Endocytotic elimination and domain-selective tethering constitute a potential mechanism of protein segregation at the axonal initial segment

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The axonal initial segment is a unique subdomain of the neuron that maintains cellular polarization and contributes to electrogensis. To obtain new insights into the mechanisms that determine protein segregation in this subdomain, we analyzed the trafficking of a reporter protein containing the cytoplasmic II–III linker sequence involved in sodium channel targeting and clustering (Garrido, J.J., P. Giraud, E. Carlier, F. Fernandes, A. Moussif, M.P. Fache, D. Debanne, and B. Dargent. 2003. Science. 300: 2091–2094). Here, we show that this reporter protein is preferentially inserted in the somatodendritic domain and is trapped at the axonal initial segment by tethering to the cytoskeleton, before its insertion in the axonal tips. The nontethered population in dendrites, soma, and the distal part of axons is subsequently eliminated by endocytosis. We provide evidence for the involvement of two independent determinants in the II–III linker of sodium channels. These findings indicate that endocytotic elimination and domain-selective tethering constitute a potential mechanism of protein segregation at the axonal initial segment of hippocampal neurons.

Introduction
The asymmetrical architecture of the neuron defines two domains: the somatodendritic domain and the axonal domain. The boundary between the soma and the axon comprises a unique and highly specialized subdomain of the neuron, the axonal initial segment (AIS). The molecular architecture of the AIS shares some homology with the organization of the nodes of Ranvier (Salzer, 2003). It is characterized by segregation of the cytoskeletal adaptor complex ankyrin G/β IV spectrin, linked to a dense network of actin, and by a high density of the voltage-gated sodium channels Na1.2 and Na1.6 and of members of the L1 cell adhesion molecule (CAM) family (Peles and Salzer, 2000; Boiko et al., 2003; Salzer, 2003). The AIS constitutes a barrier that restricts the lateral mobility of membrane proteins through differential tethering to cytoskeletal components (Kobayashi et al., 1992; Winckler et al., 1999) and blocks the diffusion of phospholipids (Nakada et al., 2003). The formation of this barrier is linked to the presence of the actin network (Winckler et al., 1999) and correlates with the accumulation of membrane proteins like sodium channels (Nakada et al., 2003). The high density of sodium channels ensures generation of the action potential, a fundamental signal in neuronal communication (Catterall, 2000). Thus, the AIS maintains neuronal polarization and contributes to electrogensis.

Little is known about the biogenesis of the AIS, but information has recently emerged indicating the pivotal role of the cytoskeletal adaptor complex ankyrin G/β IV spectrin (Bennett and Baines, 2001; Salzer, 2003). The expression of either ankyrin G (Zhou et al., 1998; Jenkins and Bennett, 2001) or β IV spectrin (Komada and Soriano, 2002) is mandatory for the assembly of molecules of the L1 CAM family and sodium channels. At the molecular level, sodium channels...
from rat brain are composed of an α subunit, the pore-forming protein Na1,1, and the auxiliary subunits, β2 or β4 and β1 or β3 (Catterall, 2000; Isom, 2001; Salzer, 2003; Yu et al., 2003). This complex molecular organization has hampered dissection of targeting and/or clustering motifs of sodium channels. Therefore, we developed an approach based on CD4 chimera expression in cultured hippocampal neurons to assess whether any of the large intracellular regions of Na1,2 contained sufficient information for sorting and specific membrane organization. Using this approach, we have recently shown that sodium channel targeting and clustering at the AIS is specified by a motif within the cytoplasmic loop linking homologous domains II–III (the II–III linker) of the Na1,1 proteins (Garrido et al., 2003). This signal was sufficient to relocalize the somatodendritic potassium channel K2.1 at the AIS of hippocampal neurons (Garrido et al., 2003). When the ankyrin binding motif of neurofascin (Davis and Bennett, 1994), a member of L1 CAMs, was replaced by the II–III linker of sodium channel Na1,2, the resulting neurofascin–sodium channel chimera was concentrated at the AIS of hippocampal neurons (Lemailliet et al., 2003). This process involves a direct association with the ankyrin repeat domain of ankyrin G (Lemailliet et al., 2003).

