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The I-II Loop of the Ca^{2+} Channel α_1 Subunit Contains an Endoplasmic Reticulum Retention Signal Antagonized by the β Subunit

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Summary

The auxiliary β subunit is essential for functional expression of high voltage-activated Ca^{2+} channels. This effect is partly mediated by a facilitation of the intracellular trafficking of α_1 subunit toward the plasma membrane. Here, we demonstrate that the I-II loop of the α_1 subunit contains an endoplasmic reticulum (ER) retention signal that severely restricts the plasma membrane incorporation of α_1 subunit. Coimmunolabeling reveals that the I-II loop restricts expression of a chimera CD8-I-II protein to the ER. The β subunit reverses the inhibition imposed by the retention signal. Extensive deletion of this retention signal in full-length α_1 subunit facilitates the cell surface expression of the channel in the absence of β subunit. Our data suggest that the β subunit favors Ca^{2+} channel plasma membrane expression by inhibiting an expression brake contained in β-binding α_1 sequences.

Introduction

High voltage-activated Ca^{2+} channels are protein complexes (Flockerzi et al., 1986; Witcher et al., 1993) formed by at least three subunits: the pore-forming subunit, α_1, and two auxiliary subunits, β and $\alpha_2\delta$, which are required for normalizing channel properties and facilitating cell membrane expression (Miller, 1992; Isom et al., 1994; Gurnett and Campbell, 1996; Walker and De Waard, 1998). Because voltage-dependent Ca^{2+} channels (VDCC) fulfill many essential cell functions such as controlling muscle contraction and neurotransmitter release, a thorough analysis of the structural organization and the role of the various subunits in channel functioning and assembly appears to be an absolute prerequisite to the understanding of VDCC physiology. The function of the auxiliary subunits seems particularly interesting, as they greatly affect channel properties. These modifications can be classified into several categories: (1) biophysical changes, which include modifications in the channel kinetics and voltage dependence of both activation and inactivation (β and $\alpha_2\delta$), (2) pharmacological changes that occur with an increase in the number of drug binding sites (β and $\alpha_2\delta$) and also in binding affinities ($\alpha_2\delta$ mainly), (3) increase in membrane trafficking, resulting in higher cell surface expression and current densities, and (4) regulatory changes, as β subunits may affect G protein or kinase regulation (Dolphin, 1998). As the increase in the number of drug binding sites may simply reflect an increase in the number of channels expressed, this classification may largely be simplified by stating that channel modification by β subunits occurs by one of two molecular processes. The first molecular impact triggered by the β subunit binding to α_1 is a functional modulation. Modifications in drug binding affinities (mostly seen with $\alpha_2\delta$ subunit) and in channel kinetics and voltage dependence all probably reflect functional modulations by conformational changes in the α_1 structure. The second change mediated by these subunits is the increase in current densities (Chien et al., 1995; Perez-Garcia et al., 1995; Josephson and Varadi, 1996; Kamp et al., 1996) and also in the number of drug/toxin binding sites (Perez-Reyes et al., 1992; Williams et al., 1992; Castellano et al., 1993; Nishimura et al., 1993; Mitterdorfer et al., 1994; Chien et al., 1995) and is likely to be mediated by an increase in the number of channels expressed at the cell surface.

Among the modifications in channel properties, the increased expression of VDCC by β subunits is one of the most interesting research topics. It is a property shared by all the β subunits tested. This increase in expression requires that the β subunit binds to a site present in the cytoplasmic loop separating the conserved domains I and II of the α_1 subunit (AID site). The observation that some stimulation in current densities could be triggered by the expression of a small β sequence (BID), which is known to bind to AID, rather than by the full-length β subunit, suggests that part of the control in channel expression level is mediated by the I-II loop of α_1 subunits (De Waard et al., 1994). In spite of the identification of these various protein-interacting sequences, the mechanism whereby β subunits induce an increase in Ca^{2+} current densities has not yet been unraveled. There are arguments in favor of functional modifications of the channel by the β subunit as well as arguments for a role in channel trafficking. Arguments in favor of functional modifications are increases in coupling efficiency between gating and opening of the channel (Neely et al., 1993), in opening probability (Wakamori et al., 1993, 1999; Shistik et al., 1995; Costantin et al., 1998), and, possibly, in channel conductance (Meir and Dolphin, 1998). A switch in channel state from silent to willing has also been proposed (Neely et al., 1995). In

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contrast, arguments in favor of a facilitated channel trafficking are the observed increases in the total amount of gating charges of various α_1 subunits (Josephson and Varadi, 1996; Strube et al., 1996; Beurg et al., 1997) and in the number of binding sites to various Ca^{2+} channel antagonists, dihydropyridines for the α_{1C} channel and ω-conotoxin GVIA for the α_{1B} channel (Lacerda et al., 1991; Brust et al., 1993). Also, immunohistochemical experiments have demonstrated a redistribution of both α_1 and β subunits as a result of their association (Gao et al., 1999). These effects are intriguing because of several apparent inconsistencies: first, most β subunits are reported to have cytoplasmic distributions, and second, all appear to lack the appropriate molecular information for a trafficking function.

The issue of β-induced stimulation is inherently complex because it implicates a combination of effects on functioning and trafficking. As a consequence, this complexity requires a thorough dissection of the mechanisms at stake. In this paper, we have tested the importance of the β subunit in the control of Ca^{2+} channel trafficking toward the plasma membrane. We demonstrate that the I-II loop acts as an expression brake by favoring the retention of the α_1 subunit in the endoplasmic reticulum (ER) and that the β subunit antagonizes this retention.

Results

The Cytoplasmic I-II Loop of α_1 Channels Acts as an Expression Brake

Several molecular determinants required for an efficient expression level of Ca^{2+} channels have already been mapped in both α_1 and β subunits. Two sites were identified: AID, located in the I-II loop of α_1 subunit, and BID, a 30 residue sequence that is within the β subunit and directly interacts with AID (De Waard et al., 1994; Pragnell et al., 1994). Though the mapping of these sites was clearly an essential step in the understanding of channel structure, it did not provide enough insights into the molecular mechanisms of subunit regulation. The major unanswered question remaining concerns the impact of β binding to AID on channel function and structure. To analyze the role of the β subunit in current stimulation, we isolated various Ca^{2+} channel structures by incorporating them into unrelated proteins. We focused on the β subunit, as it is an essential component of channel expression, and on the I-II loop of the α_1 subunit, as it is required for the observed β regulation. This procedure had the double advantage of bypassing conformational modifications in channel structure elsewhere during AID–BID association and of avoiding the functional impact of secondary α_1–β interaction sites (Walker and De Waard, 1998).

As electrophysiological recordings represent one convenient way to analyze the protein expression level at the plasma membrane, we decided to tag the carboxyl terminus of *Shaker* (*Sh*) K^+ channels with either the I-II loop of α_{1A} subunit (Sh-I-II_A) or with the entire β_3 subunit (Sh-β_3) (Figure 1A). The carboxyl terminus of the *Sh* channel was chosen as an appropriate tagging location, as it was expected to have a minimal impact on channel inactivation, contrary to the amino terminus, which is involved in fast N-type inactivation (Hoshi et al., 1990). These constructs were verified by sequencing but also by in vitro translation, with each tag appropriately increasing the molecular weight of *Sh* channels by 7 (Sh-I-II_A) or 55 (Sh-β_3) kDa (Figure 1A). We also checked the specific immunoprecipitation of these chimera proteins by polyclonal I-II_A (Sh-I-II_A) or β_3 (Sh-β_3) antibodies (Figure 1B). In control experiments, anti-I-II_A and anti-β_3 antibodies did not immunoprecipitate [^{35}S]-Sh channels (data not shown). We next investigated the effect of these Ca^{2+} channel tags on the properties of the *Sh* K^+ channel by expressing these chimera channels in *Xenopus* oocytes. As β subunits have pronounced effects on VDCC expression, we expected that the addition of the β_3 sequence on *Sh* may contribute by an unknown mechanism to an enhancement of the plasma membrane expression of this channel, as well. In fact, we found that the *Sh* current density was not modified by tagging the K^+ channel with the β_3 subunit (Figure 1C). Since one possible reason for this lack of effect could be due to a general perturbation of β conformation, we tested whether BID, an important molecular determinant for Ca^{2+} current stimulation (De Waard et al., 1994), remained active in the Sh-β_3 chimera. We found that in vitro translated [^{35}S]-Sh-β_3 was still able to bind specifically to 1 μM GST-AID_A but not to glutathione S-transferase (GST) alone (data not shown). Also, [^{35}S]-Sh does not bind to GST-AID_A, as expected by the lack of an identifiable BID sequence in K^+ channels. These results suggest that BID remains functional in the Sh-β_3 chimera and that tagging a Ca^{2+} channel β subunit onto an unrelated protein is not per se a sufficient condition for observing an increase in its plasma membrane expression. Unexpectedly, however, we repeatedly found that tagging the *Sh* channel with the I-II_A loop (amino acids 364 to 430 of BI-2; Mori et al., 1991) produces a significant decrease in the average current density of the channels expressed in *Xenopus* oocytes (Figure 1C). In this oocyte batch, average current densities were 19.3 ± 7.3 (Sh, n = 10), 2.9 ± 2.2 (Sh-I-II_A, n = 7), and 15.0 ± 2.7 μA/μF (Sh-β_3, n = 5) at 60 mV. This corresponds to an average decrease in current density of 6.7 ± 2.9-fold at 60 mV for Sh-I-II_A. Similar reductions in current densities were observed in three other batches of oocytes injected with Sh-I-II_A cRNA (4.5 ± 5.3, 4.5 ± 2.8, and 11.4 ± 8.8). This effect is not restricted to the I-II loop of the α_{1A} subunit, as a similar reduction in K^+ current density (33.5 ± 5.3-fold, n = 13) is seen with another chimera *Sh* channel tagged with the I-II loop of the α_{1C} subunit (amino acids 437 to 503 of α_{1C}) instead of α_{1A} (data not shown). Coexpressing the I-II_A loop as a separate molecular entity with *Sh*, Kv1.1, or skeletal muscle voltage-dependent Na^+ channels had no effect on their current densities (data not shown), demonstrating that the I-II loop does not decrease the expression levels of proteins to which it is not directly coupled. The current amplitude reduction observed with Sh-I-II_A channels is evident on a wide range of potentials without modification of the general shape of the current–voltage (I–V) relationship (Figure 1C). This was, however, not the case for Sh-β_3, as tagging the β_3 subunit appeared to slightly alter the shape of the I–V curve. One possible reason for this effect is that the size of the tag influences the direct molecular environment of the K^+

