRGK motif overlaps with a recently reported LCRRG motif (Zeng et al., 2013, see below for more discussion). These

data provide a foundation for future studies of the molecular mechanisms that regulate ASIC1a trafficking and function.

K/R containing motifs serve as an ER retention signal in a whole variety of membrane proteins (Teasdale and Jackson, 1996), including multiple neuronal ion channels. Examples include the RRRRR motif in AMPA receptors (Ren et al., 2003) and the RXR motif in K_{ATP} channel, NR1 and GABA_B receptors (Zerangue et al., 1999, Margeta-Mitrovic et al., 2000, Standley et al., 2000, Pagano et al., 2001, Scott et al., 2001). Here, we found that a RRGK and a KEAKR motif in the juxtamembrane region of ASIC1a play important roles in regulating its trafficking. However, in contrast to previously reported K/R motifs, which increase ER retention, these two motifs in ASIC1a are necessary for efficient surface expression of ASIC1a; mutating either the RRGK or the KEAKR motif reduced ASIC1a surface levels. The mechanism for this effect needs further investigation. However, these data suggest that, depending upon the exact motif and/or context, K/R containing motifs can also promote surface trafficking of proteins.

In a recent study, an LCRRG motif, which overlaps with the RRGK motif reported here, is required for the interaction between ASIC1a and AP2, which induces endocytosis of ASIC1a (Zeng et al., 2013). However, the authors also commented that they were unable to assess the endocytosis of the LCRRG mutants using surface biotinylation analysis, because their unpublished data suggest that the same mutants also abolished ASIC1a forward trafficking. These notations are consistent with our data which show reduced surface levels of the RRGK mutant. In addition, our results here provide one explanation for the reduced surface trafficking of the K/R mutants in ASIC1a. Mutating either RRGK or KEAKR motifs reduces the maturation of *N*-linked glycans of ASIC1a, suggesting that these motifs are involved in the biogenesis process of ASIC1a, and possibly also affect its interaction with machinery involved in forward trafficking and/or exocytosis. This speculation is consistent with our recent data showing that the *N*-glycosylation process is important for ASIC1a surface and dendritic trafficking (Jing et al., 2011, Jing et al., 2012).

Our data constitute one initial step toward a better understanding of ASIC1a regulation. However, many questions remain to be answered to elucidate the underlying molecular mechanisms. For example, it remains unclear how the K/R motifs regulate ASIC1a trafficking and channel properties. For the PDZ binding motif, although PICK1 is one known interactor of ASIC1a (Duggan et al., 2002, Hruska-Hageman et al., 2002), PICK1 increases ASIC1a surface expression (Jin et al., 2010). In contrast, mutating the PDZ binding motif here increased ASIC1a surface level. These data suggest that a PICK1-independent mechanism contributes to the increased surface expression of the GTG mutant. It will be of future interest to identify proteins interacting with these C-terminal motifs, and investigate how the identified accessory proteins regulate ASIC1a trafficking and function. Studies along this line will generate critical information for deciphering the basic biological mechanisms regulating ASIC1a, and will also provide important insights into understanding how acidosis and ASIC1a contribute to diseases. Moreover, since many proteins contain similar motifs, it also will be interesting to ask whether similar mechanisms apply to other membrane proteins. These studies will help us to better understand mechanisms regulating intracellular trafficking in general.

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HIGHLIGHTS

We performed a systematic screening of C-terminal motifs regulating ASIC1a trafficking.

Mutating two K/R rich motifs reduces surface trafficking and acid-activated current of ASIC1a.

Mutating the PDZ binding motif of ASIC1a has the opposite effect.

The mutants alter *N*-glycosylation, which may explain the changes in trafficking and channel properties of ASIC1a.

FIGURE 1. Identification of C-terminal motifs which regulate ASIC1a surface trafficking
CHO cells were transfected with wild-type ASIC1a or different C-terminal mutants as indicated. Surface proteins were labeled by surface biotinylation and isolated by NeutrAvidin pulldown. Surface and total proteins were analyzed by Western blot. (A) Diagram showing the C-terminus of ASIC1a. Numbers on top of the sequence indicate the position of the amino acids corresponding to the truncation mutants. The motif mutants generated are illustrated below the wild-type sequence. (B) Representative western blot and quantification showing the effect of serial truncation mutants on surface expression. ASIC1a and tubulin were blotted at the same time. (C–F) Western blots and quantification showing the effect of Δ6 (C), AAGA (D), AEAAG (E), and GTG (F) on surface expression.

Numbers on the bars indicate the total number of repeats. Asterisks indicate significant differences from WT ($p < 0.05$). Statistical tests used: for B, one way ANOVA with Turkey's correction, followed by Mann-Whitney test; for C-F, paired t test.

FIGURE 2. The effect of C-terminal mutants on ASIC1a current properties

(A & B) Representative traces showing acid-activated current of wild-type and mutant ASIC1a. The constructs in (A) is untagged while those in (B) are N-terminal HA tagged. (C–F) Plots showing current amplitude (C), current density (D), the rate of desensitization (E) and pH sensitivity (F). Dashed lines indicate that the data are from separate sets of experiments. Numbers on the bars (C–E) or in parenthesis (F) indicate total number of cells quantified. Asterisks indicate significant differences ($p < 0.01$ for C & D, $p < 0.05$ for E). Statistical tests used: unpaired t-test for WT vs. $\Delta 6$, one way ANOVA by a Bonferroni comparison of groups for the comparison between WT and AAGA, AEAAG, and GTG

mutants. Note that the WT controls are different between the two sets. Also note that for panel F, pH_{50} of each group was used for comparison, and the pH_{50} of $\Delta 6$ and AAGA was reduced significantly ($p < 0.05$) by 0.25 and 0.21 pH units, respectively, as compared to that of WT.

FIGURE 3. Mutating the K/R containing motifs reduces N-glycosylation of ASIC1a while mutating the PDZ binding motif has the opposite effect

CHO cells were transfected with wild-type ASIC1a or different C-terminal mutants as indicated. Cell lysates were untreated (U) or treated with Endo H (E), followed by NeutrAvidin pull-down of surface proteins. Surface and total proteins were analyzed by Western blot. (A–C) Representative Western blots and quantification showing the effect of $\Delta 6$ (A), AAGA and AEAAG (B), and GTG (C) on glycosylation status of ASIC1a. Numbers on the bar indicate the total number of repeats. Asterisks indicate significant difference from wild-type ($p < 0.05$, paired t-test).