The upstream events leading to membrane protein segregation within the AIS have yet to be dissected (Winckler and Mellman, 1999). Taking into account very recent studies on axonal sorting, several models are conceivable, that may not be mutually exclusive. One scenario is that an AIS protein is nonselectively inserted in the plasma membrane of both somatodendritic and axonal domains and subsequently eliminated in the dendrites, the soma, and in the distal part of the axon by endocytosis where it is tethered by ankyrin G at the AIS. Such a multistep process would be coordinated by independent molecular determinants, i.e., internalization and tethering motifs. It is significant that somatodendritic endocytosis has been shown to be involved in the axonal compartmentalization of a CD4 chimera bearing the COOH terminus of Na1,2 (Garrido et al., 2001) and more recently, of the synaptic protein VAMP2 (Sampo et al., 2003). In each case, the abrogation of the internalization signal impaired axonal polarization (Garrido et al., 2001; Sampo et al., 2003). A second possibility is that an AIS protein is selectively sorted and inserted in the axonal domain, as observed in the case of NgCAM, the avian homologue of L1 (Sampo et al., 2003). After lateral diffusion, a fraction is tethered at the AIS whereas the distal population is eliminated by endocytosis. Alternatively, membrane proteins preassembled with ankyrin G can be selectively sorted to the AIS, as a consequence of polarized transport along microtubules involving KIF5, a member of the kinesin super family (Nakata and Hirokawa, 2003). Finally, the possibility that segregation of a given protein at the AIS involves both direct routing and transcytosis cannot be excluded. For example, Sampo et al. (2003) have shown that NgCAM is selectively routed to axons via a targeting motif localized in its extracellular domain. Wisco et al. (2003) have shown that NgCAM is preferentially inserted in the somatodendritic domain and subsequently sorted to the axons by transcytosis, a process mediated by an internalization motif located in the cytoplasmic COOH terminus of NgCAM.

The present study was aimed at analyzing the trafficking of a CD4 chimera bearing the II–III linker of Na1,2 (CD4-Na1,2 II–III) in hippocampal neurons, to obtain new insights into the mechanisms that determine protein segregation at the AIS. At the steady-state, the surface distribution of CD4-Na1,2 II–III is restricted to the AIS of transfected hippocampal neurons (Garrido et al., 2003). We show here that CD4-Na1,2 II–III is preferentially inserted in the somatodendritic domain but is subsequently eliminated by endocytosis, whereas it is accumulated at the AIS by a diffusion trap due to the high concentration of ankyrin G. Ankyrin G tethering involves a conserved glutamate residue within the sodium channel clustering motif whereas endocytosis is governed by a segment located in the NH2 terminus of linker II–III of Na1,2.

Results

Involvement of a glutamate residue in sodium channel clustering at the AIS

The addition of the II–III linker of Na1,2 to the human CD4 receptor deleted of its cytoplasmic tail, resulted in a surface distribution of the chimera (CD4-Na1,2 II–III) markedly restricted to the AIS when expressed by transfection in hippocampal neurons (Fig. 1 A, left). The AIS was identified by the absence of the somatodendritic marker MAP2, and by staining for ankyrin G. In addition, CD4-Na1,2 II–III was resistant to Triton X-100 extraction before cell fixation (Fig. 1 A, right), a feature reflecting tethering to the cytoskeleton, presumably via ankyrin G (Winckler et al., 1999). These features are governed by a 27-residue AIS motif (Garrido et al., 2003) that contains two potential phosphorylation sites for casein kinase II and clusters of acidic residues (Fig. 1 C). To identify the critical residue(s) in this motif we decided to evaluate the contribution of acidic and serine residues by site-directed mutagenesis (Fig. 1, B–E). The substitution of both E1111 and D1113 with alanine residues drastically altered the distribution of the chimera. CD4-Na1,2 II–III E1111A-D1113A was localized in both somatodendritic and axonal domains with a loss of concentration at the AIS. In contrast, replacing E1115, EE1120-1121, E1125, and D1127A by alanine did not affect the steady-state distribution of the chimeras. When only E1111 was substituted by either an alanine (Fig. 1 B, left) or a glutamine residue, chimeras were no longer concentrated at the AIS whereas the D1113A mutation did not modify the distribution of CD4-Na1,2 II–III (Fig. 1 B, right). Consistent with these observations, mutants E1111A-D1113A and E1111A lost resistance to detergent extraction, an index of cytoskeletal tethering (Fig. 1 E). Finally mutation of each of the serine residues (S1112 and S1126) did not alter the steady-state distribution of CD4-Na1,2 II–III (unpublished data).
The II–III linker of Nav1.2 is recognized by an endocytic pathway