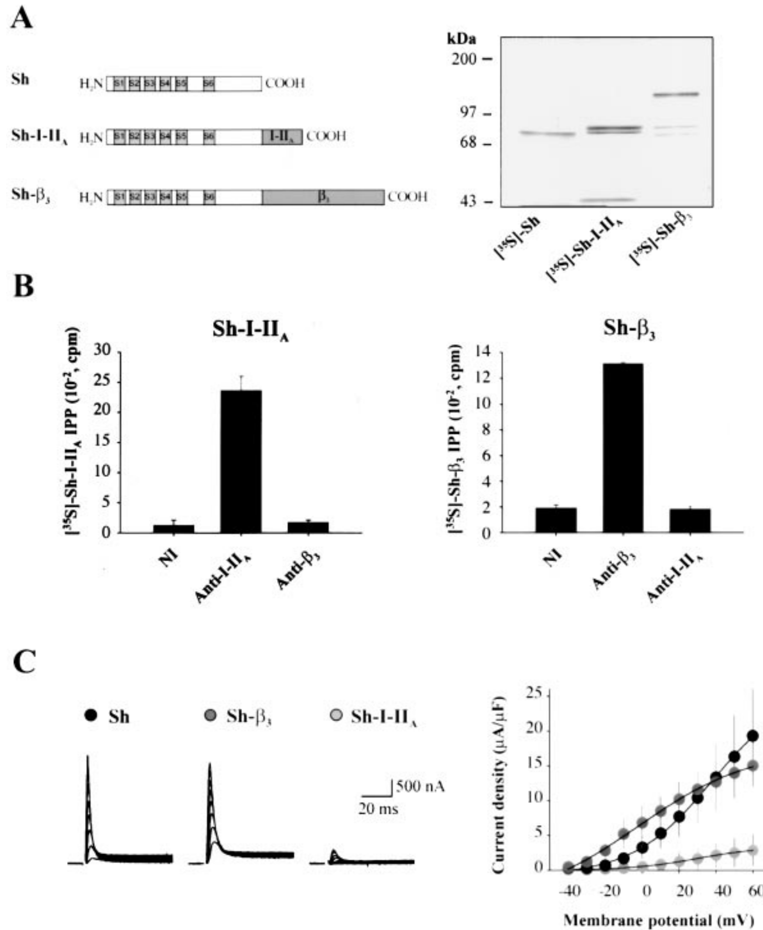

Figure 1. The I-II Loop Decreases the Membrane Expression Level of a *Sh* Chimera Channel

(A) Schematic representation of *Sh* chimera channel constructs (left) and autoradiogram of in vitro translated chimera proteins (right). The translation was performed in the presence of canine microsomal membranes. The estimated molecular weights of the chimera proteins are 73 kDa ([^{35}S]-Sh), 80 kDa ([^{35}S]-Sh-I-II$_A$), and 128 kDa ([^{35}S]-Sh-β$_3$), as expected from the size of the added sequences. In the case of [^{35}S]-Sh-β$_3$, smaller bands are also detected that appear to result mainly from proteolysis since they can be immunoprecipitated by anti-β$_3$ antibodies.

(B) Specific immunoprecipitation of [^{35}S]-Sh-I-II$_A$ and [^{35}S]-Sh-β$_3$ by the purified IgG fraction of rabbit polyclonal antibodies directed against the I-II$_A$ and β$_3$ sequence, respectively. The specificity of the antibodies is demonstrated by the lack of immunoprecipitation of [^{35}S]-Sh-I-II$_A$ by anti-β$_3$ antibodies and of [^{35}S]-Sh-β$_3$ by anti-I-II$_A$ antibodies. Also, both the I-II$_A$ and the β$_3$ antibodies were unable to immunoprecipitate [^{35}S]-Sh (data not shown). Abbreviation: NI, nonimmune serum.

(C) The tagged I-II loop decreases the K^+ current through *Sh* channels expressed in *Xenopus* oocytes.

(Left) Representative current records from oocytes expressing Sh, Sh-I-II$_A$, or Sh-β$_3$ channels. Membrane depolarization is from −20 to +50 mV (Sh and Sh-β$_3$) or from −10 to +50 mV (Sh-I-II$_A$) by 10 mV increments.

(Right) Average current voltage dependence for Sh (n = 10), Sh-I-II$_A$ (n = 7), and Sh-β$_3$ (n = 5) channels. Average membrane capacitance varied only slightly with 0.247 ± 0.029 μF (Sh, n = 8), 0.242 ± 0.037 (Sh-I-II$_A$, n = 9), and 0.239 ± 0.063 (Sh-β$_3$, n = 9), SD values.

channel. In contrast, apart from a modification in current amplitude, the tagged I-II$_A$ sequence did not significantly change other parameters of *Sh* K^+ activity. For instance, time constants for fast inactivation at 60 mV were τ = 1.8 ± 0.5 ms (Sh, n = 9), 1.6 ± 0.2 ms (Sh-I-II$_A$, n = 5), and 2.0 ± 0.3 ms (Sh-β$_3$, n = 8). Overall, these results indicate that the I-II loop of α_1 subunits acts as a plasma membrane expression brake and that, contrary to previous expectations, β subunits have no intrinsic stimulatory function in the absence of Ca^{2+} channel α_1 subunits.

Coexpression of the Ca^{2+} Channel β Subunit Reverses the Decrease in Sh-I-II$_A$ Expression

As Ca^{2+} channel β subunits are classically viewed as expression "helpers" for VDCC, we analyzed the effect of β subunit coexpression on the level of current expressed by Sh-I-II$_A$ channels (Figure 2).

We first determined whether β$_3$ is able to interact directly with the chimeric Sh-I-II$_A$ channel in a cell expression system. The Sh-I-II$_A$ channel and the β$_3$ subunit were transfected either alone or in combination in COS7 cells, labeled by the addition of [^{35}S]-methionine in the culture medium, and immunoprecipitated by anti-I-II$_A$ or anti-β$_3$ antibodies (Figure 2A). We confirmed the finding of other investigators (Berrow et al., 1997; Brice et al., 1997; Stephens et al., 1997) that untransfected COS7 cells are devoid of Ca^{2+} channel subunits since labeled proteins in the range of α_1 or β subunits were not immunoprecipitated by anti-β$_3$ or anti-I-II$_A$ antibodies (data not shown). In contrast, [^{35}S]-β$_3$ and [^{35}S]-Sh-I-II$_A$ expressed in COS7 cells could be specifically immunoprecipitated by anti-β$_3$ and anti-I-II$_A$ antibodies, respectively. As expected from a specific binding of β$_3$ onto Sh-I-II$_A$, the chimeric [^{35}S]-Sh-I-II$_A$ channel could be coimmunoprecipitated by anti-β$_3$ antibodies from cell preparations expressing both Sh-I-II$_A$ and β$_3$. These results demonstrate that the Ca^{2+} channel I-II sequence in Sh-I-II$_A$ is not only functionally inhibitory but is also able to bind the Ca^{2+} channel β$_3$ subunit.

We next investigated the effects of β$_3$ on Sh-I-II$_A$ cell surface expression. We found that, consistent with its stimulatory function in VDCC, the coexpression of β$_3$ with Sh-I-II$_A$ considerably increases the K^+ current densities in *Xenopus* oocytes (Figure 2B). Average current densities at 60 mV were increased more than 8-fold, from 4.1 ± 1.2 (Sh-I-II$_A$, n = 6) to 33.7 ± 5.4 μA/μF (Sh-I-II$_A$ + β$_3$, n = 6). The expression level of Sh-I-II$_A$ in the presence of β$_3$ was only slightly lower than the expression level of Sh alone (average current density of 44.8 ± 4.7 μA/μF [n = 6] at 60 mV) (Figure 2C). Consistent

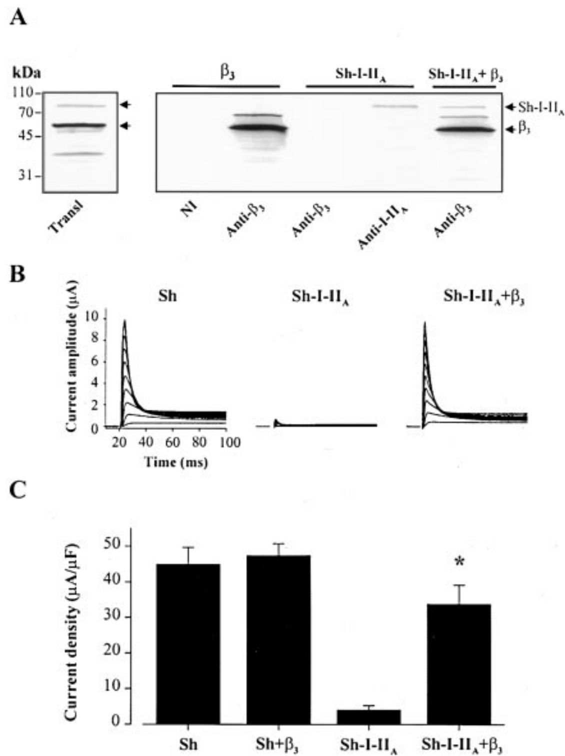

Figure 2. Expression of a β Subunit Reverses the Inhibition of K^+ Currents Imposed by the I-II Loop in Sh-I-II_A Chimera

(A) The β_3 subunit binds onto Sh-I-II_A expressed in COS7 cells.