Having demonstrated that a single mutation within the AIS motif impaired CD4-Na\textsubscript{v}1.2 II–III polarization, we examined whether this motif is sufficient for segregation of the chimera at the AIS. We replaced the COOH terminus of CD4 by a segment of 47 amino acids encompassing the AIS motif (CD4-Na\textsubscript{v}1.2 1082–1128). At the steady-state, the cell surface distribution of CD4-Na\textsubscript{v}1.2 1082–1128 differed from that of CD4-Na\textsubscript{v}1.2 II–III, displaying expression in dendrites, soma, and axons but with notably brighter staining at the AIS (Fig. 2 A). Nevertheless, CD4-Na\textsubscript{v}1.2 1082–1128 located at the AIS was still resistant to Triton X-100 extraction (Fig. 2 B). Thus, the AIS motif of Na\textsubscript{v}1.2 is necessary for tethering to the cytoskeleton but is not sufficient to restrict a CD4 chimera to the AIS. In the light of our previous study (Garrido et al., 2001) and because of the differences observed in the steady-state distribution of CD4-Na\textsubscript{v}1.2 II–III and CD4-Na\textsubscript{v}1.2 1082–1128, we evaluated the possibility that endocytosis contributes to compartmentalization at the AIS. We first applied an immunoendocytosis assay to COS-7 cells expressing CD4-Na\textsubscript{v}1.2 II–III. When the surface population of CD4-Na\textsubscript{v}1.2 II–III was prelabeled with an anti-CD4 antibody at 4°C, followed by a 20-min incubation at 37°C, it was found to be located in intracellular vesicles visualized by confocal microscopy (Fig. 3 A). The typical endocytic pattern was resistant to an acid-stripping treatment (Fig. 3 A, top). In COS-7 cells expressing CD4-Na\textsubscript{v}1.2 II–III (amino acids 428–753), antibody-labeled protein was confined at the cell surface and removed by acid stripping treatment (Fig. 3 A, bottom). We next applied a similar endocytosis assay to transfected hippocampal neurons. Antibody-labeled CD4-Na\textsubscript{v}1.2 II–III vesicles were visualized in the soma and throughout the dendrites after 30 min of endocytosis (Fig. 3 B). The presence of internalized CD4-Na\textsubscript{v}1.2 II–III in endosomes throughout the somato-dendritic domain was further confirmed by colocalization with EEA1, a marker of somatodendritic early endosomes (Wilson et al., 2000; Fig. 3 B, bottom).

Involvement of an elimination-retention mechanism in CD4-Na\textsubscript{v}1.2 II–III segregation at the AIS

We next identified at the molecular level the internalization signal in Na\textsubscript{v}1.2 II–III. We tested the internalization of several mutants in COS-7 cells. The sequential truncation of CD4-Na\textsubscript{v}1.2 up to amino acid 1030 did not impair internalization. For each mutant, the internalized antibody-prelabeled population located in intracellular vesicles was visualized by confocal microscopy. In contrast, mutants Δ1020, Δ1010, and Δ993 were confined at the cell surface (Fig. 4)
and were not resistant to acid-stripping treatment (unpublished data). Two internal deletions (Δ1010-1030 and Δ1002-1011) were further generated; Δ1010-1030 mutant impaired internalization unlike Δ1002-1011. These data indicated that 19 amino acids located in the NH₂-terminal region of Naᵥ1.2 II–III are critical for endocytosis in COS-7 cells. We next compared endocytosis of CD4-Naᵥ1.2 II–III and CD4-Naᵥ1.2 II–III Δ1010-1030 in hippocampal neurons (Fig. 5 A). Differential staining of the surface and internalized populations was performed. Positive staining for the internalized population was detected in 80% of cells expressing CD4-Naᵥ1.2 II–III (n = 184). This type of staining was only detected in 18% of cells expressing Δ1010-1030 (n = 295), indicating that the mutation impaired endocytosis. An internalized population was observed in neurons expressing Δ1098-1111, a mutant lacking the AIS motif (Fig. 5 A). The steady-state cell surface distribution of Δ1010-1030 differed from that of CD4-Naᵥ1.2 II–III, displaying expression in soma, dendrites, and axons (Fig. 5 B, top). An accumulation at the AIS was however clearly observed, that was resistant to detergent extraction (Fig. 5 B, left bottom). The distribution of the Δ1010-1030 mutant was strongly reminiscent of CD4-Naᵥ1.2 1082–1128 (Fig. 2 A). Thus, when internalization of CD4-Naᵥ1.2 II–III was impaired, its steady-state distribution was drastically altered in spite of the presence of a cytoskeletal tethering motif.