(Left) Simultaneous in vitro translation of β_3 and Sh-I-II_A, indicating their molecular weight.

(Right) Immunoprecipitation of β_3, Sh-I-II_A, and β_3 + Sh-I-II_A (cDNA ratio of 9:1) transfected into COS7 cells by nonimmune (NI), anti-β_3, and anti-I-II_A antibodies. Note that anti-β_3 antibodies immunoprecipitate a 70 kDa protein from COS7 cells transfected with the β_3 subunit, which is absent in untransfected cells. This band appears, therefore, to represent an endogenous protein that specifically associates with β_3 in COS7 cells. The molecular identity of this endogenous protein is unknown. Also, the [^{35}S]-Sh-I-II_A immunoprecipitated from COS7 cells migrates at a slightly higher molecular weight than after in vitro translation, the difference being due to glycosylation. Note that all of the expressed Sh-I-II_A protein is associated with β_3, the latter having a higher expression level.

(B) Representative current records of oocytes expressing Sh, Sh-I-II_A, and Sh-I-II_A with β_3. Membrane depolarization was elicited from −80 to −20 mV and above by 10 mV increments.

(C) Average K^+ current densities measured at a membrane depolarization to +60 mV for Sh- (n = 6), Sh with β_3– (n = 6), Sh-I-II_A- (n = 6), and Sh-I-II_A with β_3– (n = 6) expressing oocytes. Cells were all from the same cRNA injection batch. Data are the mean ± SEM.

with the lack of a β interaction site in *Sh* channels, coexpression of β_3 was without effect on the current amplitude of Sh (average current density of 47.3 ± 3.3 µA/µF [n = 6] at 60 mV). These results suggest that the β-induced reversal of the Sh-I-II_A surface expression requires β binding onto the I-II sequence of the chimeric K^+ channel.

As expression of Sh-I-II_A channels resulted in diminished K^+ current density, the most likely mechanism of action of the I-II loop is that it decreases the membrane expression of the chimeric channel. Conversely, as coexpression of β_3 increases the current density of Sh-I-II_A channels, we assumed that this effect was mediated by an increase in the plasma membrane expression of Sh-I-II_A. To further confirm the effect of the I-II loop and the β_3 subunit on the cell surface expression, we prepared new chimeric proteins in which we replaced the entire cytoplasmic sequence of CD8 by either a Myc tag (CD8-Myc), the I-II_A-Myc sequence (CD8-I-II_A-Myc), or the II-III_C-Myc sequence (CD8-II-III_C-Myc) (Figure 3A). Preliminary observations demonstrated that removal of the cytoplasmic tail of CD8 had little influence on the cell surface expression of this molecule (see also Fung-Leung et al., 1993) (data not shown). The CD8 molecule is particularly convenient to use in immunocytochemistry and in immunoprecipitation, as CD8, I-II_A, and Myc are all useful antibody epitopes (Figure 3B). We used the CD8-I-II_A-Myc construct to confirm the decrease in plasma membrane expression imposed by the I-II sequence. CD8-Myc and CD8-II-III_C-Myc were all control constructs, as both sequences lack any known characterized interaction sites with β subunits. COS7 cells transfected with either CD8, CD8-I-II_A-Myc, or CD8-II-III_C-Myc showed varied levels of membrane fluorescence after immunostaining by anti-CD8 antibody in the nonpermeabilized condition (Figure 3C). The expression level of the CD8-I-II_A-Myc protein appeared much weaker than the CD8 or CD8-II-III_C-Myc expression levels. We also found that only 0.45% of cells had a detectable level of CD8-I-II_A-Myc surface expression, which, corrected for a transfection efficiency of 19.2%, results in a cell surface expression efficiency of 2.3%. This is in fact about 44 times lower than for CD8-Myc expression, which has a 98.6% surface expression efficiency (transfection efficiency was 28.6% for cells transfected with CD8-Myc, which is equivalent to the 28.2% of cells that had a detectable surface expression of CD8-Myc). These data confirm the reduction in plasma membrane expression induced by the I-II sequence in chimera proteins. This reduction is in fact somewhat underestimated since besides a decrease in the number of cells bearing a surface expression of CD8-I-II_A-Myc, there is also a significant reduction in the intensity of CD8 staining in cells expressing this chimera that could not be quantified. These data also suggest that the decreased K^+ current densities observed with the Sh-I-II_A chimera were not due to nonspecific alterations in the opening probability of the K^+ channel. Metabolic labeling of cell cultures expressing any one of these chimeric CD8 proteins also demonstrated that their total cellular expression levels were unaltered compared with CD8-Myc, suggesting again that the reduced membrane expression of CD8-I-II_A-Myc was not due to an inhibition of protein synthesis (data not shown). Similar to the Sh-I-II_A chimera, coexpressing CD8-I-II_A-Myc with β_3 resulted in a significant upheaval of the membrane expression of the chimeric protein, whereas β_3 had no effect on the membrane expression level of either CD8-Myc or CD8-II-III_C-Myc (Figure 3C). We found that 14% of the cells had a detectable surface expression of CD8-I-II_A-Myc in the presence of β_3, which corrected for a transfection efficiency of 23.8%, and a cotransfection ratio CD8-I-II_A-Myc/β_3 of 64.3% results in a cell surface expression efficiency of 91%. This suggests that β_3 induces a 40-fold higher surface expression efficiency for CD8-I-II_A,

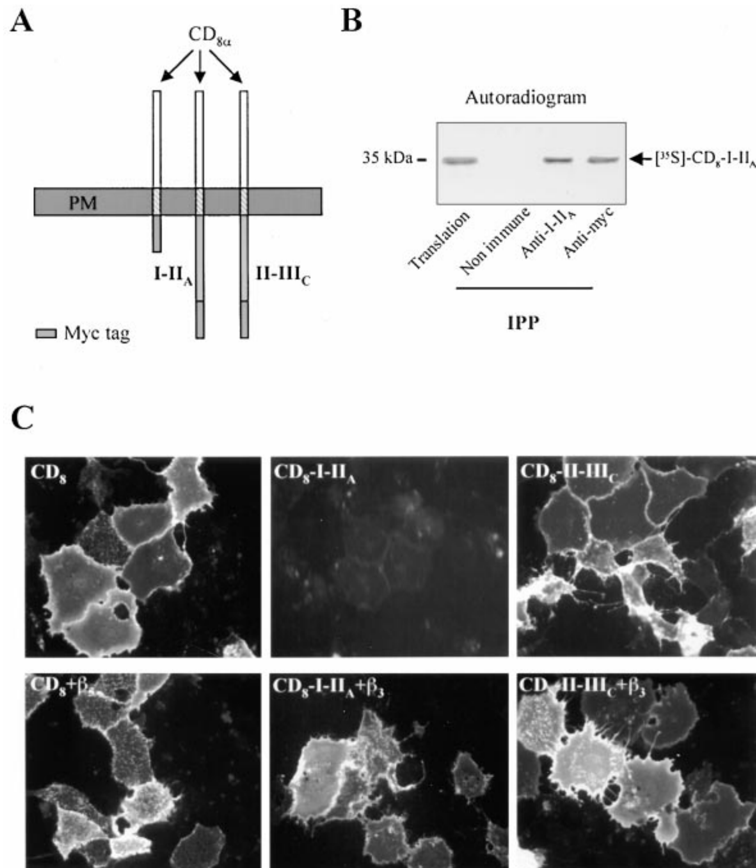

Figure 3. CD8 Chimera Proteins and Cell Surface Expression

(A) Schematic representation of various CD8 chimera proteins (CD8-Myc, CD8-I-II_A-Myc, and CD8-II-III_C-Myc). The ectodomain and transmembrane domain of CD8 were conserved, whereas the cytoplasmic domain was replaced by a Myc tag (CD8-Myc), the I-II loop of α_{1A} coupled to Myc (CD8-I-II_A-Myc), or the II-III loop of the α_{1C} subunit coupled to Myc (CD8-II-III_C-Myc).

(B) Autoradiogram showing in vitro translated [^{35}S]-CD8-I-II_A-Myc (3 μl translation reaction) and the result of its immunoprecipitation by nonimmune (NI), anti-I-II_A, and anti-Myc antibodies. Proteins were separated by SDS–PAGE electrophoresis at 9% acrylamide. The dried gel was exposed overnight.

(C) Various CD8 chimeras were expressed in COS7 cells without (upper panel) and with (lower panel) the β_3 subunit (cDNA ratio of 1:5). Surface expression of CD8 was detected by first treating the cells with a mouse anti-human CD8 (1/25 of 0.5 mg/ml), then with a GAM-rhodamine antibody and observed with a nonconfocal microscope. To be able to detect some membrane expression of CD8-I-II_A-Myc when expressed alone, the exposure time was increased 4-fold compared with the five other conditions. There were no differences in staining intensities observed between CD8-II-III_C-Myc and CD8-II-III_A-Myc constructs (data not shown).

in close agreement with the surface expression efficiency of CD8 alone. Specific association of β_3 to CD8-I-II_A-Myc was shown in cell metabolic labeling and coimmunoprecipitation experiments (data not shown). Also, we found that β_3 had no effect on the total cell expression level of CD8-I-II_A-Myc (data not shown).

Chimeric Proteins Expressing the I-II Loop Are Associated with the ER

As the membrane expression of chimeric proteins expressing the I-II loop is significantly decreased, it is assumed that the proteins remain within the cell, somewhere along the protein trafficking pathway. We therefore wanted to determine the intracellular fate of chimera proteins harboring the I-II loop. As expected, we observed that the CD8-I-II_A-Myc protein was mainly localized within the cell (Figure 4), contrary to CD8-Myc, which has a predominant plasma membrane localization (data not shown; see Figure 5Aa for CD8-Myc coexpressed with β_3). The distribution of CD8-I-II_A-Myc was reticulate and coincided well with the staining obtained with an anti-BiP antibody. As BiP is an ER resident protein, these data suggest that the I-II loop retains the CD8-I-II_A-Myc chimera in the ER. In contrast, expression of CD8 alone did not coincide with the ER (data not shown). We also analyzed the distribution of the β_3 subunit expressed in COS7 cells. As several β subunits (β_{1b} and β_{2a}) are proposed to reach the plasma membrane in the absence of any α_1 subunit (Chien et al., 1995; Brice et al., 1997), the effect of β_3 subunit on Sh-I-II_A and CD-I-II_A-Myc membrane expression could have been due to the binding of these chimera proteins to the plasma membrane–associated fraction of β subunit. This hypothesis turns out to be very unlikely, however, since we find that β_3 has a mostly cytoplasmic localization when expressed alone (Figure 4d). Costaining with an antibody against the ER resident protein BiP further demonstrates that β_3 does not colocalize with the ER compartment (Figures 4e and 4f).

Expression of β_3 with CD8-I-II_A-Myc Favors a Cell Redistribution of Both Proteins

We next determined the effect of β_3 expression and association with CD8-I-II_A-Myc on the intracellular distribution of these two proteins. Coexpression of CD8-Myc with β_3 did not change the cell distribution of CD8-Myc or β_3, consistent with the lack of association between these two molecules (Figure 5A). CD8 distribution appears mainly in the plasma membrane, with some punctuate intracellular distribution. This distribution never coincided with the distribution of β_3, which remained intracellular and diffuse. Similar observations were made when β_3 was cotransfected with CD8-II-III_C-Myc or CD8-II-III_A-Myc, also suggesting the absence of association between β_3 and these various II-III sequences (data not shown). In contrast, when β_3 was expressed with CD8-I-II_A-Myc, the distribution of both proteins was modified (Figure 5B). As shown in Figure 5Ba, CD8-I-II_A-Myc appeared present both intracellularly and in (or at least close to) the plasma membrane as a result of

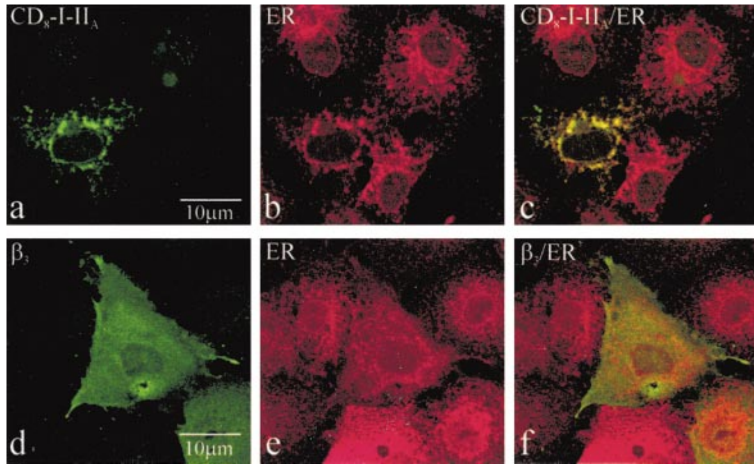

Figure 4. The I-II_A Sequence Restricts the CD8-I-II_A-Myc Chimera to the ER

Subcellular localization of CD8-I-II_A-Myc (upper panel) and β_3 (lower panel). Permeabilized COS7 cells were incubated with a polyclonal anti-Myc antibody ([a], CD8-I-II_A-Myc), a monoclonal anti-BiP antibody ([b and e], ER), and a rabbit polyclonal anti-β_3 antibody ([d], β_3). Immunostaining was visualized with a second antibody coupled to FITC (CD8-I-II_A-Myc and β_3) or TRITC (ER). Superimposed labeling is also shown for CD8-I-II_A-Myc and ER (c), and β_3 and ER (f).

β_3 expression. In contrast to CD8-I-II_A-Myc, which was partially redistributed, the entire pool of β_3 was now present close to the plasma membrane (Figure 5Bb). The plasma membrane pools of CD8-I-II_A-Myc and β_3 are colocalized as a result of their association (Figure 5Bc). These results are consistent with the observation that β_3 increases the plasma membrane expression of both Sh-I-II_A and CD8-I-II_A-Myc chimera proteins. The reasons for a complete redistribution of β_3 compared with CD8-I-II_A-Myc are unclear at this stage.

As another indication of a molecular association between CD8-I-II_A-Myc and β_3, we tested by pulse-chase experiments where in the cell both molecules may interact immediately after synthesis. Figure 5C demonstrates that even with very short metabolic labeling time (5 min) and short chase time (5 min), immunoprecipitation with

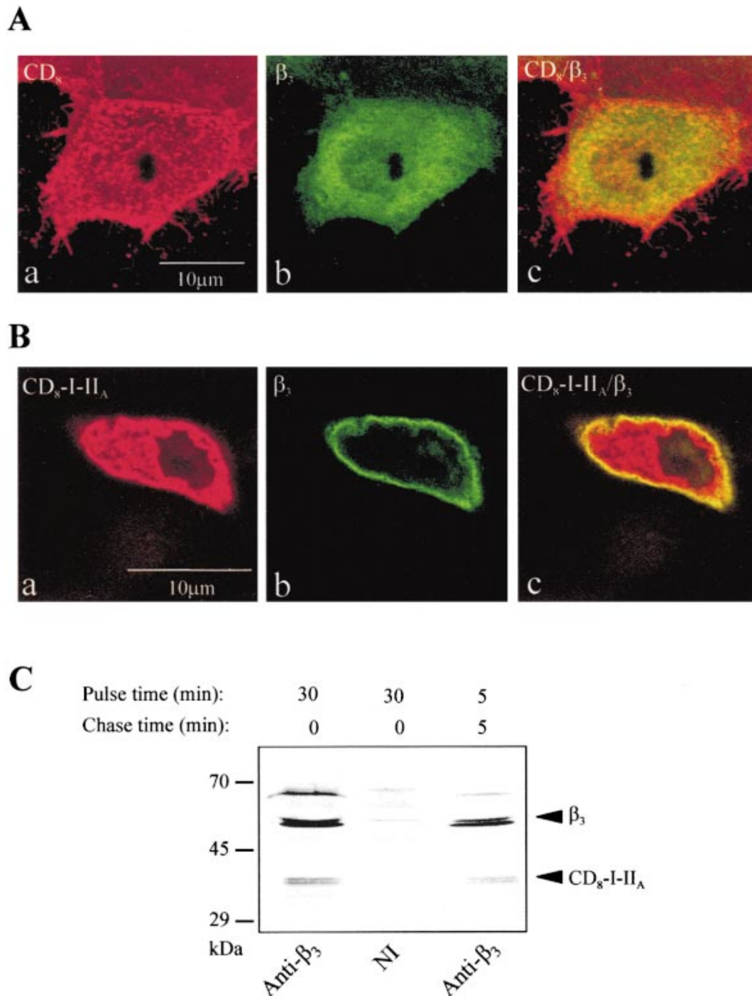

Figure 5. Cellular Redistribution of CD8-I-II_A-Myc and β_3 When Coexpressed in COS7 Cells

(A) Coexpression of CD8-Myc and β_3 (cDNA ratio of 1:5). Permeabilized COS7 cells were incubated with a monoclonal anti-CD8 antibody ([Aa], CD8-Myc) or a rabbit polyclonal anti-β_3 antibody ([Ab], β_3). Immunostaining was visualized with a GAM-TRITC (CD8-Myc) or GAR-FITC antibody (β_3). Superimposed labeling is also shown in (Ac).

(B) Coexpression of CD8-I-II_A-Myc and β_3 (cDNA ratio of 1:5). Permeabilized COS7 cells were incubated with a monoclonal anti-Myc antibody ([Ba], CD8-I-II_A-Myc) or a rabbit polyclonal anti-β_3 antibody ([Bb], β_3). Immunostaining was visualized with a GAM-TRITC (CD8-I-II_A-Myc) or a GAR-FITC antibody (β_3). Superimposed labeling is again shown in (Bc). In (A) and (B), confocal images represent the sum of eight successive sections.

(C) Metabolic labeling of COS7 cells expressing CD8-I-II_A-Myc and β_3 subunit (cDNA ratio of 1:5). Cells expressing both CD8-I-II_A-Myc and β_3 subunit were incubated for either 30 min with [^{35}S]-methionine in the culture medium with no chase or 5 min with [^{35}S]-methionine with 5 min chase. Cells were collected, and proteins were solubilized and immunoprecipitated with either nonimmune (NI) of anti-β_3 antibodies (β_3).

anti-β_3 antibodies reveals an association between β_3 and CD8-I-II_A-Myc. The level of this interaction was not increased by a 30 min pulse. These data strongly suggest that the association of β_3 with CD8-I-II_A-Myc occurs early in the biosynthesis pathway, presumably at the ER level. Our immunofluorescence data illustrating that the complex CD8-I-II_A-Myc/β_3 is close to the plasma membrane (Figure 5B) are not in contradiction with this interpretation since this technique addresses mostly the molecules that have been synthesized long ago. These results indicate that upon binding of β_3 onto the I-II loop of CD8-I-II_A-Myc in the ER, the resulting molecular complex leaves the ER and moves toward the plasma membrane. It should be noted that only a fraction of the entire pool of CD8-I-II_A-Myc could be displaced by the coexpression of β_3. Altering the transfected cDNA ratio between CD8-I-II_A-Myc and β_3 did not change this observation. The reasons for this partial effect are unclear at this stage, but it is possible that some other cell regulatory components binding to the I-II loop act as inhibitors of β_3 association or that binding of β subunit onto the I-II sequence should occur before retention of the chimeric protein takes place. Whatever the case, these results clearly demonstrate that the binding of β_3 to the I-II loop triggers a signal, allowing the chimeric proteins to continue their cell trafficking process beyond the ER.