A comparison of the sequences of the II–III linker region in different sodium channel types revealed that the segment involved in endocytosis is less conserved in Naᵥ1.8 and 1.9 (see Fig. S1). In view of these differences, we generated a CD4 chimera containing the II–III linker of Naᵥ1.8. In COS-7 cells, CD4-Naᵥ1.8 II–III was not endocytosed, unlike CD4-Naᵥ1.2 II–III (see Fig. S2). When expressed in hippocampal neurons, CD4-Naᵥ1.8 II–III was distributed at the surface of the somatodendritic and axonal domains with an accumulation at the AIS (arrow). Bars, 20 μm.

A determinant of the linker II–III of Naᵥ1.2 is involved in endocytosis of CD4-Naᵥ1.2 II–III. (A) Schematic representation of mutations generated in Naᵥ1.2 II–III. The position of the AIS motif is delineated between amino acid 1102 and 1128. Internalization in COS-7 cells is indicated (+ or −). (B) COS-7 cells expressing the indicated mutants were submitted to an immunoendocytosis assay for 20 min at 37°C. Mutants Δ1030 and Δ1002-1011 underwent endocytosis whereas Δ1010 and Δ1010-1030 were confined to the plasma membrane. Bars, 20 μm.
Kinetics of insertion of CD4-Na\(_{1.2}\) II–III in the plasma membrane of hippocampal neurons

We next examined whether the insertion of newly synthesized CD4-Na\(_{1.2}\) II–III occurs preferentially in the somatodendritic domain or in the axonal domain. With this aim, we looked at the time course of cell surface insertion of CD4-Na\(_{1.2}\) II–III in hippocampal neurons by using brefeldin A (BFA) to block Golgi apparatus trafficking (Cid-Arregui et al., 1995; Jareb and Banker, 1997). After a 4-h posttransfection period, hippocampal neurons were treated overnight with BFA, resulting in protein accumulation in the Golgi apparatus. After removal of BFA, the cell surface distribution of CD4-Na\(_{1.2}\) II–III was visualized by immunostaining at different time intervals (Fig. 6 A). At \(t = 0\), upon BFA removal and after cell permeabilization, an intracellular accumulation was observed in the perinuclear region (unpublished data) and staining that was consistent with BFA block. After 30 min of recovery, CD4 surface staining was visualized on the somatodendritic membrane with an equivalent signal at the AIS in 81% of cells (cell population designated SD-AIS). At this stage of recovery, no signal was visualized on the distal part of axons, similar to that observed with CD4-Na\(_{1.2}\) II–III (Fig. 6 A). A surface distribution in both domains (SD-A), but with no enrichment at the AIS was largely predominant after 2 h of recovery (77%). These observations indicated that CD4-Na\(_{1.2}\) II–III is preferentially inserted in the somatodendritic membrane and in the AIS, where it is enriched, and subsequently in the distal part of the axons.

Discussion

In the present study, we analyzed the trafficking of CD4-Na\(_{1.2}\) II–III, a chimera bearing the cytoplasmic region that determines sodium channel targeting and clustering at the AIS (Garrido et al., 2003), to explore potential mechanisms involved in protein accumulation in this membrane subdomain. Evidence has been obtained for the following scenario. CD4-Na\(_{1.2}\) II–III is first preferentially inserted in the plasma membrane of the soma and dendrites, and is concentrated at the AIS, before its insertion in axonal tips. The high density of ankyrin G-B IV spectrin complex presumably acts as a diffusion trap at the AIS. The untrapped population in soma, dendrites, and in the distal part of axons is subsequently eliminated by endocytosis. However, our findings do not exclude the possibility that additional mechanisms contribute to the accumulation of proteins at the AIS.