Channel Expression Is Facilitated by Partial Deletions in the I-II Loop

We wanted to test the possibility that a disruption of the I-II expression brake could produce a stronger channel expression in the absence of any β subunit. We constructed three deleted derivatives of the rabbit α_{1A} subunit (BI-2 Δ1, BI-2 Δ2, and BI-2 Δ3) that lacked parts of the I-II loop (Figure 6A). The first construct that was made, BI-2 Δ1 (Δ372–456), lacked most of the sequence that we tagged in the Sh-I-II_A chimera (amino acids 364 to 430). Unfortunately, we found that both BI-2 Δ1 and BI-2 Δ3 were nonfunctional when expressed alone or with β_3 subunit in oocytes (Figure 6C). The lack of effect of β_3 is consistent with the complete deletion of the AID sequence in these constructs. Average current densities for these constructs were 0.04 ± 0.03 μA/μF (without β_3, n = 5) and 0.02 ± 0.02 μA/μF (with β_3, n = 3) at 20 mV for BI-2 Δ1, whereas they were 0.12 ± 0.02 μA/μF (without β_3, n = 6) and 0.11 ± 0.05 μA/μF (with β_3, n = 3) for the BI-2 Δ3 channel. In contrast, there was a marked channel activity in cells expressing BI-2 Δ2 alone, with an average current density of 1.86 ± 0.22 μA/μF (n = 12) (Figures 6B and 6C). Also, consistent with the deletion of the three critical β-binding residues of AID (Y392, W395, and I396), BI-2 Δ2 currents were not enhanced by β_3 subunit coexpression (average current density of 1.99 ± 0.24 μA/μF, n = 12). Interestingly, we found that BI-2 Δ2 channels inactivate more rapidly than BI-2 channels, which is consistent with a role of the I-II loop in inactivation. At 20 mV, BI-2 Δ2 channels inactivated along two components with time constants of τ_1 = 17.2 ± 5.7 ms (54.7% ± 7.5% of total current, n = 11, SD) and τ_2 = 123 ± 30 ms (45.3% ± 7.6% of current) compared with τ_1 = 21.3 ± 6.4 ms (27% ± 7% of total current, n = 9, SD) and τ_2 = 226 ± 48 ms (73 ± 7% of current) for BI-2 channels. In spite of the removal of a large portion of the retention sequence in the I-II loop, the current densities expressed by BI-2 Δ2 were not significantly higher than those of BI-2 in this injection set (average current density of 1.8 ± 0.4 μA/μF, n = 9). However, it has been hypothesized that oocytes contain small amounts of an endogenous β_3-like subunit that may be responsible for the weak current densities detected when expressing exogenous α_1 subunits alone (Tareilus et al., 1997). To test this hypothesis, we coexpressed BI-2 along with the I-II_A loop as a separate molecular entity. Because of the small size of the I-II_A loop compared with the full-length α_1 subunit, it was hoped that it would be synthesized at higher concentrations and that it would act as a sink by depleting endogenous free β subunits. We found that the coexpression of the I-II_A loop indeed produced a more than 10-fold decrease in the average current density of BI-2 (0.16 ± 0.05 μA/μF, n = 10), suggesting that currents produced by cells injected with BI-2 cRNA alone were the result of an association between the endogenous β and exogenous BI-2 subunit. Consistent with this interpretation is the finding that the coexpression of a mutant form (Y392S) of the I-II_A loop, known for its weaker interaction with β subunits (Pragnell et al., 1994), did not alter to any extend the current density of BI-2-expressing oocytes (1.77 ± 0.45 μA/μF, n = 11), demonstrating the involvement of β subunits in this process. It should be emphasized that in spite of its involvement in the expression of BI-2, the interaction of this endogenous β subunit with BI-2 is probably only transient, as the channel properties of BI-2-expressing oocytes are quite different from the channel properties of exogenous BI-2- and β-expressing oocytes (De Waard and Campbell, 1995). Also, Neuhuber and collaborators (1998a) suggested that transient interactions between α_1 and β are sufficient for membrane expression of the channel. Interestingly, the I-II_A loop had no effect on the current density of BI-2 Δ2 channels (2.24 ± 0.39 μA/μF, n = 10), a result that was expected since β_3 has no effect, and the channel lacks the well-defined residues for β binding (Figure 6C). These results suggest that BI-2 Δ2, contrary to BI-2, was able to reach the plasma membrane in the absence of any functional β subunit interaction, presumably because of a weaker retention of the channel at the ER. The level of BI-2 Δ2 channel expression was not comparable to the level reached by BI-2/β_3-expressing oocytes (7.88 ± 1.42 μA/μF, n = 11), possibly because of residual retention. However, these data may also suggest that, besides retention by the I-II loop and inhibition of retention by the β subunit, additional and nonconflicting β-modulated mechanisms come into play in the increase in current density. Various possibilities include (1) an increased half-life for channels permanently associated with β subunits, (2) a faster rate of plasma membrane incorporation by β subunits or, (3) as shown by many investigators, an increased channel opening probability in the presence of β subunits.

Discussion

Contribution of β Subunits to Cell Trafficking of Voltage-Dependent Ca^{2+} Channels

There have been numerous publications illustrating that β subunits increase voltage-gated Ca^{2+} channel current

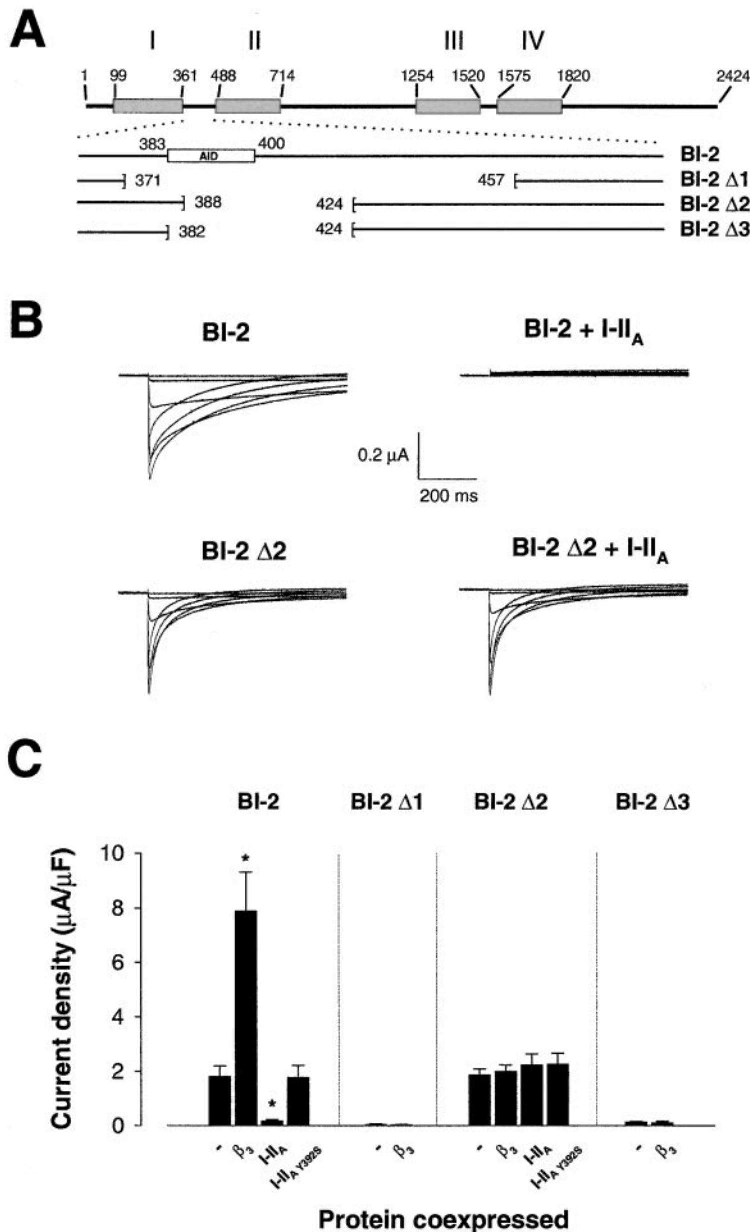

Figure 6. Expression of I-II Deleted Derivatives of the α_{1A} Subunit

(A) Schematic representation of the rabbit α_{1A} subunit (BI-2 isoform) and corresponding sequence deletions in the I-II loop. BI-2 Δ1, deletion of amino acids 372 to 456; BI-2 Δ2, deletion of amino acids 389 to 423; and BI-2 Δ3, deletion of amino acids 383 to 423. All three constructs removed the three critical residues required for β subunit binding (Y392, W395, and I396).

(B) Current traces elicited by membrane depolarization from −20 to 40 mV (10 mV increments) for BI-2 alone, BI-2 coexpressed with I-II$_A$, BI-2 Δ2, and BI-2 Δ2 coexpressed with I-II$_A$.

(C) Average current densities for the various BI-2 constructs expressed alone or in combination with β_3, the I-II$_A$ loop, or the non-β-interacting I-II$_A$ Y392S loop. Current densities were measured at a membrane depolarization of 20 mV, and all result from the same cRNA-injected oocyte batch; n = 6 to 12 oocytes for each condition. Average membrane capacitance was 0.18 ± 0.02 μF.

density at the cell surface. It can be concluded from these studies that this increase occurs by a combination of two major events: functional modulation (channel availability, opening probability, and single channel conductance) and changes in the membrane trafficking of voltage-dependent Ca^{2+} channels. Recent evidence in favor of a membrane-trafficking action of β subunits are increases in the number of drug binding sites at the cell surface (Lacerda et al., 1991; Brust et al., 1993) and in the total amount of gating charge (Josephson and Varadi, 1996; Beurg et al., 1997), which is a reliable indication of the number of α_1 molecules present in the plasma membrane, even if they are not functionally permeating Ca^{2+} ions. More recently, immunohistochemical observations all show that β subunits contribute to a cell surface redistribution of various α_1 subunits (Chien et al., 1995; Krizanova et al., 1995; Brice et al., 1997; Neuhuber et al., 1998b; Yamaguchi et al., 1998; Gao et al., 1999). Such an extended function of β subunit in both modulation and trafficking is not limited to voltage-dependent Ca^{2+} channels since similar mechanisms of action have been proposed for the K^+ channel Kvβ_2 subunit (Shi et al., 1996) and the Na^+ channel β subunits (Qu et al., 1995).

There are two major difficulties in analyzing the mechanisms implicated in the enhancement of current density by β subunits. The first one is to determine the extend to which each mechanism (functional modulation versus trafficking changes) contributes to this process. The second difficulty is to identify the molecular determinants involved in each one of these two forms of current enhancements and to dissect the underlying molecular events. This task is further increased in complexity if the molecular determinants identified are implicated in both functional and trafficking types of regulation. This is particularly true for all those α_1 sequences that are

known to bind to the β subunit and that contribute to the increase in current density. For instance, the I-II loop of the α_1 subunit contains such a site, one that is responsible for anchoring the β subunit in the channel complex. This region is also under severe regulatory constraint and, besides binding β subunit, it controls channel inactivation (Zhang et al., 1994) and integrates the regulatory input of protein kinase C (PKC) and G proteins (De Waard et al., 1997; Zamponi et al., 1997). All of these regulatory processes are able to modulate current amplitude dynamically, contributing to the idea that this region may also be regulated by β subunits via functional changes. Besides the primary α_1–β interaction, there are also weaker secondary interactions between these two subunits, involving the amino and carboxyl termini of α_1 subunit, two structures known for their implication in the control of channel expression and opening probability (Wei et al., 1994, 1996; Walker et al., 1998). It is further assumed that several other interaction points need to be uncovered to explain several other regulatory properties of β subunits (Walker and De Waard, 1998). Another difficulty when working with the full-length α_1 subunit is that multiple attachment points of β onto α_1 may retain the channel in a new conformation, which could impact both the function and the trafficking of the channel. Therefore, the major difficulty in distinguishing between a role of β subunit in channel conformation and cell trafficking lies within the Ca^{2+} channel structure itself, i.e., the localization of the various α_1–β interaction sites and their impact on channel structure, gating, and cell processing.

We were able to circumvent these problems by integrating several molecular determinants that were potentially responsible for the control of trafficking channels outside their molecular context (the α_1 subunit), into appropriate chimeras, thereby bypassing a conformational impact of the β subunit on Ca^{2+} channel structure and functioning. This approach was successful since the stimulatory impact of β subunit on channel expression in the plasma membrane could be reconstituted and analyzed by the use of appropriate chimeras (*Sh* and CD8).

The I-II Loop Contains an ER Retention Signal

Our data demonstrate that the addition of the I-II loop to *Sh* channels or to the α chain of the CD8 receptor results in an important reduction in the plasma membrane expression of these chimera proteins. Both proteins are affected in a similar way by the addition of the I-II loop, suggesting that the alteration in cell surface expression is not due to nonspecific changes in protein conformation. This is further confirmed by the lack of effect of the II-III tag (II-III_C or II-III_A) on the plasma membrane expression of CD8. The reduction in plasma membrane expression triggered by the I-II loop appears to be caused by an increased retention of these proteins in the ER, as chimera proteins containing the I-II sequence localize to this compartment. Such a conclusion further unravels the importance of using the gain-of-function approach that is provided with the CD8 and *Sh* chimeric proteins, as both molecules normally reach the plasma membrane in the absence of the I-II tag, which may not necessarily be the case for the Ca^{2+} channel α_1 subunit itself.

At this stage, we speculate that the sequence of the Ca^{2+} channel I-II loop binds to an ER chaperone protein whose normal function is to retain incompletely assembled multisubunit receptors in the ER (Figure 7). ER retention proteins appear to participate in a "quality control" function at the ER level by retaining unassembled chains and also by insuring the proper folding of the molecules. For instance, calnexin is a typical ER retention protein that retains incompletely assembled T cell receptor components in the ER (Rajagopalan et al., 1994). Evidence in favor of this interpretation is the fact that injection of an excess of a synthetic I-II_A peptide into oocytes expressing Sh-I-II_A resulted in an increased level of surface expression of this chimeric channel, suggesting the existence of a saturable retention system (data not shown). It is likely that the full-length α_1 subunit undergoes the same process of retention in the ER, as α_1 subunits are found associated to ER membranes when expressed alone (Gao et al., 1999). An analysis of the sequence of the I-II_A loop reveals that it contains five diarginine and two dilysine motifs, none being present in the AID sequence. Dilysine motifs are normally localized toward the carboxy-terminal end of proteins, whereas diarginine motifs have been found close to the amino-terminal end. These signals ensure the ER localization of these proteins by continuous retrieval from post-ER compartments. Their contribution in the ER retention of the I-II loop is, however, unlikely since none of these motifs are located at either the amino or carboxyl terminus of the protein, and out of these seven motifs, only two are also present in α_{1C} sequence. Recently, a novel RXR ER retention/retrieval sequence was identified in the α (Kir6.1/2) and β (SUR1) subunits of ATP-sensitive K^+ channels that is required at multiple stages of correct channel assembly and surface expression (Zerangue et al., 1999). Importantly, this sequence does not require proximity to either the amino or carboxyl terminus, and it can function in a cytoplasmic loop. We found two such sequences in the I-II_A loop, one (RER) located 16 amino acids before the AID sequence and a second (RMR) 74 amino acids after AID. Their implication in ER retention of the Ca^{2+} channel α_1 subunit is also uncertain since both sequences are still present in the BI-2 Δ2 construct, which presents a facilitated surface expression. Whatever the exact motif implicated, a retention system based on a block of the cell trafficking of the main ion-permeating subunit presents clearly the advantage of severely restricting the surface expression of immature voltage-dependent Ca^{2+} channels, which otherwise would be detrimental to normal cellular function and trophic Ca^{2+} entry.

Interestingly, the I-II_A sequence contains both a β subunit binding domain (AID; Pragnell et al., 1994) and two Gβγ binding domains (De Waard et al., 1997; Zamponi et al., 1997; Dolphin, 1998). As β subunits were absent from the expression systems used, at least in COS7 cells, they are not likely to contribute to the retention process itself. Also, we can probably rule out a contribution of the Gβγ complex in retention, as we found that tagging the I-II loop of α_{1C}, known for not binding Gβγ, is also able to produce intracellular retention of *Sh* chimera.

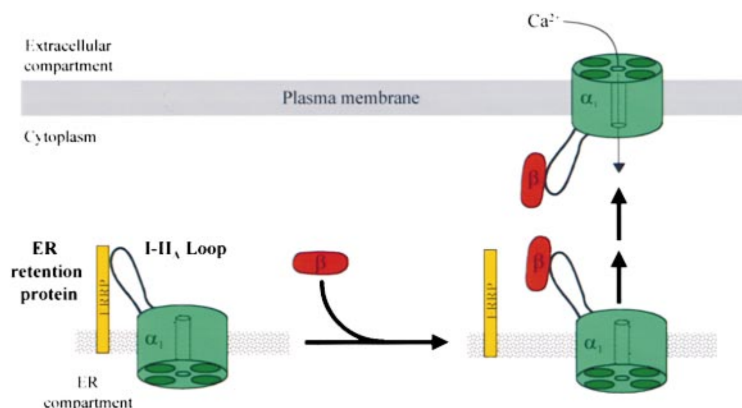

Figure 7. A Cell Model for β-Induced Increase in Ca^{2+} Channel Expression at the Plasma Membrane

β subunit plays a role in the cell trafficking of α_1 subunits. In the absence of β subunit, the α_1 subunit remains trapped within the ER by binding via the I-II loop onto an ER retention protein (ERRP) of unknown identity. Expression and subsequent binding of the β subunit to the I-II loop relieves the trafficking clamp imposed by the I-II sequence and allows fully matured Ca^{2+} channel complexes to be incorporated into the plasma membrane. For simplification purposes, the $\alpha_2\delta$ subunit has not been drawn but may facilitate the function of β subunit.

Binding of the β Subunit to the I-II Loop Unlocks the Brake on Channel Expression

We have shown a considerable decrease in the surface expression of chimeric proteins harboring the I-II loop on their carboxyl-terminal tail. For two different chimeras, this reduced plasma membrane expression can largely be overcome by coexpressing a Ca^{2+} channel β subunit. We have also demonstrated by pulse-chase experiments that the β subunit retains the ability to bind to the I-II loop in spite of the fact that it is tagged onto the carboxyl termini of proteins of widely different structure. In agreement with a binding of β subunit to the I-II sequence of these chimeric proteins, we found that both the CD8-I-II_A protein and the β_3 subunit underwent a considerable cell redistribution toward the plasma membrane. The most impressive redistribution concerns the β_3 subunit, whose entire pool was almost exclusively present under the cell surface. A similar observation was made by the coexpression of various β subunits with the full-length α_{1C} channel by Gao and collaborators (1999). Interestingly, pulse-chase experiments demonstrate that the association of β_3 to CD8-I-II_A-Myc occurs very rapidly after synthesis, most likely at the ER level, which is consistent with the almost exclusive ER localization of this chimeric protein (Figure 3C). Importantly, the absence of β_3 subunit localization at the ER level in CD8-I-II_A/β_3 coexpression experiments suggests that the binding of β_3 onto the I-II sequence triggers a rapid departure of the CD8-I-II_A/β_3 complex from this cell compartment. Further analyses will be required to examine the effect of the β subunit on the residency time of α_1 in the ER. Overall, these experiments demonstrate that the β subunit facilitates the plasma membrane incorporation of these chimeric proteins by actively removing an inhibitory clamp contained in the I-II loop. By extension, we conclude that the disruption of an ER retention signal during channel assembly represents one mechanism whereby the β subunit acts to increase VDCC expression (Figure 7). In future experiments, it will also be of interest to determine the regulatory contribution of the Gβγ complex in VDCC retention. Also, because Gβγ and β subunits have an antagonistic relationship in functional modulation (Dolphin, 1998), it may be that Gβγ affects the β subunit inhibitory ER retention potency by direct competition. Obviously, much remains to be gained by more precisely defining the I-II residues implicated in ER retention and the mechanism whereby the β subunit inhibits retention.