The time course of the appearance of CD4-Na\(_{1.2}\) II–III is consistent with the temporal profile of membrane segregation at the AIS. The kinetics of membrane segregation at the AIS are consistent with the hypothesis that the high density of ankyrin G-B IV spectrin complex acts as a diffusion trap at the AIS. The untrapped population in soma, dendrites, and in the distal part of axons is subsequently eliminated by endocytosis. However, our findings do not exclude the possibility that additional mechanisms contribute to the accumulation of proteins at the AIS.
at the cell surface revealed that the neo-synthesized protein is preferentially inserted in the somatodendritic domain rather than in distal regions of axons. This finding is unlikely to result from a side effect of BFA block because it has been shown recently that the initial insertion of membrane proteins in axonal tips is not perturbed by this type of treatment (Wisco et al., 2003). We also observed that an uniform somatodendritic distribution preceded enrichment at the AIS. Previous studies have indicated that the accumulation of membrane proteins like sodium channels and neurofascin at the AIS is coordinated by ankyrin G (Jenkins and Bennett, 2001) and is impaired by inactivation of either ankyrin G or /H9252 IV spectrin gene expression (Zhou et al., 1998; Komada and Soriano, 2002). When the II–III linker of Na v1.2 was fused to a cytosolic protein like the green fluorescent protein, the resulting chimera was trapped within the AIS (Garrido et al., 2003). Moreover, the AIS motif of Na v1.2 contains a 9-amino acid ankyrin binding site, highly conserved within the sodium channel Na v1 family (Lemaillet et al., 2003). A single glutamate residue (Na v1.2 E 1111) within this motif plays a critical role in CD4-Na v1.2 II–III tethering to cytoskeleton at the AIS. A similar mechanism could be involved in Ng-CAM accumulation. This axonal protein, that possesses the highly conserved ankyrin binding site of the L1 family (Garver et al., 1997), is initially incorporated in the somatodendritic domain of hippocampal neurons (Wisco et al., 2003). This interpretation is consistent with the fact that perturbation of the diffusion barrier formed by the AIS results in a somatic localization of NgCAM and sodium channels (Winckler et al., 1999; Nakada et al., 2003).

Despite the importance of anchoring, we show here that cytoskeleton tethering, presumably by ankyrin G–B IV spectrin, is not sufficient for CD4-Na v1.2 II–III segregation at the AIS, endocytosis is also required. The internalized population of CD4-Na v1.2 II–III was observed throughout the soma and dendrites and to a lesser extent, in the distal part of axons. It was never seen within the AIS, in all observed cells. The presence of endocytic vesicles in these regions is consistent with the steady-state distribution of CD4-Na v1.2 II–III. The requirement for endocytosis was further demonstrated by fact that a mutation (Δ1010-1030) that abolished internalization of CD4-Na v1.2 II–III altered its steady-state distr-
bution without inhibiting accumulation at the AIS. Consistent with this conclusion is the observation that CD4-Na_{1.8} II–III, which was not internalized either in COS-7 cells or in hippocampal neurons, was distributed at the steady-state in soma, dendrites, and axons, and was enriched at the AIS. These findings also indicate that the internalization of CD4-Na_{1.2} II–III is not involved in its accumulation at the AIS but rather in its elimination in the somatodendritic domain and in the distal part of axons. They imply that transcytosis is unlikely to be involved in the accumulation of CD4-Na_{1.2} II–III at the AIS. However, we cannot exclude the possibility that the presence of internalized CD4-Na_{1.2} II–III in the distal part of axons may reflect transcytosis from the somato-dendritic domain to the axonal tips, as described for NgCAM (Wisco et al., 2003). The sequence that governs endocytosis of CD4-Na_{1.2} II–III does not encompass a canonical internalization signal such as the di-leucine or tyrosine-based motifs (YxxΦ) recognized by the clathrin-mediated endocytotic pathway (Bonifacino and Traub, 2003). Hence, further investigation will be required to analyze the endocytic pathway recognized by the II–III linker of Na_{1.2}. A differential regulation of endocytosis in the somatodendritic versus the axonal domain cannot be excluded.

In conclusion, our present study shows that endocytosis and domain-selective tethering confers CD4-Na_{1.2} II–III segregation at the AIS. However, whether the Na_{1.2} somatic channel follows a similar trafficking pathway remains to be explored. Multiple mechanisms probably play a role in establishing polarized sorting to the AIS. For instance, it is conceivable that ankyrin G-β IV spectrin may also act during sorting, involving preassembly with sodium channels and the CAM family. Consistent with this hypothesis is the recent observation that when RNAi was used to eliminate accumulation at the AIS.
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