Potential Mechanisms of β Subunit Action

In common with several other oligomeric membrane proteins (Hendershot et al., 1987), including voltage-dependent K^+ channels (Nagaya and Papazian, 1997), the assembly of voltage-dependent Ca^{2+} channels appears to take place in the ER. Signals that can cause retention in the ER have been found in the cytoplasmic domains of individual subunits of multimeric receptors destined to the cell surface. Interestingly, these ER retention signals can be masked during the assembly of oligomeric receptors, like, for instance, in the case of the human high-affinity receptor for immunoglobulin E (IgE) (Letourneur et al., 1995). Similarly, we found that the β subunit plays an essential role as a chaperone protein in the expression of voltage-dependent Ca^{2+} channels and represents the rate-limiting step in de novo biosynthesis of these structures. We discuss several mechanisms that can be considered for β subunits in disrupting the ER retention signal after assembly: (1) during α_1–β assembly, the ER retention signal in α_1 is masked sterically by β, (2) the ER retention signal specifically interacts with an antisignal present in β and is made nonfunctional, (3) a dominant export signal in β causes transport of α_1–β complexes independently from the presence of a retention signal in α_1, or (4) a combination of these various possibilities.

There is obviously evidence in favor of the two first possibilities, as we found that a deletion in the I-II loop, including AID, the sequence required for β binding, favors a higher surface expression level of the α_1 subunit. In terms of molecular mechanisms implicated, it is possible that the β subunit acts as a direct competitor by occupying the critical residues of the I-II loop required for α_1 subunit retention. In this scenario, the residues implicated in ER retention are expected to be strictly identical to those required for β binding. Alternatively, the β subunit may act as an allosteric competitor that triggers a remote conformational switch elsewhere in the I-II loop. This change in conformation would in turn be sufficient for releasing the molecular complex from the ER and for favoring its cell trafficking toward the plasma membrane. The implication of this second possibility is that at least some of the I-II sequence responsible for ER retention is different from that required for β binding. It would also provide significant opportunities for additional regulation mechanisms to take place, as the I-II loop is also a target for G protein binding and PKC regulation.

The third possibility, that β subunit contains a dominant export signal, would at first glance appear to be unlikely since we found that β_3 has a cytosolic distribution and, contrary to certain isoforms of β_2 that are palmitoylated on their amino terminus (Chien et al., 1995), does not locate itself to the plasma membrane. Also, it was found that this unique property of β_2 to localize to the plasma membrane, even when expressed in the absence of the α_1 subunit, is not required to target the α_1 to the plasma membrane (Brice et al., 1997; Gao et al., 1999). Finally, we found that the surface expression levels of *Sh* channels tagged with the β_3 subunit were not increased in spite of the functionality of the BID sequence. However, it should be noted that, in spite of its cytosolic distribution, a significant fraction of the β_3 subunit is associated with intracellular membrane systems, as suggested by metabolic labeling and subcellular fractionation experiments (see also Chien et al., 1995; Brice et al., 1997; data not shown). Therefore, an additional contribution of this subunit in the cell trafficking of VDCC besides the inhibition of ER retention should not be ruled out, as we will discuss later.

Additional Molecular Determinants Regulating VDCC Expression Level

To study the role of ER retention in the cell trafficking of α_1 subunits, we attempted to delete most of the retention signal present in the I-II loop of full-length α_1 channel. This approach was based on an analogous case concerning the secretion of Igs. It is known that the transport out of the ER of unassembled Ig molecules is blocked by the binding of Ig heavy chains to BiP, an ER resident chaperone protein. When this association with BiP does not occur, as induced by mutations, incompletely assembled heavy chains can be secreted (Hendershot et al., 1987). A major difficulty in exploring the role of retention in Ca^{2+} channel expression level is the possibility that shortened I-II sequences alter the function of the channel. In line with this idea is the fact that two of our deletion channels (BI-2 Δ1 and BI-2 Δ3) were nonfunctional. Since the deleted sequences include several sites of PKC regulation and of β subunit and $G\beta\gamma$ binding, it is possible that regulation and binding of these proteins are both required for normal channel processing and activity. Alternatively, it is also possible that the lack of retention produces misfolded proteins that are degraded. In spite of these difficulties inherent to the Ca^{2+} channel structure itself, we were able to detect some facilitated expression in the absence of a β subunit for a milder deletion channel (BI-2 Δ2). These data suggest that part of the increase in channel expression by β subunit is due to an antagonizing role of β subunit in channel retention at the ER level.

Our findings also suggest that a major deletion of the retention signal in the I-II loop of α_{1A} does not produce a current density that is equivalent to that of $\alpha_{1A}\beta_3$ cells. One possibility is that there is residual retention left in the I-II loop and that this approach cannot be pursued much further because excessive deletion produces lack of functionality or misfolding of the channel. However, it is also possible that other mechanisms govern the increase in current density by β subunits besides the inhibition of retention. We briefly discuss several future research avenues.

First, one can envisage the existence of several β-dependent ER retention signals located in various domains within the α_1 subunit. Since the β dependency is required to explain the β-induced increase in channel expression and because the β subunit is entirely cytoplasmic, these other ER retention signals may bind the β subunit and be located in β-accessible cytoplasmic α_1 sequences. This possibility is in agreement with the notion that there are other α_1–β interaction sites identified (Walker et al., 1998, 1999) and several others to map (Walker and De Waard, 1998). It will be interesting to determine if these other points of interaction also are molecular determinants in the control of channel trafficking toward the plasma membrane. Additional interaction sites could be helpful in precisely distributing the various VDCC along the cell surface (soma, dendrites, and axons).

Second, it is possible that, in addition to relieving the I-II retention signal, binding of β to α_1 is required for the proper channel conformation, allowing it to pass the "quality check" imposed by the ER. This operation could be realized by a subtle conformational modification in channel structure. Once β has bound to AID, it binds to other secondary sites on α_1, imposing a conformational constraint on α_1 that allows it to pass the ER. Note that this molecular constraint may also be responsible for changes in the gating patterns that were reported for β subunits (Costantin et al., 1998; Wakamori et al., 1999). Alternatively, binding on the I-II loop triggers only a discrete local conformational change without additional α_1–β binding requirements. This discrete conformation change may impact closely located channel structures (such as the IS6 or IIS1 transmembrane segments). In favor of conformational changes is the observation that the $\alpha_2\delta$ subunit, which contacts the α_1 subunit exclusively via transmembrane regions or extracellular epitopes (Felix et al., 1997), potentiates the effects of β subunit on channel expression (De Waard and Campbell, 1995; Shistik et al., 1995; Brice et al., 1997).

Third, binding of the I-II loop to the β_3 subunit may favor the emergence of new β competencies, including the transport of the channel to the plasma membrane. This may seem unlikely because of the predominantly cytoplasmic localization of the β_3 subunit used in our studies. However, even the β_3 subunit appears to be membrane associated to some extent, and it is possible that this subunit changes conformation upon binding to the I-II sequence. If this is the case, then the β subunit has a double competence, first by relieving the ER retention imposed by the I-II loop on α_1 subunit and second by transporting the α_1 to the plasma membrane after anchoring itself to this subunit. Further extensive experiments would be required to test this hypothesis.

Finally, part of the increase in current density occurs via functional modulation of the channel and is outside the scope of this study. Other viable possibilities include an increase in the length of the half-life of the α_1 subunit.

Conclusion

The oligomerization of ion channel subunits is a particularly complex assembly event because subunit composition, stoichiometry, and relative position are all critical to normal function. There is considerable advantage that this process occurs within the ER. First, there are kinetic reasons

since the proper attachment of β subunit to α_1 and the subsequent folding or postranslational events that follow may be slow processes that would perturb normal channel function if it had to occur at the level of the plasma membrane. Second, the mechanisms of β subunit regulation that we unravel ensure that only fully matured Ca^{2+} channel complexes can reach the plasma membrane, incomplete channels remaining in the ER until β subunits are synthesized by the cell.

Experimental Procedures

Antibodies and Antisera Production

Rabbit anti-β_3 antibodies were as previously described (Raymond et al., 1999). Polyclonal antibodies against α_{1A} subunit (BI-2) were produced by injecting 100 μg of purified GST protein in fusion with part of the I-II loop of α_{1A} (amino acids 369 to 418). After boosting the immune response of the rabbit three times, the rabbit was bled, and the presence of anti-I-II_A antibodies was tested by ELISA against GST-I-II_A, immunoblots of brain synaptosomes, and immunoprecipitation of [^{35}S]-labeled proteins containing the I-II_A sequence. The rabbit polyclonal anti-CD8 (H-160) was from Santa Cruz Biotechnology and recognizes the amino-terminal region (amino acids 22 to 182) of the α subunit of human CD8. The monoclonal mouse anti-human CD8 antibody (HIT8a) is from Pharmingen and is also directed against the α subunit. The monoclonal anti-Myc antibody (9E10) is from Boehringer, whereas the polyclonal anti-Myc antibody (8A12) is a generous gift from Dr. M. Takahashi (Mitsubishi Kasei Institute, Tokyo, Japan). The monoclonal anti-BiP antibody raised against the carboxyl terminus rat BiP (10C3) is from StressGen Biotechnologies. Goat anti-mouse conjugated to rhodamine (GAM-Rhod) or TRITC (GAM-TRITC) and goat anti-rabbit conjugated to FITC (GAR-FITC) were from Jackson Immunoresearch Laboratories (Immunotech, France).

Plasmid Constructions

The *Sh* B T449V was amplified by the polymerase chain reaction (PCR; forward primer, TTTGGTACCCTAACCATGGCCGCCGTTGCC, and reverse primer, GCGGAATTCAACGTCGGTCTCGATACTAACG GCCAGGGCATT) and subcloned into the KpnI–EcoR1 sites of pcDNA3 (Sh B construct). Next, chimera constructs (Sh-I-II_A, Sh-I-II_C, and Sh-β_3, respectively) were obtained by subcloning either the PCR product of the I-II_A loop of BI-2 (forward primer, GGGGAAT TCGCCAAAGAAAGGGAGCGGGTGGAGAAC, and reverse primer, TTTGAATTCTTACTTCTTGATAGTGGCTCTCCG), the PCR product of the I-II_C loop of α_{1C} (forward primer, GGGGAATTCGAGTTTTC CAAAGAGAGGGAGAAGGCCAA, and reverse primer, TTTGAATT CTTAAGATTCGGTCTCACTTGTGGGCATGC), or the β_3 subunit (forward primer, CCGGAATTCTATGACGACTCCTACGTGCCCGGGT TTGAGGAC, and reverse primer, TTTGAATTCTGGTCAGTAGCTGT CCTTAGGCCA) into the EcoR1 site of pcDNA3-Sh B. For chimeras between CD8 and the I-II or II-III loops of α_{1A} or α_{1C}, we used the pcDNA3-CD8-βARK-Myc vector, generously provided by Dr. J. Lang (Geneva University, Switzerland), replaced the βARK sequence (BamH1–EcoR1 sites) by in-frame PCR products coding for the entire α_1 loops, I-II_A (amino acids 360 to 487; forward primer, CGCGGATCCTCAGGGGAGTTTGCCAAAGAAAGGGAG, and reverse primer, CCGGAATTCCTGAGTTTTGACCATGCGACGGATGTA), or II-III_C (forward primer, GGAAGGATCCGACAACCTGGCTGATGCTGAG, and reverse primer, ACGTGAATTCCGTGTCGTTGACGATACGGTG) (CD8-I-II_A-Myc and CD8-II-III_C-Myc constructs). In these two constructs, the cytoplasmic sequence of CD8 was totally removed, but the transmembrane domain was conserved. For experiments with CD8, we used the plasmid clone of human antigen CD8A from ATCC or a pcDNA3-CD8-Myc construct. Finally, the PCR products coding for the I-II_A loop and a mutant form of it (I-II_A Y392S) were also subcloned in-frame in pcDNA3.1/His-C (Invitrogen; same primers as for CD8-I-II_A-Myc).

All constructs were checked by restriction site mapping and sequencing (Genome express).

Electrophysiological Recordings

Stage V or VI oocytes, obtained from *Xenopus laevis* frogs, were microinjected with 50 nl of various cRNA mixtures (0.3 μg/μl BI-2, BI-2 Δ1, BI-2 Δ2, or BI-2 Δ3; 0.2 μg/μl wild-type and chimera Sh B channels; 0.15 μg/μl I-II_A or I-II_A Y392S; and 0.1 μg/μl β_3). Cells were then incubated in defined nutrient oocyte medium (Eppig and Dumont, 1976) for 3–4 days prior to recordings. Two-electrode voltage clamp was performed with a GeneClamp amplifier (Axon Instruments, Foster City, CA). The following extracellular recording solution was used (in mM): NaCl, 96; KCl, 2; $MgCl_2$, 1; $CaCl_2$, 0.5; HEPES, 10; and niflumic acid, 1 (pH 7.4). Electrodes filled with (in mM) KCl, 140; EGTA, 10; and HEPES, 10 (pH 7.2) had resistances between 0.5 and 1.5 MΩ. Current records were filtered at 2 kHz, leak subtracted online by a P/6 protocol, and sampled at 5–10 kHz. Data were analyzed using pCLAMP version 6.02 (Axon Instruments).

Cell Cultures and Transfection

COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM glutamax, GIBCO-BRL) containing 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin at 37°C in 5% CO_2. Transfections were performed using the lipofectamine method from GIBCO-BRL. Basically, for coverslips of 18 mm diameter, 1.5 μg cDNA in 125 μl optimem was incubated for 20 min with 5 μl lipofectamine in 125 μl opti-MEM. The 250 μl opti-MEM mixture was then added for 4–5 hr at 37°C to COS7 cells (final opti-MEM volume of 1 ml). Next, cells were washed with DMEM, incubated at 37°C, and tested 48 hr after transfection. In the case of multiple cDNA transfection, the cDNA ratio used is given in the figure legends. When appropriate, the addition of insert-free pcDNA3 vector was used to bring the cDNA concentration up to 1.5 μg. Cells grown in 60 mm petri dishes were transfected similarly, except that the total cDNA concentration was increased 2-fold.

Metabolic Labeling

Metabolic labeling was performed essentially as described (Shi et al., 1996). Briefly, cells grown on plastic dishes (35 mm diameter) were preincubated in methionine-free DMEM for 30 min at 37°C, later supplemented with 200 μCi/ml [^{35}S]-methionine at 37°C for 1 hr. Cells were washed twice in phosphate-buffered saline (PBS) and lysed, and proteins were solubilized for immunoprecipitation.

In Vitro Translation

The ^{35}S-labeled probe was synthesized by coupled in vitro transcription and translation using the TNT system (Promega) and in the presence of canine microsomal membranes. Nonincorporated [^{35}S]-methionine was removed by purification on a PD10 column (Pharmacia). Tranlated proteins were loaded onto 3%–12% SDS–PAGE gels.

Binding of GST Fusion Proteins

Purified GST fusion proteins were coupled to glutathione-agarose beads by a 30 min incubation in TBS (25 mM Tris, 150 mM NaCl [pH 7.4]), 0.1% Triton X-100. Binding was initiated by the addition of the translation product (2–10 pMol final concentration of translated protein), and the mixture was incubated at 4°C for 6 hr unless otherwise stated. Beads were washed four times in binding buffer, and the radioactivity associated was analyzed either by SDS–PAGE and autoradiography or by scintillation counting. Nonspecific values were determined as the radioactivity associated to GST-glutathione-agarose beads.

Immunocytochemistry

For cell surface labeling, COS7 cells transfected and grown on coverslips for 2 days were washed once in DMEM and incubated for 45 min at 4°C with the primary monoclonal anti-CD8 antibody (20 μg/ml) in DMEM, 10 mM HEPES, and 0.1% bovine serum albumin (BSA) (pH 7.4 with NaOH). The cells were washed three times for 5 min with PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (buffer 1 [pH 7.4]), fixed with 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 20 min at 25°C, rinsed three times with PB buffer, then incubated for 10 min in 50 mM NH_4Cl in PB and 30 min in 5% BSA PB buffer. Next, cells were incubated for 45 min with the secondary antibody (GAM-Rhod 1/300) in PB buffer supplemented with 1%

BSA at room temperature followed by three washes of 5 min each in PB buffer.

For intracellular labeling, transfected cells were washed three times in buffer 1, fixed with 4% paraformaldehyde in 0.1 M PB for 20 min at 25°C, rinsed three times in PB buffer, then incubated for 10 min in 50 mM NH_4Cl in PB. Next, cells were permeabilized with 0.066% saponin and 0.2% gelatin in PB (buffer 2 [pH 7.4]) for 30 min and incubated for 45 min at room temperature with the primary antibody (monoclonal anti-Myc, 4 μg/ml; monoclonal anti-BiP, 10 μg/ml; polyclonal anti-β_3 subunit, 14 μg/ml) in buffer 2. Cells were then washed three times for 5 min with buffer 2 and incubated with the secondary antibody for 45 min (GAM-TRITC 1/400 and/or GAR-FITC 1/250) in buffer 2. The cells were washed three times for 5 min each with buffer 2.

Coverslips were mounted in Mowiol and observed in confocal (Leica TCS, Leica Lasertechnik, Heidelberg, Germany) or nonconfocal fluorescence microscopy (Zeiss). For fluorescence microscopy, images were acquired with a SenSys digital camera (Photometrix, France) and Image Pro Plus software (Media Cybermetrics). For confocal microscopy, particular attention was accorded to possible contamination of one signal by another, so ascending contamination was checked at each acquisition by cross-talk control.

Cell surface expression efficiency of CD8 and CD8 chimera molecules were determined in the following manner. First, COS7 cells were transfected with either CD8, CD8-I-II_A-Myc, or CD8-II-III_C-Myc with or without β_3 subunit. Two days later, cells were then separated into two groups, one for the determination of transfection efficiency (anti-Myc labeling after permeabilization), and another for the determination of the percentage of cells having a surface expression (anti-CD8 labeling on nonpermeabilized cells). The efficiency of cell surface expression corresponds to the ratio of these two values and has been expressed as a percentage. This ratio was corrected by the percentage of β_3 cotransfection when applicable. The number of cells counted was between 200 and 700 for each experimental condition.

Immunoprecipitation

Immunoprecipitation of proteins metabolically labeled in transfected COS7 cells was performed as follows. Cells were lysed in TBS, 0.5% Triton X-100 (pH 7.4) supplemented with a cocktail of protease inhibitors (complete; Boehringer). The lysates were centrifuged at 1500 rpm for 15 min at 4°C, and supernatants were kept for immunoprecipitation. Labeled proteins present in the supernatant were immunoprecipitated for 3 hr at 4°C with 10 μg (anti-I-II_A or anti-β_3) or 4 μg of IgG (anti-Myc) coupled to protein A–Sepharose. Immunoprecipitates were washed three times with solubilization buffer and once without detergent added. In vitro translation products were immunoprecipitated similarly, but in TBS, 0.5% Triton X-100, 0.1% BSA (pH 7.4). Immunoprecipitated proteins were loaded onto a 9% SDS–PAGE gel, and the gel was dried and exposed for autoradiography.

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